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Advanced biological processes for nitrogen removal from agricultural digestate

Doctoral dissertation of: Davide Scaglione

Matriculation number: 738549

Supervisor: prof. Roberto Canziani

Co-supervisor: Dr. Elena Ficara

Tutor: prof. Roberto Canziani

Chair of the doctoral program: prof. Fernando Sansò

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A Valentina

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ABSTRACT

Anaerobic digestion is more and more applied with the double purpose of effectively treating livestock waste-water and as a mean to produce renewable energy as biogas. The liquid fraction of the digested material is rich in ammonium nitrogen; however, its disposal on agricultural soil is often not possible as intensive-breeding farms do not have enough arable land available to comply with the stringent limits on allowed nitrogen loads for land application of manure and other animal wastes. These limits derive from the enforcement of the European directive on nitrates (91/676/CEE) and other recent national and regional regulations, aiming at protecting groundwater from nitrate pollution.

This has stimulated the search for cost-effective nitrogen removal techniques. This thesis is part of a bigger project ("BRAIN", founded by the Italian Ministry of Forestry and Agriculture) that has the general aim of exploring the feasibility of advanced biological processes to reduce nitrogen from agricultural digestate. The main challenges in treating these type of wastewaters with innovative biological nitrogen removal processes concern the wide variability in the characteristics of agricultural digestate, due to (i) the seasonality of the digested matrixes, (ii) the variable operating conditions of the digesters and (iii) the occasional or permanent occurrence of inhibitors such as recalcitrant organics (antibiotics, humic and fulvic acids) or heavy metals.

The BRAIN project considers two biological processes to treat the liquid fraction of agricultural digestate:

I) the nitritation-denitritation process (here called "DENO2") and
II) the fully autotrophic nitrogen removal process.

The DENO2 process is based on the fact that nitrite is an intermediate compound in both nitrification and denitrification steps, and therefore both nitrate production and reduction can be bypassed. In the fully autotrophic nitrogen removal process, partial nitritation is followed by anaerobic ammonium oxidation via the anammox process, resulting in the production of dinitrogen gas. This thesis deals with the DENO2 process and with the anammox process, while the partial nitritation is not part of this work.

A pilot-scale SBR (800L) has been operated according to the nitritation-denitritation mode. It is located at a piggery farm (20000 pigs) in Lombardy, treated the supernatant from a full scale digester, fed on thickened piggery manure, poultry manure and agro-wastes (maize, wheat). High influent variability in terms of C/N ratio was registered depending on seasonal variation in the piggery waste production, variation in the co-substrates fed to the digester in addition to the piggery wastewater (maize, wheat, poultry manure) and variable anaerobic digestion efficiency. The initial inoculum was already rich in AOB and a stable nitritation efficiency has been maintained: in fact, the NO_2/NOx ratio at the end of the aeration phase remained always around 80-90% at temperature of both 25°C and 30°C, SRT up to 30 d and oxygen concentration of 0.75-1 $mgO_2\ L^{-1}$. Operation at high SRT (20-25d) allowed to maintain nitrogen removal efficiency in the range 60% to 95%, in spite of the high influent COD/N variability (from 1.3 to 5). When COD/N ratio in the influent was higher than 3, the nitrification process in the reactor was less efficient, mainly because of oxygen limitation, which was due to concurrent heterotrophic activity and the thick flocculent biomass matrix. Respirometric tests and microbiological analyses confirmed the hypothesis on oxygen limitation, which was probably due to diffusion through the thick flocculent biomass matrix. Free ammonia and free nitrous acid inhibitions on nitritation activity were assessed: IC50 values were found to be 148±5 $mgNH_3\text{-}N\ L^{-1}$ and 0.16±0.02 $mgHNO_2\text{-}N\ L^{-1}$, respectively. During the experimentation, free ammonia affected AOB activity more than free nitrous acid. However, under stable operation, the overall FA and FNA inhibition was calculated to be averagely 8±4% and always lower than 20%. Relevant N_2O emissions, accounting for 14-20% of the N treated, have been detected in preliminary tests.

In this thesis three different lab-scale studies on the anammox process are included, namely:

- anammox enrichment from conventional sludge samples;

- tests to assess the applicability of anammox to treat the liquid fraction of agro-waste digestate;

- nitrite inhibition and recovery of anammox biomass.

Although anammox microorganisms are widely diffused in both natural and man-made environments, these microorganisms grow very slowly, and the availability of a suitable biomass inoculum is important both for research and applicative purposes. Enrichment from environmental sludge samples is a way to fulfill this need. A simple fed-batch method was applied to enrich six sludge samples collected from Italian wastewater treatment plants (either from the anaerobic or anoxic stage) treating municipal, yeast-production or swine effluents. All samples were found to be adequate for anammox microorganism enrichment. The length of the lag-phase before such anaerobic ammonium oxidation was observed was around 100 days for most samples, thus similar to that measured in previous attempts. Three of the enriched samples were subsequently mixed and used to inoculate a SBR reactor fed with mineral medium. A 10 fold increase in the nitrogen removal rate was achieved in 80 days, reaching 0.22 $gN\ L^{-1}d^{-1}$.

In the following experimental phase, an highly enriched granular biomass taken from a full scale reactor was used. The stability of the anammox process when treating agricultural digestate after

solid/liquid separation and aerobic pre-treatment at different dilution levels was evaluated in a lab-scale SBR. The study was conducted at stable influent nitrogen concentrations comparable to those expected in a full-scale plant. The nitrogen loading rate applied was maintained around 0.6 kg N $m^{-3}d^{-1}$ during the whole experimentation. No pretreatment was applied and the fraction of real wastewater in the influent was gradually increased up to 100% (v/v). Anammox process was stable and could efficiently remove the applied nitrogen load when the percentage of real wastewater in the feed was lower than 70%. The maximum nitrogen removal capacity in the reactor increased 4 times in around 60 days reaching 5.1 kg N $m^{-3}d^{-1}$ despite the fraction of real wastewater blended in the SBR was increased from 10% to 70%. Later on, anammox activity dropped. The causes are still unclear. The most probable reasons may be: the presence of one or more compounds that are already present in the real waste water (e.g. antibiotics, humic/fulvic acids) or one or more compounds which may have been produced in the reactor by hydrolysis of slowly degradable organic compounds. Nevertheless, the anammox process appears to be applicable to this real wastewater after a moderate dilution of 0.5:1, without any further pretreatment. This result need to be confirmed by a future long term experimentation under stable conditions.

Finally the nitrite inhibition effect on anammox biomass was evaluated. Anammox granules from two SBRs fed on very different wastewaters (synthetic medium and landfill leachate) shared many similarities in terms of microbial population and kinetics, resulting in similar response to increasing concentrations of nitrite (100, 200, 300, 500 mgN-NO_2^- L^{-1}). Anammox granules from both reactors were proven to be quite tolerant to moderate to high nitrite concentrations, as long as the exposure time was limited to 3-4 hours with less than 40% activity loss at 500 mgNO_2^--N L^{-1}. However, after prolonged exposure (24 h), the activity loss was substantial (IC50: 173 mgNO_2^--N L^{-1} and 171 mg mgNO_2^--N L^{-1} for Anammox granules grown on synthetic medium and on leachate, respectively). After washing the granules with nitrite-free medium, anammox activity recovered substantially from both SBRs, reaching 60-80% of the initial maximum specific activity. This confirms that major activity losses in anammox reactors can be avoided by a timely identification of process operational conditions causing nitrite build-up in the reactor.

SUMMARY

1 INTRODUCTION

1.1 Nitrogen in agriculture

Nitrogen is crucial to life on earth, and the nitrogen cycle is one of the most important nutrient cycles for natural ecosystems. Plants absorb nitrogen from the soil, and animals eat the plants. When they die and decompose, the nitrogen returns to the soil, where bacteria convert it and the cycle starts again.

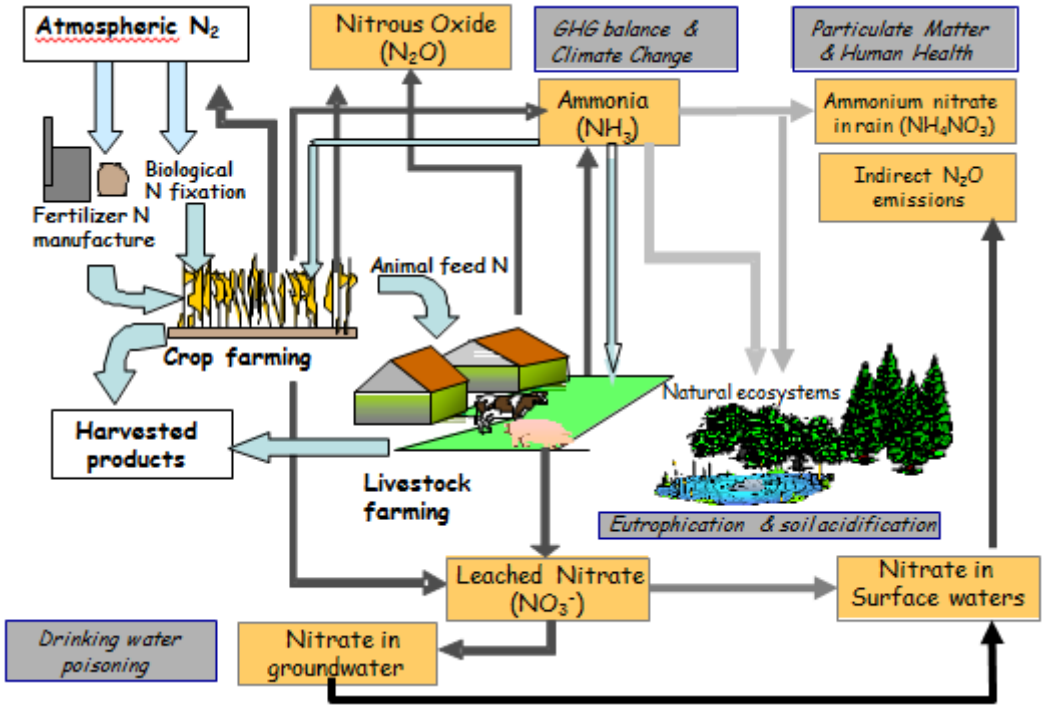


Figure 1.1 - Nitrogen cycle in agriculture

Excessive fertilizers application on soil (e.g. to enhance the production of livestock feeding) is one of the main reasons for water pollution and eutrophication. Livestock breeding produces gaseous emissions (i.e. N_2O, NH_3, CH_4), causing acidification and greenhouse effects, and wastewaters highly concentrated in nitrogen. A schematic overview of the role that livestock farming plays in the N-Cycle is given in Figure 1.1.

Livestock manure represents one of the most significant contributions to the nitrogen (N) sources in Europe and, therefore, the recovery of N is strategic for economical and environmental reasons. Nevertheless, agronomic N recovery by manure land application has to be regulated according to good agricultural practices and vulnerability of the water bodies. In several EU regions, manure nutrients exceed the amount that can be utilized on land (170 kg N $ha^{-1}y^{-1}$ in Nitrate Vulnerable Zones -NVZ) according to the criteria of good agricultural practice set out by the Nitrates Directive (Commission of the European Communities, 1991). A large area of Europe (40.9%) has been designated as NVZ (Commission of European Communities, 2007). In the Lombardia region – Italy, NVZ account for the 56% (5823 km^2) of the overall available arable land.

To reduce the environmental impact of livestock farming, manure (and wastewater originating from manure processing) has to be handled and/or treated in proper ways.

1.2 Technologies for agricultural digestate treatment

Anaerobic digestion is more and more applied with the double purpose of effectively treating livestock wastewater and as a mean to produce biogas as renewable energy. Digestate is the sludge that remains after the anaerobic digestion process. The overall nitrogen content of the substrates fed to the digester does not change during digestion, because it is only partly transferred to the gas phase as gaseous ammonia and a small fraction is consumed for bacterial growth, which leaves the digester together with the digested material. The main difference is that most of the organic nitrogen is mineralized, so that the fraction of nitrogen as ammonia is higher in the digestate.

There are different technologies for digestate treatment. Besides solid/liquid separation technologies, that allow for a better management of digestate, there are two different groups of technologies:

- physical-chemical processes which separate ammonia and concentrate it in other flows, either solid, liquid or gaseous, that can be further processed to recover ammonium salts;
- biological processes which convert ammonia into nitrogen gas that returns to the atmosphere.

Nitrogen recovery is a desirable option, but its sustainability and the possibility of large-scale applications has yet to be demonstrated, both from the technical (for example the need for additional treatments to purify the concentrated flows), environmental (for example by comparing alternative products and technologies through a Life Cycle Assessment), administrative/regulatory, and finally, economic aspects. The economic feasibility is closely linked to the market for recovered materials and the ability to compete with traditional chemical fertilizers. Moreover, Maurer et al. (2003) compared the energy consumption of different technologies for N recovery from source separated urine and calculated that:

- N recovery by air stripping followed by acid condensation and $(NH_4)_2SO_4$ production needs 90 MJ/kgN;
- biological removal with nitrification/denitrification with methanol as substrate needs 109 MJ/kgN;
- biological removal with the completely autotrophic process (partial nitritation + anaerobic ammonia oxidation) needs only 19 MJ/kgN;
- the Haber-Bosch process to produce ammonia from N_2 needs 45 MJ/kgN.

It follows that the combination of the conventional biological removal process with the Haber-Bosch process (154 MJ/kgN) results less convenient than the N recovery with air stripping (90 MJ/kgN), but completely autotrophic processes plus the Haber-Bosch process could be more energetically convenient (64 MJ/kgN).

1.2.1 Solid-liquid separation

Solid/liquid (S/L) separation technologies allow for a better management of digestate, but do not change the overall amount of nitrogen in the digestate. The separation, however, can modify the N/P ratio of the two fractions produced: most of the phosphorus remains in the solid fraction, while the liquid fraction is rich in soluble nitrogen as ammonia.

There are different kind of technologies for solid/liquid separation with different costs, purposes and efficiencies (Moller et al. 2000). The highest efficiency in S/L separation is achieved by the decanter centrifuge (Moller er al. 2002).
Burton (2007) well summarizes what separation processes can achieve:
- to make the liquid manure easier to handle, reducing risks of blockages in pipelines;
- to produce solid by-products containing most of the original solid material;
- to produce clarified liquids with greatly reduced levels of insoluble organic matter, phosphorous and heavy metals;
- to make it easier to apply livestock effluents to farm lands and to transport the solid concentrates outside the farm;
- to enhance anaerobic digestion performance by producing a concentrated feed from a dilute effluent;
- to produce a compostable solid fraction from liquid manures/digestate.

An effective S/L separation is also a necessary step before all the following technologies that can be applied for nitrogen removal or recovery from digestate.

1.2.2 Physical and chemical treatments

Air stripping in combination with absorption, can be used to remove and recover ammonia from livestock wastewaters and agricultural digestate. Ammonia is transferred from the waste stream to the air, and then absorbed from the air into a strong acid solution (typically sulfuric acid), thereby generating an ammonium-salt, which can be crystallized. To be efficient, the process has to be run at high pH (Liao et al. 1995) or high temperature. The main limiting factor for ammonia air stripping at high temperature is the availability of a cheap thermal energy source. When combining anaerobic digestion with a stripping/absorption process, the biogas produced during anaerobic digestion can partially or totally provide the heat needed for stripping at high temperature. Bonmatì and Flotats (2003) confirmed better results in terms of efficiency and salt quality for ammonia high temperature stripping in case of digested pig slurry compared with fresh slurry.
Another technology, that could be combined with ammonia stripping and can be used to concentrate the nutrients is the evaporation and subsequent condensation in a mechanical vapour recompression (MVR) unit (Melse and Verdoes, 2005). Drying is an alternative technology to be applied to digestate, and it is often combined to condensation or acid absorption thus producing an ammonium concentrated flux and a solid fraction.

Membrane filtration represents a suitable technology for nutrient concentrate production (Masse et al., 2007). Selective reverse osmosis (RO) membranes can also produce water of relatively high quality that could be reused at the farm. Water reuse would be especially advantageous for the large pig farms situated in areas experiencing water-shortage problems. RO requires extensive manure pre-treatments to prevent fouling, maximise membrane life, and increase fluxes. The use of reverse osmosis for nutrient concentration and manure treatment is technically feasible with proper pre-treatment, but high costs remain the main impediment to the application of the system.
Combination of technologies such as membrane and stripping, are proposed by some companies to treat agricultural digestate. Up to now, no long-term data are available about operational costs and maintenance needs. Preez et al. (2005) proposed a scheme (BIOREK) that includes processes (tested at pilot-scale) such as anaerobic digestion, ammonia stripping, and advanced membrane technology based on ultrafiltration and reverse osmosis. The main drawback of such a system is the high running cost and some operational problems such as membrane clogging.
Struvite precipitation can also be considered a physical-chemical technology useful and advisable with the main purpose of phosphorus recovery, but this technology is not discussed here, since its N-removal efficiency is low and it cannot be applied as a stand-alone N-removing process.

1.2.3 Biological processes

In most modern wastewater treatment plants (WWTP), nitrogen, which enters the plant in the form of ammonium or organic nitrogen, is removed by biological nitrification/denitrification. As a first step, ammonium is converted to nitrate (nitrification, reaction 1) which is converted to nitrogen gas in the second step (denitrification, reaction 2). Benefits of the process are the high potential removal efficiency, high process stability and reliability.

$$NH_4^+ + 2O_2 + 2HCO_3^- \rightarrow NO_3^- + 3H_2O + 2CO_2 \quad (1)$$

$$5C + 4NO_3^- + 2H_2O \rightarrow CO_2 + 4HCO_3^- + 2N_2 \quad (2)$$

Among the alternatives to improve manure management, biological processes are often seen as (a part of) the solution due to their natural occurrence in the N, C and P cycles. Studies on biological treatment of livestock effluents initially dealt with organic matter degradation and odour reduction, but lately nitrogen removal (Evans et al., 1986; Oleszkiewicz, 1986) and the energy recovery by anaerobic digestion are also gaining more and more attention. Different technologies have been proposed for the biological treatment of livestock effluents:
(a) Aerated lagoons (Oleszkiewicz, 1986);
(b) Fixed-bed reactors (Westerman et al., 2000);
(c) Activated sludge with anoxic tank (Willers et al., 1993);
(d) Activated sludge with intermittent aeration (Bicudo and Svoboda, 1995);
(e) Sequencing batch reactors (Bortone et al., 1992; Mace and Mata-Alvarez, 2002);

Activated sludge with anoxic tank gives good results and can be applied to the treatment of swine effluent (Vanotti et al., 2007). However, processes including intermittent feeding and aeration are generally considered to be technically and economically more efficient. Consequently, most full-scale processes are single tank technologies (Bicudo and Svoboda, 1995), based on intermittent aeration to achieve a good nitrogen removal through nitrification–denitrification, including SBR systems (Mace and Mata-Alvarez, 2002). Such a process is well suited to variable influent loads.
The main issue when applying conventional nitrification-denitrification process to digestate treatment is the low C/N ratio in the influent, causing the need for external carbon dosage to support denitrification. Obaja et al. (2005) and Deng et al. (2008) have shown that the dosage of fresh pig slurry as carbon-source can be an economic alternative.
Advanced biological processes such as the nitritation-denitritation process and the completely autotrophic process (partial nitritation and anammox process) are suitable for digestate treatment and allow economical saving thanks to lower aeration request and lower external carbon source need (Table 1.1). Nitritation/denitritation can save half of the energy cost for a WWTP, while partial-nitritation and anammox up to 80%.
Nitritation-denitritation and anammox processes will be described in the following paragraphs.

Table 1.1 – Oxygen and COD needs for different biological processes (from Van Hulle et al. 2010)

Process	Oxygen need ($gO_2\ gN^{-1}$)	Dissimilatory COD need ($gCOD\ gN^{-1}$)	Assimilatory COD need ($gCOD\ gN^{-1}$)
Nitrification-denitrification	4.57	2.86	4.0
Nitritation-denitritation	3.43	1.72	2.4
Partial nitritation-Anammox	1.72	-	-

The cost of the autotrophic nitrogen removal process (applied to urban digester supernatant) was estimated to be 1 euro per kg N removed, while other conventional nitrogen removing techniques cost from 2 to 4 € per kg N removed (van Hulle et al., 2010). In practice, the selection of either a biological or a physical-chemical method for nitrogen elimination is also determined by the nitrogen concentration of the wastewater. According to Mulder (2003) biological treatment by autotrophic nitrogen removal is to be preferred for concentrated wastewater streams with ammonium concentrations in the range of 100–5000 mg NH_4^+-N L^{-1}. By considering the revenue from recovered ammonium salts selling and the thermal energy recovered from biogas production, the maximum ammonium concentration for biological digestate treatment could be

adjusted to 3000 mg NH_4^+-N L^{-1}; above this threshold value ammonia stripping may become more convenient.

1.3 Nitritation-Denitritation process (DENO2)

The DENO2 process is based on the fact that nitrite is an intermediate compound in both nitrification and denitrification steps, and therefore both nitrate production and reduction processes can be bypassed. In this process, a partial nitrification up to nitrite is performed followed by nitrite denitrification as shown in Figure 1.2.

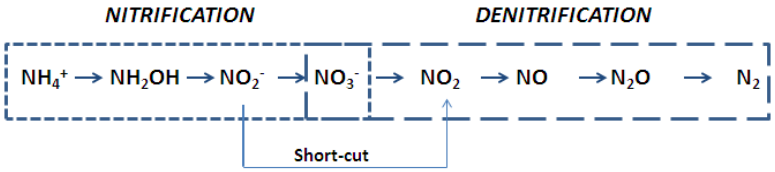


Figure 1.2 - Biological nitrification–denitrification via nitrite pathway (DENO2)

1.3.1 Principle of the DENO2 process

Nitrification is a biological oxidation process, which involves two different groups of bacteria. The first step of nitrification is carried out by ammonia-oxidizing bacteria (AOB) and consists in the oxidation of ammonia to nitrite via hydroxylamine (NH_2OH), involving the membrane-bound ammonia mono-oxygenase (AMO) and the hydroxylamine oxidoreductase (HAO); the second group consist in nitrite-oxidizing bacteria (NOB), and further oxidizes nitrite to nitrate. Under typical conditions (diluted wastewater, ambient temperature, neutral pH and non-limiting oxygen values), the first step is the rate-limiting one; in contrast, nitrite is oxidized rapidly to nitrate, so nitrite is seldom accumulated in nitrifying reactors. In the partial nitrification process, however, nitrite accumulation is required, and the second step must be restrained so as to accumulate AOB and washout NOB.

Nitrogen removal via nitrite has been previously recognized as economically beneficial in biological wastewater treatment plants (Turk and Mavinic 1986). The DENO2 process saves the oxygen required for nitrite oxidation (25% of the total oxygen demand) and 40% of the carbon demand (Figure 1.3).

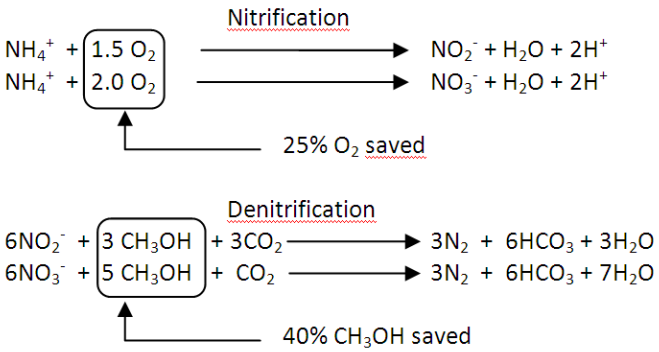


Figure 1.3 – Comparison between nitrification-denitrification and nitritation-denitritation process

However, the difficulty in applying nitrogen removal via nitrite lies in retaining the ammonia oxidizing bacteria while eliminating the nitrite oxidizing bacteria.

1.3.2 Key parameters to obtain a stable nitritation process

To date, researchers have developed many control methods and strategies to achieve partial nitrification (Sinha et al., 2007). The main objective of these methods was to accumulate AOB and washout NOB through different strategies. The main strategies are listed below.

- **High T(°C) and low SRT**: Selective wash out of NOBs from continuous flow reactors (such as a Chemostat) has been achieved at elevated temperatures (30–40°C), coupled with a dilution rate that is less than the growth rate of NOBs but greater than the AOB growth rate (about 1–0.5 $days^{-1}$) The most known application of this strategy is the single reactor high activity ammonia removal over nitrite (SHARON) process (Hellinga et al. 1998, Mosquera-Corral, 2005). Hellinga et al. 1998, reported that it is enough to keep the temperature at 25 °C when reactor's temperature and sludge age are the sole control parameters. Anyway, several experiences report successful partial nitrification under longer sludge age (e.g.: Pollice et al., 2002; Fux and Siegrist, 2004).

- **Dissolved oxygen concentration**: The dissolved oxygen half-saturation coefficients of AOB and NOB are 0.2–0.4 mg L^{-1} and 1.2–1.5 mg L^{-1}, respectively (Picioreanu et al. 1997). Therefore, low DO concentration limits the growth of NOB more that than that of AOB. Stable nitritation with the use of low dissolved oxygen (DO) concentration has been observed in a variety of reactor configurations (Sliekers et al. 2005; Wyffels et al. 2004; Canziani et al., 2006).

- **FA and FNA concentrations**: inhibitions have been confirmed by many authors as key parameters to achieve a stable nitritation process. Concentrations of Free Ammonia (FA) and Free Nitrous Acid (FNA) can be calculated as a function of pH, temperature, and Total Ammoniacal Nitrogen (TAN) for FA, or Total Nitrite (TNO_2) for FNA, according to Anthonisen et al. (1976). FA and FNA inhibit at different concentrations both AOB and NOB but FA has a most significant inhibitory effect on NOB (Jubany, 2009). Abeling and Seyfried (1992) found that FA

concentrations between 1 and 5 mg NH_3–N L^{-1} inhibit the nitratation but not the nitritation. Jubany (2007) found IC_{50} values for NOB of 0.45 mgHNO_2-N L^{-1} and 9.5 mgNH_3-N L^{-1}. However, the threshold inhibition concentrations of free ammonia found in the literature vary within a wide range, depending also on biomass acclimatization (Jubany, 2007).

Other authors reported (Peng and Zhou, 2006) that nitrification can be inhibited by free hydroxylamine, heavy metals, organic compounds, fulvic acids, strong oxidants, volatile fatty acids, and halide. Heavy metals, such as chromium, nickel, copper, zinc, lead, and cadmium, might inhibit both steps of nitrification reaction, but the inhibition thresholds are different.
Wett and Rauch (2003) reported that bicarbonate substrate limitation can also reduce NOB activity. Tukotomi et al. (2010) reported that nitrite-oxidizing bacteria (NOB) were eliminated in a reactor when $NaHCO_3$ was used as the alkalinity source. From the kinetic data, they inferred that high IC concentrations drive stable nitritation by promoting a higher growth rate for AOB than for NOB.
Park et al. (2010) proposed a model that provides a method to identify good combinations of pH, DO, and total ammonium nitrogen (TAN) to support shortcut nitritation. They demonstrate that the effect of DO-alone and the effect of DO plus direct pH inhibition cannot give a strong enough selection against nitrite oxidizing bacteria. However, by adding the FA and FNA effects a much stronger selection effect is achieved that is most effective at pH values around 8. Thus, a generalized conclusion is that having pH around 8 is favourable in many situations.
While investigating the causes of a stable nitritation, in a nitritation-denitritation taking place in an SBR, by making use of a calibrated ammonium and nitrite oxidation model, the aerobic phase duration was found to be a key factor leading to nitritation (Blackburne et al., 2008).
The Sequencing Batch Reactor (SBR) has been proven to be an appropriate configuration to obtain a stable nitritation-denitritation process (Fux et al. 2004, Fux and Siegrist, 2004; Dosta et al., 2007). Dosta et al. (2008) applied this system to treat piggery digestate.

1.3.3 N_2O emissions

N_2O is a strong greenhouse gas (1t = 298 tCO_2). Agriculture and manure management are involved in gas emissions: ammonia (NH_3) and two Green House Gases (GHG), nitrous oxide (N_2O) and methane (CH_4). Livestocks are responsible for 64% of NH_3, 37% of CH_4 and 65% of N_2O anthropogenic emissions. On the overall, about 30% of the GHG produced by livestock production are attributed to manure management (Bernet et al., 2009).
Other researchers found high N_2O emissions from the biological treatment of animal slurry ranging from 0.8 to 20% (Bernet et al., 2009). Ahn et al. (2011) registered a statistically higher N_2O emission (0.57±0.17% of the total influent N- load) in a partial nitritation reactor compared to full nitritation process. From full scale partial nitritation facilities. N_2O emissions ranged from 0.24-3.8% of total influent nitrogen load (Gustavsson et al., 2011).
Kampschreur et al. (2009) concluded that the main parameters that cause N_2O production are low dissolved oxygen concentration and increased nitrite concentrations in both nitrification and

denitrification stages and low COD/N ratio in the denitrification stage. Itokawa et al. (2001) observed that during steady-state operation of an intermittently aerated bioreactor treating high-strength wastewater, as much as the 20–30% of the nitrogen load was emitted as N_2O when the COD/N ratio was below 3.5.

Foley et al. (2010) made a comprehensive N_2O emission and formation study on seven full-scale WWTP founding a wide range of N_2O generation ranging from 0.006 to 0.253 kgN_2O-N per kgN denitrified. They concluded that higher N_2O generation was shown to generally correspond with higher nitrite concentrations, but with many competing and parallel nitrogen transformation reactions occurring, it was very difficult to clearly identify the predominant mechanism of N_2O production.

Based on their results, Ahn et al. (2010) argued that activated sludge processes that minimize transient or permanent build up of ammonium or nitrite, especially in the presence of dissolved oxygen, are expected to have lower N_2O emissions. From the literature, it still remains unclear whether nitrifying or denitrifying microorganisms are the main source of N_2O emissions.

The tendency of WWTPs to decrease their energy consumption by reducing the aeration rate could adversely affects the greenhouse effect: even though this strategy decreases CO_2 emission, this could be counteracted by the increased N_2O emission due to its 300-fold stronger greenhouse effect.

Many of the factors that are reported to favour N_2O emissions are present in $DENO_2$ process configuration, therefore this aspect needs to be considered and evaluated.

1.4 Anammox process

At the beginning of the 20th century the N-Cycle was assumed to be fully understood. However, there was still no reaction in this N-Cycle that accounted for the possibility of the anaerobic oxidation of ammonium, even though this reaction is associated with a considerable release of Gibbs free energy, (ΔG^0=-358 kJ mol^{-1}), with nitrite as electron acceptor (Broda, 1977).

The first evidence for the existence of anaerobic ammonium oxidation (anammox) was from a pilot-scale denitrifying reactor at the baker's yeast factory Gist-Brocades in Delft, The Netherlands (Mulder et al., 1995). Succeeding this evidence, numerous attempts to isolate the organism responsible for this process failed in the following years (Strous et al., 1999). Also in Germany (Hippen et al., 1997) and Switzerland (Siegrist et al., 1998) the production of dinitrogen gas instead of nitrate ("nitrogen losses") was reported in full-scale rotating disc contactors treating (ammonium-rich) wastewater originating from landfill leachates. Almost ten years after the first evidence for the existence of anaerobic ammonium oxidation, scientists succeeded in the molecular identification of the bacteria responsible for the anammox reaction by means of a novel experimental approach based on the introduction of molecular tools and modern bioreactor engineering in/to microbial ecology (Strous et al., 2002). By density gradient centrifugation, physically separated cells (purified up to 99.6%) of the enrichment culture of a representative of the phylum Planctomicetes, Candidatus "Brocadia anammoxidans" were shown to oxidize

ammonium to dinitrogen gas, with nitrite as electron acceptor under strictly anoxic conditions (Strous et al., 1999).

This novel process was named anoxic deammonification, or ANAMMOX (from ANoxic AMMonium OXidation) and was applied in combination with the partial conversion of ammonium to nitrite (in a proportion of about 1:1) performed by aerobic ammonia oxidizing bacteria (AOB). A first approximation of the overall process (Van Dongen, 2001) was therefore:

$$NH_4^+ + NO_2^- \rightarrow 2N_2 + 2\ H_2O$$

If compared to the conventional nitrification/denitrification process, the combination of partial nitritation and anoxic deammonification (or ANAMMOX) allows to reduce the oxygen demand to one half, the sludge production to one tenth and the need of external carbon to zero. The operational costs of reagents and of sludge treatment and disposal are therefore reduced to one tenth of those of a conventional process. If personnel, maintenance and capital costs are also considered, the overall costs are 1.5 times lower than conventional processes (Fux, 2003).

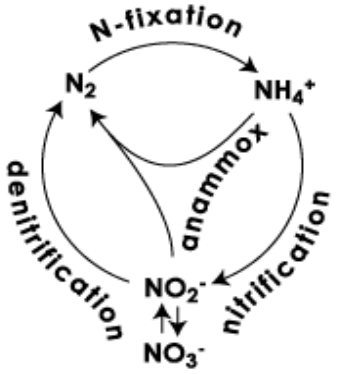


Figure 1.4 – Simplified scheme of the N-cycle with the anammox process

1.4.1 Anammox stoichiometry and kinetics

Although anammox bacteria are autotrophs with a similar energy gain from their catabolic reaction as AOB, their maximum specific growth rate is considerably lower: instead of 1 to 1.2 day^{-1} (typical for AOB Anthonisen et al., 1976), the growth rate of anammox bacteria is from 0.05 to 0.2 day^{-1} (Strous et al., 1999; Tsushima et al., 2007; Van der Star et al., 2008b; Lotti, 2011).
By making the mass balances over different Anammox enrichment cultures, the overall stoichiometry of the Anammox reaction was determined as expressed in the following equation (Strous et al.,1998):

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

The nitrate production in the anammox process stems from nitrite oxidation, which functions as the electron-donating redox reaction for the CO_2 fixation (van de Graaf et al., 1996). Nitrate production is an inevitable part of the overall anammox reaction and can be used to measure the growth of anammox bacteria. The stoichiometric conversion ratios of nitrite to ammonium and nitrate to ammonium are of (circa) 1.1 to 1.3 and 0.1 to 0.25, respectively. A higher ratio between nitrite and ammonium and/or the reduced nitrate production generally is an indication of the (co)occurrence of heterotrophic denitrification.

Besides conversion of ammonium and nitrite, anammox bacteria are also capable of reducing nitrate to nitrite and nitrite to ammonium with fatty acids as electron donor. The produced nitrite and/or ammonium serves again as a substrate for the normal anammox catabolism (Kartal et al., 2007) but completely changes the stoichiometry described above.

The decay rate of anammox bacteria in slow-growing organisms is not easy to assess because, like growth, decay is also slow. Recently, it was estimated at 0.0048 d^{-1} at 35°C (Scaglione et al., 2009) under anaerobic conditions, which is equivalent to a “anammox biomass half-life” of 145 days. The decay is thus about a 10-fold lower than the maximum specific growth rate, which is in line with observations for faster-growing organisms.

Anammox bacteria are strictly anaerobic and are inhibited by dissolved oxygen. However, inhibition caused by low concentrations of oxygen was demonstrated to be reversible. Egli et al. (2001) stated that oxygen inhibits Anammox metabolism reversibly at low oxygen levels (air saturation of 0.25–2%) but probably irreversibly at high levels (>18% air saturation). Strous et al. (1997) concluded from experiments with intermittent oxygen supply that the Anammox process was reversible inhibited by oxygen, making partial nitritation and Anammox possible in one reactor.

1.4.2 Anammox enrichment

As anammox bacteria grow very slowly (Strous et al., 1999), the availability of a suitable biomass inoculum is very important.

The start-up of a new installation is usually performed with an inoculum taken from the few existing plants which apply patented processes such as SHARON-Anammox, CANON and OLAND (Li et al., 2008). Alternatively, one can try to grow ANAMMOX cultures on the specific substrate by gradual enrichment of a mixed culture taken from sludge digesters or activated sludge reactors and develop site-specific solutions. There are several evidences of the quite wide diffusion of the Anammox microorganisms both in natural environments and in man-made ones, such as wastewater treatment plants (Kuenen, 2008). Enrichment techniques mean anammox biomass to grow from environmental sludge samples within a reasonable time frame (3 to 6 months, e.g. Pynaert et al., 2004, Noophan et al., 2009), to be used as inoculum for the start-up of reactors.

Different types of reactors have been suggested to be suitable for anammox enrichment (Egli et al., 2001; Tang et al., 2010), although the use of a sequencing batch reactor (SBR) has been recommended (Strous et al., 1998) and applied in most cases (e.g. Dapena-Mora et al., 2004, Lopez et al., 2008). Nevertheless, simple and low cost fed-batch strategies can be suitable when testing an array of several sludge inocula (Sánchez-Melsió et al., 2009).

This possibility is particularly attractive as, on the one hand, it allows to cultivate a specifically adapted biomass on the specific substrate to be treated and, on the other hand, it overcomes the need for biomass specimen purchased abroad.

1.4.3 Implementation of the anammox process

The removal of ammonium with the anammox process always consists of partial nitritation followed by the anammox process. The partial nitritation process consists in the conversion of about half of the incoming ammonium to nitrite by AOBs. The nitrite produced is then used in the anammox process to convert the remaining ammonium to dinitrogen gas. Both processes can take place in one reactor or in two reactors operated in series. An extensive review on the engineering aspects and practical applications of the anammox technology has been published recently (van Hulle et al., 2010). A brief introduction to the two different configurations is presented below.

One reactor configuration

When the nitritation and the anammox process take place in the same reactor, oxygen acts both as a substrate (for AOB) and as an inhibitor (for anammox bacteria). Since even very low oxygen levels cause (reversible) inhibition of anammox bacteria, they require truly anoxic conditions in the reactor, whereas, for allowing the growth of AOB, aerobic conditions are needed. In addition, the SRT should be sufficiently high (several weeks) to allow anammox bacteria to growth. To obtain both aerobic and anoxic conditions in the same reactor, three different approaches can be distinguished:

1. Continuous operation, in which the oxygen levels are governed by gradients in biofilm systems (Hippen et al., 1997; Kuai and Verstraete, 1998; Sliekers et al., 2002; Szatkowska et al., 2007). In such systems, oxygen is consumed in the outer layer of the biofilm and thus does not penetrate the biofilm completely. The anammox process can thus be performed in the anoxic inner layers making use of the produced nitrite that diffuses further into the biofilm. The same approach has been performed in suspended growth systems (Joss et al. 2009).
2. Intermittent aeration, in which oxygen levels vary in time (Third et al., 2005; Wett, 2006). In such systems, nitritation takes place during the aerated periods and the anammox process during the non-aerated periods. However, when the low penetration depth of oxygen in biofilm systems is taken into account, according to the first approach (previously described) the anammox process is likely to play a role during aerated periods as well.
3. Physical transportation of the biomass between the aerobic and the anoxic zone, by either (I) alternating between submerged and emerged phases as in rotating biofilm contactors (Kuai and Verstraete, 1998) or (II) moving the biomass between aerated and non-aerated zones of a reactor .

Besides conditions that favour the growth of AOBs and anammox bacteria, successful operation of single-stage nitritation-anammox reactors also requires that NOBs are outcompeted. Since these three groups share common substrates, competition can potentially occur on oxygen (AOB-NOB), ammonium (anammox-AOB) and nitrite (anammox-NOB). Several reactor types can be used to perform the one-reactor nitritation-anammox process. These include granular sludge systems

(sequencing batch reactors, air lifts, bubble columns) as well as systems with biomass growing on carrier (moving or fixed bed reactors). The main common feature is the ability to achieve high SRTs and efficient mixing.

Joss et al. (2009) have demonstrated the applicability of stable process of nitritation/ANAMMOX in different single stage full-scale SBRs in Switzerland. Ammonium oxidation capacities of up to 500 gN m^{-3} d^{-1} with conversion to N_2 of over 90% of the influent nitrogen are achieved by keeping the concentration of dissolved oxygen in the reactor at less than 1 mg L^{-1} with continuous aeration and by controlling the process by an ion-selective ammonium electrode or alternatively by the conductivity signal.

Two-reactor configuration

In two-reactor configurations, the nitritation and the anammox process are taking place separately in an aerated and a non-aerated reactor, respectively. The characteristics and requirements for the two-reactor set-up differ completely from the one-reactor operation. Both reactors are discussed separately below.

Nitritation Reactor

In the nitritation reactor, about half (55% as optimum) of the ammonium needs to be converted to nitrite to produce the desired reaction mixture for the anammox process. The challenge in this type of reactor is to prevent the growth of NOB, which would lead to production of nitrate rather than nitrite, and ensure that only half of the ammonium is converted. Many routes for production of nitrite rather than nitrate are applicable as described in par. 1.3.2. The counter-ion of the ammonium in the waste stream is the main factor ensuring that only half of the ammonium is converted. If this is bicarbonate (as it is the case in most waste streams), nitritation is pH-limited, rather than ammonium limited, as only 50% of the produced protons can be balanced by the bicarbonate buffering capacity.

Depending on the ratio between bicarbonate and ammonium contained in the wastewater (usually 1-1.2 for sewage sludge digestates), an equilibrium pH will be reached (6.3 to 6.6) at which 50 to 60% conversion of ammonia occurs (Van Dongen et al., 2001).

Anammox Reactor

Key factors for the stable operation of anammox reactors are sufficiently long biomass retention and good mixing. The latter is mainly important at the reactor inlet, as the concentrations of nitrite in the influent are generally high enough to be toxic. Biomass retention is important with regard to the slow growth of anammox bacteria. It should be noted that the required sludge age in typical cases is not extremely long, due to the higher temperatures at which most reactors are operated. In principle, a SRT of 30 days is sufficient. Especially in discontinuously operated systems with low biomass densities, flotation is a possible concern (Dapena-Mora et al., 2004), as are sudden changes in mixing or a too high exposure to shear stress (Arrojo et al., 2008).

The requirements for anammox reactors (good mixing and high biomass retention) are met in full-scale applications in granular sludge reactors where a selective pressure (i.e. settling ability) is used for the formation of granules. This type of reactor consists of separate mixing and settler

zones. In the latter zone, a stable upflow velocity (>1 m h^{-1}) strongly selects dense granules. The advantage of this type of reactor is the very high volumetric loading rate achievable when modern reactor designs are used (i.e. internal circulation and biofilm gas-lift suspension reactors), the use of the produced gas as a free/cheap mixing agent (Van der Star et al., 2007). The availability of specific biofilm surface area is a crucial factor to obtain high volumetric conversions.

The main advantages of working with a two reactors configuration compared with one reactor are:
- the possibility to treat wastewaters with higher BOD concentrations, higher solid content or higher variability: the organic carbon in excess is consumed in the partial nitritation reactor, thus avoiding excess of heterotrophic growth in the anammox reactor;
- up to 10 times higher rates for the anaerobic step (van der Star et al., 2007).

Nevertheless, the combined nitritation-anammox solution has other considerable advantages (Joss et al. 2009):
- operating a one-step reactor results in considerable simplification of reactor control and operation (e.g.: no pH control);
- under steady-state operation, nitrite is continuously depleted, reducing the risk of nitrite accumulation.

1.4.4 Treatable wastewaters

The application of the anammox process is mainly associated with wastewaters with a high nitrogen content (>200 mgN L^{-1}) and a low C/N ratio. Since no organic material is a requirement in the nitritation-anammox process, wastewaters with a higher C/N ratio can be treated advantageously by combining the nitritation-anammox process with an anaerobic pretreatment.
The anammox process has been successfully tested and used mainly in the treatment of reject waters (sludge digester liquids). These water come from the anaerobic digestion of waste sludge and typically contain 500 to 1,500 mgN L^{-1} of ammonium (as ammonium bicarbonate). Although reject water flows are quantitatively low (typically 0.5 to 2% of the main influent flow rate), they contain 5 to 20% of the overall nitrogen load. Both the one-reactor (Hippen et al., 1997) and two-reactor (Van Dongen et al., 2001) configurations were tested and are now applied in full scale plants (Van der Star et al., 2007; Wett, 2007; Joss et al. 2009; Ling 2009) either with granular or suspended biomass. In Sweden, the application of partial nitritation/anammox process for the treatment of digester supernatant on moving-bed biofilm system, was also studied (Gut el al., 2006).
Wastewaters from food industries are generally high loaded and protein-rich (and thus nitrogen-rich), and can be efficiently digested. The remaining waste stream can be used in the nitritation-anammox process. A digested potato wastewater is currently treated at full scale in a one-reactor nitritation-anammox process (Abma et al., 2009), as it is the digested wastewater of a tannery in a two-reactor configuration. Other effluents treated are digested seafood and fish canning effluents (Dapena-Mora et al., 2006)

Also the source-separated urine treatment with nitritation-anammox process has been tested (Sliekers et al.,2004). Black water (toilet water) is another source-separated N-reach waste stream. After anaerobic digestion of black water, an ammonium-rich (1 to 1.5 gN L^{-1}) waste stream remains, which can be treated with one-reactor (Vlaeminck et al., 2009) or two-reactor (De Graaff et al., 2009) nitritation-anammox process.
In landfill leachates, ammonium concentration can be as high as 5 gN L^{-1} and variability is site-specific and related to the landfill age. Few experiences of old-landfill leachate treatment with anammox process are available (Liang et al., 2008; Ruscalleda et al., 2008; Wang et al., 2010).

More than 20 full-scale plants are now in operation in Netherlands, Switzerland, Germany, Belgium, Austria, Sweden, Japan, and China (Van der Star et al. 2011). The start-up time decreased in later start-ups, as a result of the availability of biomass for inoculation and of the better understanding of the process.

1.4.5 Anammox process treating piggery waste waters

Recently, the applicability of the anammox process to livestock wastewater has gained interest. First, lab tests have been performed in the USA (Vanotti et al., 2006; Szogi et al., 2007), Korea (Dong and Tollner, 2003; Ahn et al., 2004; Choi et al., 2004), Japan (Yamamoto et al. 2008), and in Northern Europe (Molinuevo et al., 2009). Feasibility of livestock wastewater treatment have been confirmed only applying a dilution ratio or intensive pre-treatments.
Hwang et al. (2005) tested a lab-scale combined SHARON-ANAMMOX process. In the Anammox reactor, nitrogen conversion rate and specific nitrogen removal rate were 0.72 kgN $m^{-3}_{reactor}$ d^{-1} and 0.44 kgN $kgVSS^{-1}$ d^{-1}, respectively at a loading rate of 1.36 kgN $m^{-3}_{reactor}$ d^{-1}.
Karakashev et al. (2008) proposed the PIGMAN concept for the treatment of high strength organic wastes such as piggery manure. The scheme included full-scale anaerobic digestion, centrifuge decantation, UASB post-digestion, partial oxidation to decrease residual COD and convert part of the ammonium to nitrite and finally the OLAND (oxygen-limited autotrophic nitrification–denitrification) process for autotrophic nitrogen removal in one reactor. The proposed treatment train resulted in a removal efficiency for total organic matter, nitrogen and phosphorus of 96%, 88% and 81%, respectively when treating piggery manure. However, more investigations are needed to clarify the economical and environmental sustainability of such a process scheme at pilot-scale and full-scale.
Besides the huge variability in the quality and quantity, a relevant issue when applying the anammox process to piggery manure could be the excess of biodegradable organic carbon, as reported by Molinuevo et al. (2009). They treated the pig manure effluent after UASB-post-digestion and partial oxidation in a granular anammox reactor. After increasing the fraction of digestate blended with synthetic wastewater up to 12% v/v in the influent (corresponding to 242 mg L^{-1} COD) they stopped because denitrification became the dominant process. No details are given about the biodegradable fraction of the influent COD.
Yamamoto et al. (2008) tested a partial nitritation and a subsequent treatment by anammox process (up-flow fixed bed column) to treat swine digestate. The liquid fraction of digestate was

pre-treated by clari-flocculation and diluted. The anammox nitrogen removal rates decreased to 0.22 kgN $m^{-3}_{reactor}$ d^{-1} corresponding to 10-20% of the NRR obtained with a synthetic influent. In a following research, Yamamoto et al. (2011), while treating piggery digestate, obtained a relatively high anammox nitrogen removal rate of 2.0 kgN $m^{-3}_{reactor}$ d^{-1} under a NLR of 2.2 kgN $m^{-3}_{reactor}$ d^{-1}. However, the partial nitritation effluent was filtered and diluted 7-10 times before being fed to the anammox reactor.

Qiao et al. (2010) reported a combined lab-scale partial nitritation reactor and a granular anammox reactor treating the liquid fraction of digested piggery waste. By diluting a minimum of 1.5 times the partially nitrified effluent, the NRR of the anammox reactor reached 3.1 kgN $m^{-3}_{reactor}$ d^{-1} under a NLR of 4.1 kgN $m^{-3}_{reactor}$ d^{-1}.

Relevant issues when treating livestock wastewaters could be the presence of antibiotics or heavy metals. Fernandez et al. (2009) reported a decrease of 75% of the specific anammox activity when 20 mg L^{-1} of cloramphenicol were continuously fed to an anammox SBR. These authors also observed similar effects when 50 mg L^{-1} of tetracycline hydrochloride were continuously fed.

Lotti et al. (2011) made some inhibition batch tests on copper, zinc and antibiotics. Presence of increasing concentrations and prolonged exposure to Copper and Zinc leads to a decreasing specific anammox activity (SAA). Similarly, the inhibiting effect of oxytetracycline and sulfathiazole on the specific anammox activity increased with increasing concentrations and longer exposure time. However, short term exposure results showed a negligible loss of activity after 24 hours exposure at concentrations up to 100 mg L^{-1} of oxytetracycline (IC_{50} = 1100 mg L^{-1}) and sulfathiazole (IC_{50} = 650 mg L^{-1}). After 14 days exposure to 100 mg L^{-1} of oxytetracycline and sulfathiazole, the anammox activity decreased till 75% and 50% relatively to the unexposed culture, respectively. The authors concluded that the inhibitors studied do not represent a real hazard for the application of the anammox process, since a lower specific activity can be counterbalanced by a higher biomass concentration in the reactor.

Most of the references suggest the feasibility of applying the anammox process to piggery wastewaters but, to our knowledge, no published data are available on the treatment of real wastewaters without any dilution and all researches are still at the laboratory scale. Moreover, experimental data are limited to piggery wastewaters and no results are available on the treatment of digestates with mixed composition (piggery manure, poultry manure, energy crops).

1.5 Anammox activity and inhibitions

The most critical aspects in the process scaling-up are related, on the one hand, to the slow growth rate of Anammox bacteria, with a doubling time as long as 14 days and, on the other hand, to their sensitivity to moderate concentrations of their own substrate (nitrite, Strous et al., 1999); these aspects make the process potentially unstable and difficult to start-up. Simple monitoring techniques for the assessment of the specific Anammox activity are therefore needed to regulate the loading pattern thus avoiding potentially dangerous overloading conditions. Available techniques are hereafter discussed.

1.5.1 Anammox activity tests

According to the reaction stoichiometry, the Anammox bacterial activity can be evaluated in batch tests by tracking:

- ammonium, nitrite and nitrate concentrations in time; this is a simple and conventional but time consuming method, requiring manual sampling and analysis;
- the N_2 production rate;
- the acidity requested to maintain the suspension pH at a constant level (pH-stat titration); this is an automatic technique, which however requires a controlling unit for the dosage of the acidic titrant (Remigi, 2001); moreover, the stoichiometric relationship between the alkaline titration rate and the reaction rate is pH dependent (Ficara et al., 2002);
- the heat power generation, measuring the reaction enthalpy (e.g. for nitrification is 359 kJ $mol^{-1}NH_4^+$); very sophisticated instruments (named micro-calorimeters) are required to achieve adequate sensitivities (5-10 mW L^{-1}) because biological reactions produce very low heat power (Aulenta et al., 2002) compared to other chemical reactions (e.g. combustion). Scaglione et al. (2009) applied this technique to track anammox activity.

Regarding the first option, the analysis of ammonium, nitrite and nitrate concentrations in time is the simplest and more direct method giving information also about the $\Delta NO_2^-/\Delta NH_4^+$ and $\Delta NO_3^-/\Delta NH_4^-$ consumption/production.
As for the second option, the N_2 production rate can be easily quantified by monitoring the pressure increase that occurs when the process takes place in closed bottles; this is feasible by using simple manometric devices. Manometric methods have a quite wide spectrum of potential applications since the majority of bioreactions that take place in the liquid phase implies the production/consumption of a poorly soluble gaseous species. Common applications include the heterotrophic aerobic degradation of organic matter (e.g. the assessment of the biological oxygen demand BOD) and anaerobic digestion (e.g. the assessment of biological methane potential, BMP). The applicability of this method to study the Anammox process was already proposed (Dapena-Mora et al., 2007) and its accuracy verified by comparing the N_2 gas production to the nitrogen consumption in the liquid phase (Caffaz et al., 2008). A similar method was also applied to estimate the Anammox decay constant (Scaglione et al., 2009).

1.5.2 Inhibition of anammox activity

Processes depending on slow growing microorganisms suffer from prolonged recovery periods when microorganisms get inhibited and lose activity. In this sense, the Anammox process can be especially prone to this issue due to its sensitivity to various exogenous compounds, such as methanol, NaCl, flocculants, antibiotics (van de Graaf et al., 1996; Dapena-Mora et al., 2007; Isaka et al., 2008). Nevertheless, the adaptation of many Anammox populations to high salinity (Kartal, 2006) and organic carbon concentrations has also been demonstrated (Hu et al., 2010). However, the interesting issue from an engineering point of view is the severe inhibition caused by nitrite,

one of the process substrates serving as the terminal electron acceptor. Therefore, a key point for proper and safe operation of the fully autotrophic nitrogen removal is how to avoid accumulation of nitrite above the threshold level causing severe inhibition.

Unlike inhibition of AOB and NOB, there are indications that the ion itself (NO_2^-) is more toxic to the organisms (Strous, 2000) than nitrous acid (HNO_2) the undissociated form that is most easily transported through the cell wall by passive transport.

The level at which toxicity occurs and its reversibility remains unclear and seems strongly dependent on the exposure time. Nitrite inhibition was detected in a SBR system at concentrations higher than 100 $mgNO_2^-$-N L^{-1} (Strous et al., 1999), and a complete loss of activity was observed at 185 $mgNO_2^-$-N L^{-1} (Egli et al., 2001). Some other studies reported data about the inhibitory effect of ammonia and nitrite both in batch and in continuously fed reactors: Fux et al. (2004) reported serious inhibition at concentration of 30-50 $mgNO_2^-$-N L^{-1} during six days; Jung et al. (2007) reported an activity decrease above 70 $mgNO_2^-$-N L^{-1}. Nitrite inhibition has been considered irreversible (Strous et al. 1999). In a short term batch test, Bettazzi et al. (2010) reported 25% activity decrease with 60 $mgNO_2^-$-N L^{-1} while the 50% inhibition concentration (IC_{50}) was found to be 350 $mgNO_2^-$-N L^{-1} by Dapena Mora (2007). Fernandez et al. (2010), after evaluating the loss of activity in two SBRs with long term continuous feeding of different concentrations of ammonium and nitrite, reported a subsequent restoration of the activity in about one month. Kimura et al. (2010) tested short term and long term inhibition of ammonium and nitrite to anammox entrapped in gel carriers using batch and continuous feeding tests. In the batch experiment, the anammox activity started decreasing at concentrations higher than 274 $mgNO_2^-$-N L^{-1}, while in continuous feeding tests the anammox activity decreased by 10% when the nitrite concentration increased up to 750 $mgNO_2^-$-N L^{-1}. The activity was completely recovered within 3 days by decreasing the influent nitrite concentration. This last finding is in contrast with Strous et al. (1999).

At the moment, it is still not clear whether nitrite exerts a toxic (irreversible) or an inhibitory (reversible) effect and whether the inhibitory (or toxic) level depends on the type of wastewater the Anammox bacteria are exposed to.

2 OBJECTIVES AND OUTLINE

Anaerobic digestion is more and more applied with the double purpose of effectively treating livestock waste-water and as a mean to produce renewable energy as biogas. The liquid fraction of the digested material is very rich in ammoniacal nitrogen and is usually returned to the agricultural soil. However, digestate land disposal may be an issue in intensive-breeding farms that do not have sufficient arable land to comply with the European directive on nitrates, which poses stringent limits on nitrogen discharge in order to protect groundwater from nitrate pollution.
This issue has prompted much attention to novel processes performing nitrogen removal which should be much cheaper than conventional biological processes in order to make nitrogen removal at farm level economically sustainable.

This thesis is part of a bigger project, named BRAIN (Biotechnologies for nitrogen reduction from digestates by applying innovative processes and for promoting the economical and environmental sustainability of biogas production), founded by Italian Ministery for Agriculture. The aim of this project is to explore the feasibility to remove nitrogen from agricultural digestates by applying advanced biological processes.
In the BRAIN project, two processes have been considered to treat the liquid fraction of agricultural digestate:
- the nitritation-denitritation process (hereafter named DENO2): a partial nitrification up to nitrite followed by nitrite heterotrophic denitrification. This process allows to reduce 25% the oxygen demand and 40% the external carbon if compared to conventional nitrification/denitrification process. The difficulty in applying nitrogen removal via nitrite lies in retaining the ammonia oxidizing bacteria while eliminating the nitrite oxidizing bacteria.
- the completely autotrophic process (partial nitritation and anammox), performed in two separate reactors. If compared to the conventional nitrification/denitrification process, the complete autotrophic process allows to reduce the oxygen demand to one half, the sludge production to one tenth and the need of external carbon to zero. The choice of using two reactors instead of the all-in-one option is due to the high variability of the influent quality especially in terms of BOD/N ratio. So the idea behind the use of the two reactors configuration was to make sure that all biodegradable carbon was removed in the first reactor prior to feeding the anammox reactor.
This thesis deals with the DENO2 process and with the anammox process, while the partial nitritation is not part of this work.

The main challenges in the application of biological processes to these type of wastewaters concern the wide variability in the characteristics of agricultural digestate associated to the seasonality of the digested matrixes and the occasional or permanent occurrence of inhibitors such as recalcitrant organics (antibiotics, humic and fulvic acids) or heavy metals.

The specific objectives of these studies were to evaluate:
-the technical feasibility, in terms of process stability under variable influent characteristics, of the DENO2 process to treat the liquid fraction of agricultural digestate;

-the feasibility to enrich anammox biomass from different conventional sludges within a reasonable time-frame using a simple fed-batch procedure or a sequencing batch reactor;
-the stability of the anammox process when treating agricultural digestate after solid/liquid separation and aerobic pre-treatment at different dilution levels as a first step toward the assessment of its technical applicability;
-the reversibility of nitrite inhibition of anammox granular biomass in two reactors treating synthetic and real wastewater.

In this thesis, a pilot scale study on the application of the DENO2 process and three different lab-scale studies on the anammox process are presented. These experimental campaigns are presented in the following chapters as summarized below.

Chapter 4: This chapter is dedicated to the assessment of the applicapility of the DENO2 process to treat the liquid fraction of agricultural digestate. Results of a 205-days experimentation on a 800L-SBR performing the DENO2 process are presented. The pilot plant was located at a piggery farm and treated the liquid fraction of the agricultural digestate produced by a full-scale digester. The digester was fed on a mixture of piggery primary sludge, poultry manure and variable amounts of energy crops (maize or wheat). The influent and the operational paramaters of the SBR varied during the course of the experimentation, therefore the overall experimentation was divided into different experimental periods. For each period, the effluent quality and process efficiency are presented. Batch respirometric tests allowed to study the specific nitrification activity under different conditions: the oxygen limitation phenomenon was studied and free ammonia (FA) and free nitrous acid (FNA) inhibition functions were assessed. Preliminary results on N_2O emission and on microbiological analyses are also presented.

Chapter 5: This chapter deals with the enrichment of anammox biomass from conventional sludges. First, the efficacy of a simple, low-tech fed-batch procedure of anammox enrichment coupled with activity measures is evaluated. Results from an experimental campaign on 6 sludge samples collected from Italian wastewater treatment plants are here presented. Three of the samples were later mixed and used to inoculate a SBR reactor fed with synthetic wastewater for further enrichment.

Chapter 6: In this chapter, an experimental study run on a 7L SBR with granular anammox biomass is presented. During the first period, the reactor was fed with a synthetic wastewater; then, increasing fractions (from 10% up to 100% v/v) of real wastewater were blended to the synthetic one. The real wastewater was the effluent of the DENO2 process described in chapter 4 with ammonium and nitrite salts added to achieve the desired nitrogen load. Besides conventional monitoring parameters, results from FISH analyses and activity tests are also presented.

Chapter 7: The results presented in this chapter were obtained during my stay at the LEQUIA laboratory of the University of Girona (Spain). The aim of this work was the evaluation of the inhibition extent at various nitrite concentrations on two granular anammox biomasses, started

from the same inoculum, and cultivated on a synthetic medium (SBR-1) and on urban landfill leachate (SBR-2). In particular, we were interested in evaluating whether the inhibitory response to high nitrite levels was affected by the nature of the wastewater that the Anammox bacteria were used to treat. Moreover, the occurrence and relevance of the following activity recovery was also investigated on both Anammox biomasses.

3 MATERIALS AND METHODS

In this chapter, pilot-scale and the lab-scale reactors used during the various experimentations presented in this thesis are described. Moreover, the measuring principle, the set-up and equipments used to perform biological activity tests on nitrifiers and anammox biomass are also presented, although details related to specific procedures used according to the aim of each activity test are described within each dedicated chapter. Analytical and microbiological methods and equipments are finally outlined, with the exception of those carried out at the LEQUIA laboratories, which are detailed in chapter 7.

3.1 Reactors

3.1.1 Pilot scale nitritation-denitritation SBR

The pilot scale SBR was located at a piggery farm (20000 pigs) in the Lombardy region which produces from a minimum of 150 to a maximum of 400 m^3 d^{-1} of piggery slurry, according to the number and age of the breeding pigs.

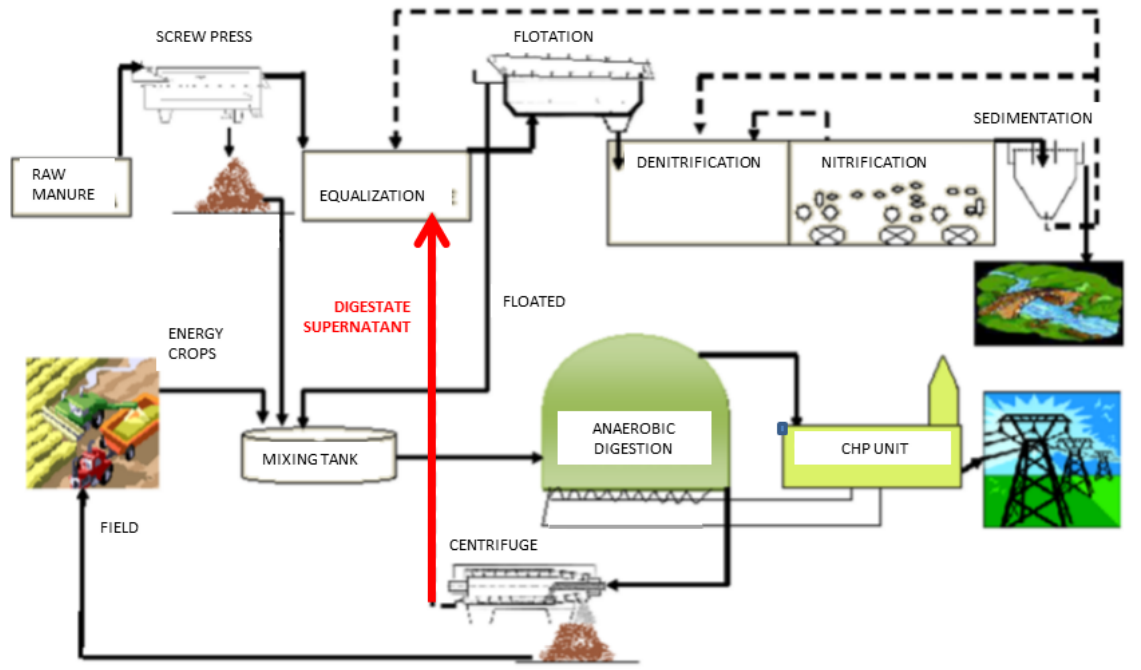


Figure 3.1 – Simplified flows scheme of the manure treatment plant

After a preliminary rough solid/liquid separation (screw-press), the semi-liquid fraction of the piggery slurry is sent to a conventional wastewater treatment plant (WWTP). After floatation, the clarified water is sent to an activated sludge process which provides biological nitrogen removal by conventional heterotrophic pre-denitrification and autotrophic nitrification. The floated primary sludge is sent to two anaerobic digesters (total volume 2600 m^3), and the digestate is centrifuged.

The liquid fraction is recycled back to the biological section of the WWTP, while the solid fraction is used for land spreading (Figure 3.1).

Figure 3.2 – Location of the pilot plant in the piggery-farm area

The pilot plant treated part of the digestate supernatant after centrifugation. Figure 3.2 shows the location of the pilot plant, while
Figure 3.3 depicts the plant and reports a schematics. The pilot plant is made of a buffer tank divided into 2 connected sections with a total volume of 1.8 m^3 and an SBR with a maximum volume of 0.8 m^3. During the experimentation, the maximum working volume was 0.65 m^3. Two adjustable-flow dosing pumps for bicarbonate and sodium acetate addition were installed and allowed to adjust pH during nitrification and to provide readily biodegradable COD during post-denitrification.
The sensors in the SBR are Temperature, Dissolved Oxygen (DO), Oxidation Reduction Potential (ORP) and pH. All signals have been monitored and recorded. Remote monitoring was possible via an internet connection.

(a)

(b)

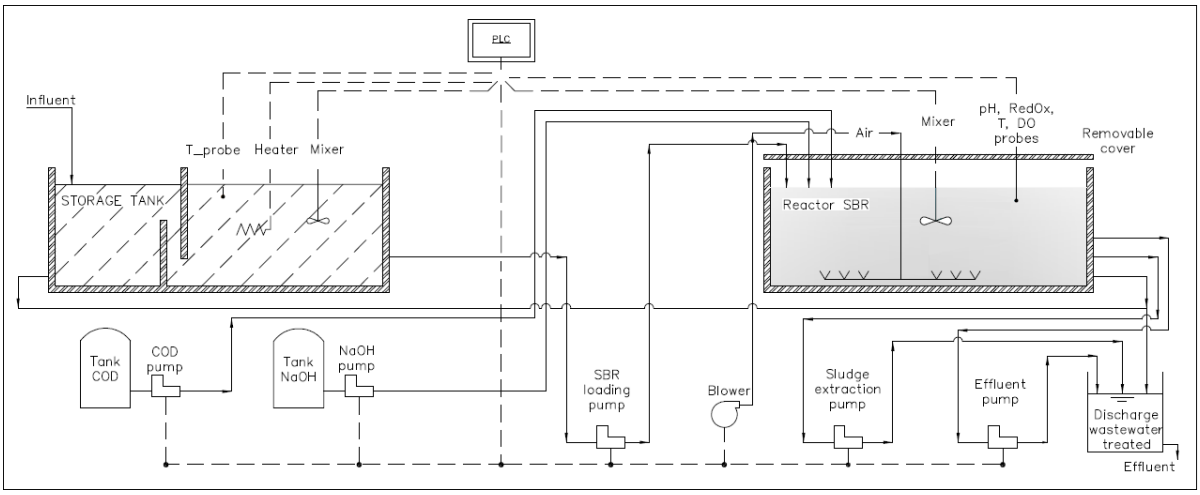


Figure 3.3 – Picture (a) and scheme (b) of the pilot plant

3.1.2 SBR lab-scale reactor

The reactor supplied by Diachrom (Mendrisio, CH) is made by a control unit (main body and control panel), a fermenter (glass reactor) and a computer connected to the control unit via a USB port. The functional diagram in Figure 2 shows the flows into and out of the fermenter, all controlled by the controller.

The different parts of the SBR are:

- 7L glass reactor (fermenter) closed by a flanged stainless steel top plate with gas-tight openings for probes and sampling ports.
- monitoring probes (pH, O_2, temperature),
- feeding, withdrawal, and sampling ports;
- vertical mechanical mixing at variable speed (100-1000 rpm);
- bottom heating plate and cooling water circulation system controlled by the control unit;
- 4 peristaltic pumps with variable flow (range 1-80 ml/ min) used for acid or alkaline dosage, influent feeding and effluent discharge.

The control unit allows to implement the following functions:

- To switch on/off the feeding and discharge pumps;
- To regulate the mixing level;
- To set the operating parameters (pH range, and temperature);
- the acquisition of the probes signals for their storage and transmission to the PC connected to the control system;
- the definition, recording and saving of the cycle phases.

The reactor headspace is connected to a N_2 (or mix 95% N_2/5% CO_2) gas cylinder and is equipped with a backpressure valve to keep the pressure at 1.1 bar in the reactor. Pressure was monitored

throughout the experimentation by a pressure transducer installed on the reactor head (Leo Record Pressure Data Logger, SensorONE Ltd, UK).

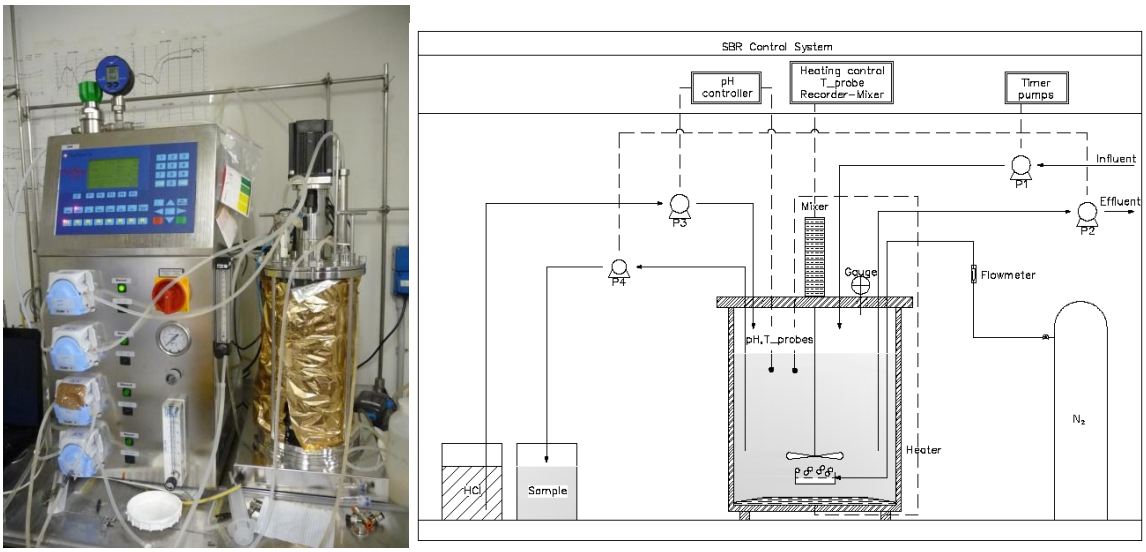


Figure 3.4 - Lab-scale reactor picture (left) and functional scheme (right)

3.2 Activity batch tests

3.2.1 Automatic pH/DO-stat titration to assess nitrification kinetics

Nitratation and nitritation rates were determined by applying the pH/DO-stat titration technique (Ficara et al., 2000; Artiga et al., 2005). The measuring principle of set-point titration is the following: controlled amounts of an appropriate titrant are added to a sludge sample to maintain constant the level of a chemical species which takes part in the bioreaction under study. The reaction rate is proportional to the measured titration rate via the reaction stoichiometry, while the amount of titrant dosed is proportional to the amount of substrate converted.

When dealing with nitrification, dissolved oxygen-stat (DO-stat) and pH-stat titration are especially useful. The reason is twofold: (i) the level of DO and pH are carefully controlled to the desired level and (ii) the reaction rates are easily calculated from their stoichiometric relation with titration rates. Specifically, the NaOH addition rate (r_{NaOH} in mmol/min) is used to assess the ammonium oxidation rate (r_{NH} in mgN/min) by remembering the ratio between ammonium oxidation and alkalinity consumption, that is, according to the two-step nitrification model:

$$r_{NH} = r_{NaOH} \cdot 14 \cdot \frac{i_{XB} + \frac{1}{Y_{AOB}}}{i_{XB} + \frac{2}{Y_{AOB}}},$$

where, following the IWA-ASM (Henze et al., 1987) usual notation:

i_{XB} = fraction of N in bacterial cells (0.086);

Y_{AOB} = growth yield coefficient for AOB (0.21).

Similarly, nitrite oxidation rate (r_{NO2}) is assessed from O_2 addition rate (r_O), from the respirometric ratio:

$$r_{NO2} = r_O \cdot \frac{\frac{1}{Y_{NOB}}}{\frac{1.14 - Y_{NOB}}{Y_{NOB}}} = r_O \cdot \frac{1}{1.14 - Y_{NOB}},$$

where:

Y_{NOB} = growth yield coefficient for NOB (0.06).

The specific ammonium oxidation rate and nitrite oxidation rate of the sludge (r_{AOB}, r_{NOB}, in mgN $gVSS^{-1}$ h^{-1}), are calculated by taking into account the biomass content of the sludge sample in terms of volatile suspended solid (VSS, in gVSS):

$$r_{AOB} = \frac{r_{NH} \cdot 60}{VSS} \qquad r_{NOB} = \frac{r_{NO2} \cdot 60}{VSS}$$

Tests were performed at constant temperature by means of a thermostated bath. Sodium hydroxide (0.05 M) and hydrogen peroxide (0.1 M) were used as titrants.

Similar titration tests for the study of nitrification were already described and validated (e.g. Ficara et al. 2000 and Artiga et al. 2005 for pH/DO-stat titration).

Titration tests were performed by the MARTINA authomated titration system provided by SPES s.c.p.a (Fabriano, AN, Italy).

The same instrument was also used to perform conventional respirometric tests.

3.2.2 Manometric batch test to assess specific anammox activity

To assess the specific anammox activity, batch tests were performed in gas-tight bottles in which the bioprocess was monitored by tracking the overpressure growth. This manometric method is based on the principle that the rate of a bioprocess that consumes/produces a poorly soluble gaseous component is proportional to the rate of decrease/increase in pressure. As for the anaerobic ammonium oxidation, the N_2 production can be assessed by applying the ideal gas law to pressure data collected during a batch test. This measuring principle was already proven to be applicable and advantageous in the monitoring of anaerobic ammonia oxidation (Dapena Mora et. al, 2007; Scaglione et al. 2009; Bettazzi et al., 2010).

From the overpressure data during time, the moles of nitrogen produced $n_{N2}(t)$ can be calculated by using the ideal gas law:

$$n_{N_2}(t) = \frac{P(t) \cdot V_{HS}}{R \cdot T}$$

being V_{HS} the volume of the gaseous phase (L), R the ideal gas coefficient (atm L mol^{-1} K^{-1}) and T the temperature (K) and P(t) the overpressure (atm).
The corresponding produced normal volume of gas can be easily estimated knowing that 1 gas mol = 22.4 L at standard conditions (T=0°C and P=1atm).
For each test, the maximum or average N_2 production rate dN_2/dt (molN h^{-1}) can be estimated from the maximum or average slope of the cumulated nitrogen production curve.
The maximum specific Anammox activity, SAA_{max} (gN_2-N $gVSS^{-1}$ d^{-1}) can also be assessed by referring the nitrogen production rate to the amount of biomass in the bottle:

$$SAA = \frac{dN_2/dt}{gVSS} \cdot \frac{28gN}{molN_2} \cdot \frac{24h}{d}$$

In the experimentations performed in this work, manometric determinations of the N_2 production rate were performed by means of two different experimental set-up, depending on the equipments that were available in the laboratory were these tests were carried out. Hereafter, the equipment used in the experimentations described in chapters 4 and 5 that were performed at Politecnico di Milano are presented. The equipment used in the experimentation carried out at the LEQUIA laboratory are described in chapter 7.

3.2.3 Manometric tests set-up

Manometric measures were performed by using the OxiTop® Control system (WTW, DE) (Figure 3.5). This is a convenient manometric device consisting of a pressure transducer and data logger located inside a measuring head that allows for an automated and frequent pressure-data acquisition. The measuring head is mounted onto the main opening of a glass bottle of 1140 ml in volume.

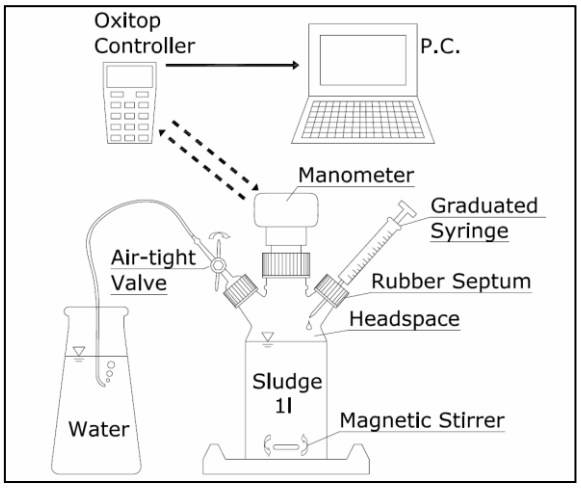


Figure 3.5 - Set-up of the manometric equipment for the assessment of the N_2 production rate

The bottle is featured with two lateral openings; one is sealed by a rubber septum and is used for nitrogenous substrate injections; the second opening was modified from the original WTW set-up by equipping it with a teflon airtight valve that was used for biogas discharge into a water bath for effective de-oxygenation of the bottle headspace.
Manometric determinations of the anammox activity were performed according to the following procedure. Each bottle was filled with anammox biomass and diluted with mineral medium (or wastewater) to reach the working volume of 800-1000 mL. The pH was controlled at 7.6 with HCl addiction. The headspace was flushed with N_2 gas to ensure anaerobic conditions. Bottles were located in a thermostated chamber at 35±0.5°C and agitated by a magnetic mixer. After an initial phase of headspace pressure stabilization (temperature and vapor pressure stabilization), substrates were added by spike injections through the rubber septum. Substrates were injected by using NH_4Cl, $NaNO_2$ and KNO_3 (10 gN L^{-1}) concentrated solutions. The NO_2^--N/NH_4^+-N ratio in the injection was around 1 in all tests, in order to work under ammonium non-limiting conditions.

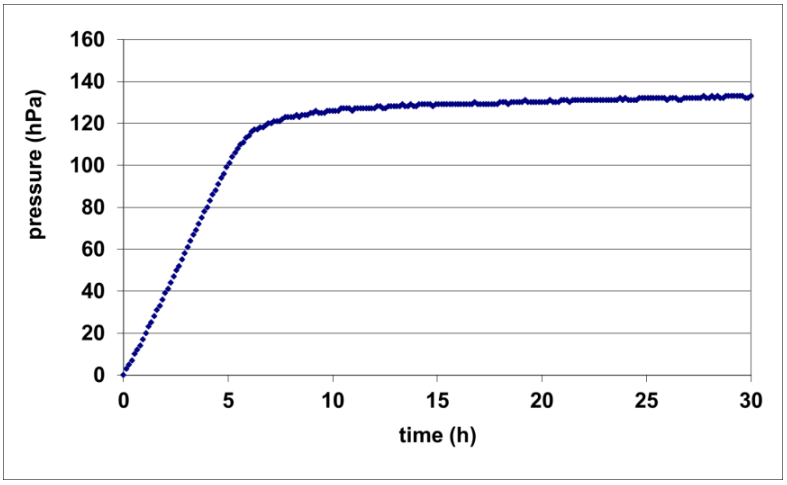


Figure 3.6 - Output of a typical manometric tests on Anammox biomass

The accuracy of the method was previously evaluated by running several sets of tests. By comparing the expected stoichiometric N_2 production with that one estimated from pressure data, an average relative error of 4.5±3.3% was assessed (Caffaz et al., 2008). The same method was previously used to determine the anammox decay coefficient (Scaglione et al., 2009).

3.3 Microbiological analyses

Samples for microbiological analyses were taken from the reactors described in chapters 3, 4 and 5. Around 12 mL of biomass from each reactor were fixed with paraformaldehyde and hybridization with fluorescent probes was performed as described by Daims et al. (2005). Details about the probes used in this study are reported in Table 3.1. In addition, samples were

counterstained with 4',6-diamidino-2-phenylindole (DAPI) as control. FISH images were taken with an optical microscope Axioskop 40 (Zeiss, DE).

Table 3.1 – Characteristics of FISH probes

Probe	Target	Stringency	Flurochrome	Reference
Eub338I + Eub338II + Eub338III	most Eubacteria	0-50	fluorescein	Amann et al., 1990 Daims et al., 1999
Amx1900	most anammox bacteria (23S rRNA)	30	Cy3	Schmid et al., 2001
Amx820	most anammox bacteria (16S rRNA)	40	Cy3	Schmid et al., 2001
Apr820	Cand. "Anammoxoglobus propionicus"	40	Cy3	Kartal et al., 2007
Kst157	Cand. "Kuenenia stuttgartiensis"	25	Cy3	Schmid et al., 2001
Ban162	Cand. "Brocadia anammoxidans"	40	Cy3	Schmid et al., 2001
Nso1225	AOB β-Proteobacteria	35	Cy3	Mobarry et al., 1996
Nso190	AOB β-Proteobacteria	55	Cy3	Mobarry et al., 1996
Nsm156	genus Nitrosomonas	5	Cy3	Mobarry et al., 1996
Nsv443	genus Nitrosospira	30	Cy3	Mobarry et al., 1996
NIT3	genus Nitrobacter (NOB)	40	Cy3	Wagner et al., 1996
Ntspa662	genus Nitrospira (NOB)	35	Cy3	Daims et al., 2001

3.4 Analytical methods

Commercial photochemical test kits (Hach Lange GmbH, Dusseldorf, Germany, Test LCK303, LCK304, LCK339, LCK340, LCK341, LCK342; LCK514; LCK314 spectrophotometer type LANGE Xion500) were used for ammonium, nitrite, nitrate and COD measurements. Ammonium, nitrite and nitrate as well as soluble COD were measured after 0.45 μm filtration.

Total Kjeldahl Nitrogen (TKN), Total suspended solids (TSS), Volatile suspended solids (VSS), Sludge Volumetric Index (SVI) and BOD were all measured according to the APHA Standard Methods for the Examination of Water and Wastewater (APHA, 2005).

Alkalinity was measured by potentiometric titration to end point pH according to APHA Standard Methods for the Examination of Water and Wastewater (APHA, 2005). Metals analyses were measured by Inductively Coupled Plasma –Mass Spectrometry (ICP-MS) with a ICPMS model 7700X (Agilent Technologies, USA) according to the US-EPA method 200.8 EMMC version revision 5.4 (1994).

Conductivity was measured with multimeter dual 3420 (WTW, DE).

3.5 N_2O emissions measuring campaign

Preliminary N_2O emissions analyses were performed on the pilot SBR. The SBR was equipped with a removable cover featured with a sampling port. The sampling port was connected to a 1L Cali-5-Bond™ gas- bag. For each test, 5 grab samples were taken during the first hour of aeration.

Figure 3.7 – Picture of set-up for the off-gas N_2O sampling

N_2O concentrations were analysed with FTIR spectroscopy and micro gaschromatography. The FTIR spectrometer used was a MultiGas™ 2030 FTIR Continuous Gas Analyzer (MKS, USA).
The microgaschromatograph used was a MICROGC 3000 A (Agilent, USA) equipped with PLOT Q column, (8mt x 0,32mm). Column temperature and pressure during the measurement were 60 °C and 15 psi respectively.

4 NITRITATION / DENITRITATION PROCESS (DENO2) TO TREAT THE LIQUID FRACTION OF AGRICULTURAL DIGESTATE

The main objective of this part is the evaluation of the feasibility of the nitritation-denitritation biological process (short-cut nitrification-denitrification, here defined as "DENO2 process") to remove nitrogen from the liquid fraction of the digestate.
Results on a pilot scale experimentation are presented. Details on the pilot SBR, located in a piggery farm, are reported in par 3.1.1.

4.1 Influent characteristics

The characteristics of the influent to the SBR have been quite variable during the course of the experimentation depending on the management of both the piggery farm and the digesters.
Table 4.1 summarizes the main characteristics of the influent during the experimentation, which spanned over 205 days.

Table 4.1 – Influent characteristics during the experiemtation

Parameter		min-max	Mean± ST. DEV	Number of data
pH	-	8.2-7.8	8.0±0.1	36
Conductivity	mS cm^{-1}	10.1-15.7	14.2±2.1	6
NH_4^+-N/ TKN	%	87-97	92±5	4
NH_4^+-N	mgN L^{-1}	619-1616	1151±251	50
COD	mg L^{-1}	1325-7500	2634±1178	50
COD/N	gCOD $gTKN^{-1}$	0.9-6.3	2.2±1.2	50
BOD_5/COD	-	0.34-0.57	0.44±0.1	5
BOD_{20}/COD	-	0.44-0-62	0.54±0.07	4
solubleCOD/COD	-	0.41-0.77	0.60±0.17	4
TSS	gTSS L^{-1}	0.09-1.15	0.43±0.22	37
Alkalinity	mg$CaCO_3$ L^{-1}	4400-14300	7128±2932	17

The high variability of the influent is mainly due to:

- seasonal variation in the livestock management (quantity and age of the pigs present in the farm);
- different co-substrates fed to the digester mixed with the piggery wastes (maize silage, or poultry manure, Table 4.2);
- inefficient management of the digester in some periods (short SRT and temperature variation);
- variation in the efficiency of the solid/liquid separation system applied to the digestate.

The influent is characterized by a moderate solid content (0.43±0.22 gTSS L^{-1}) confirming a good average efficiency of solid separation by the centrifuge.

Alkalinity was high (7128±2932mg$CaCO_3$ L^{-1}) and the influent pH remained around 8, so that, during the experimentation, pH in the reactor never dropped below 7.5. The alkalinity/NH_4^+-N ratio varied between 0.8 and 2 (mol Alk/mol N), thus it was not necessary to add bicarbonate to complete the DENO2 process.

Salinity was quite high, though not extreme, as conductivity ranged between 10.1 and 15.7 mS cm^{-1}.

The BOD_5/COD ratio was relatively high: this was mainly related to the way the digesters were managed, as their hydraulic retention time did not allow complete methanization of the biodegradable organic carbon of the substrates.

Table 4.2 - Fractions of the different substrates fed to the digester during the experimentation (% in terms of volatile solids)

	min-max (%)	Mean± ST. DEV (%)
Piggery manure	32-100	60±12
Maize	0-51	15±16
Wheat	0-36	7±14
Poultry manure	0-44	15±11

Concentrations of ammonium and COD in the influent during the experimentation are reported in Figure 4.1.

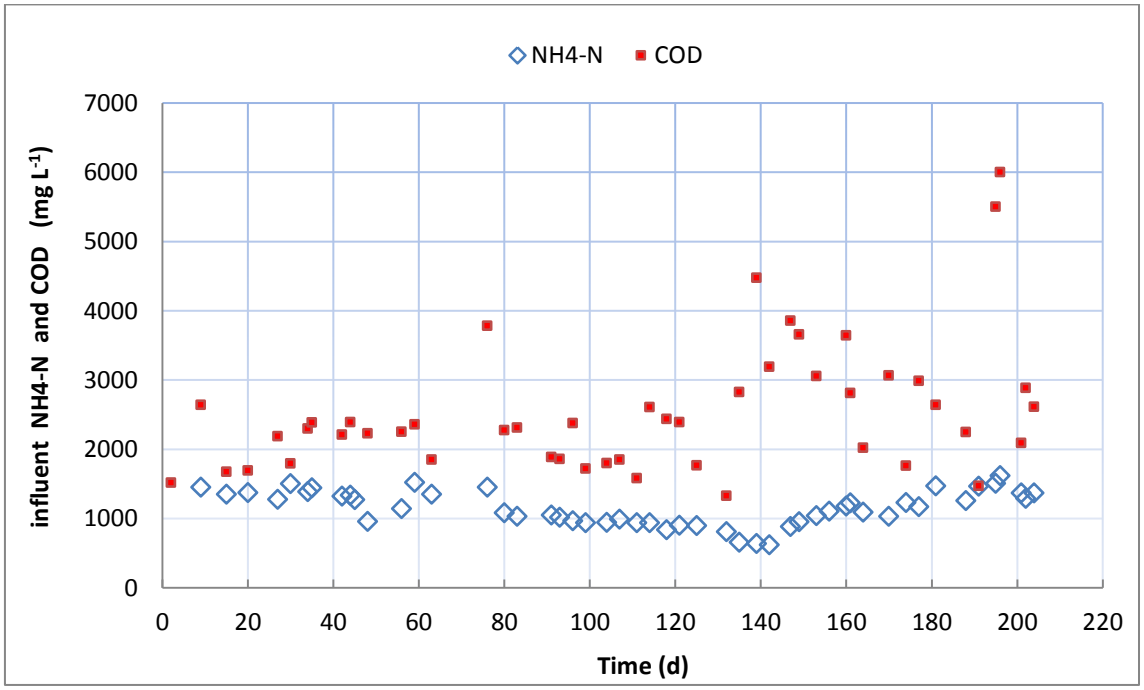


Figure 4.1 - Ammonium and COD in the digester supernatant fed to the SBR

The increase in the organic carbon content especially after day 130 caused a subsequent increase in the COD/N ratio from 1-2 to up 5-6 (Figure 4.2). The peaks over 4 gCOD/gN in the influent are related to problems in the digesters that caused an incomplete digestion.

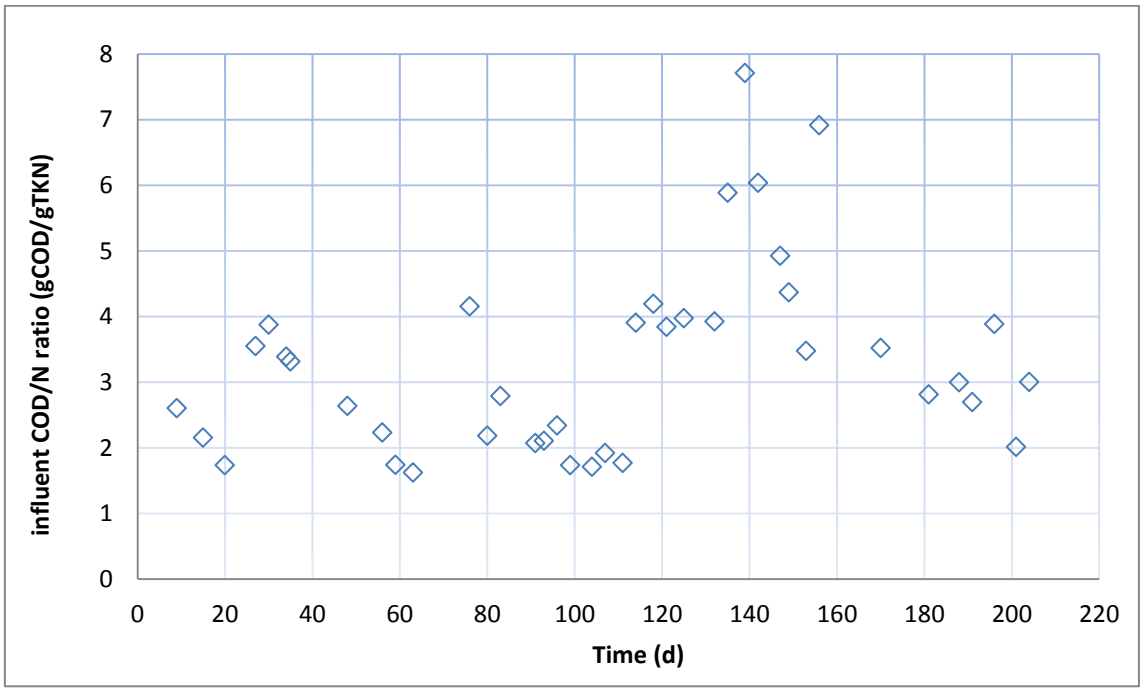


Figure 4.2 - COD/N ratio in the influent fed to the SBR during the experimentation

4.2 Plant operation

4.2.1 Start-up

The pilot plant was inoculated with 200 L of concentrated activated sludge (6 gTSS L^{-1}) taken from the recycle stream of the main WWTP. The concentrated sludge was diluted with 400 L of the digester supernatant so that the initial TSS concentration in the SBR was 2 gTSS L^{-1}.
In Table 4.3, the main operating parameters of the SBR during start-up are summarized.

Table 4.3 – Main SBR operating parameters of the SBR during start-up

Parameter	Range/Mean value
SBR working volume min-max	600 – 680 L
Temperature	30 ± 2°C
pH (min – max)	7.5 – 8.5
Dissolved Oxygen (aeration phase)	0.5 mgO_2 L^{-1}

Then, the plant was run in batch mode for 2 days under continuous aeration, to promote nitrification. After nitrification was established, cycles were started. The SBR has been managed with cycles of 6 hours with initial HRT of 3d, NLR of 0.4 gN $L_{react}^{-1}d^{-1}$ and SRT 5d. The lengths of the different phases varied during the various experimental periods (see paragraph 4.3 below for further details).

4.2.2 Process monitoring and calculations

Influent and effluent N compounds, COD and solids, MLVSS and MLTSS as well as intra-cycle analyses have been performed on a weekly bases.
Periodically, respirometric tests by pH/DO-stat titration (see paragraph 3.2.1) were performed to estimate nitrification specific activity. Dedicated tests allowed to assess the FA and FNA inhibition models, and the oxygen half saturations constant for heterotrophs and ammonium oxidisers.
Analyses of the off-gas were performed during the aeration phase (5 samples every 10-15 min) in two cycles.
Every 15-20 days, samples from the reactor have been collected, fixed and hybridized with the method reported in par. 3.3 to perform FISH analyses to track ammonium oxidisers and nitrite oxidisers abundance.
Nitrogen loading rates (NLR) and Nitrogen removal rates are calculated as:

$$NLR = \frac{NH_{4\,in} \times V_d}{V_{react}}$$

$$NRR = \frac{NH_{4\,in} \times V_d - (NH_{4\,out} + NO_{2\,out} + NO_{3\,out}) \times V_d}{V_{react}}$$

where:

NH_{4in}, NH_{4out}, NO_{2out} , NO_{3out} are the soluble nitrogen species concentrations (gN L^{-1}) in the influent and in the effluent,

V_d = volume of supernatant treated daily (L),

V_{react}=maximum reactor volume.

Nitrification and denitrification efficiencies are calculated making the N mass balance as:

$$\%NIT = \frac{N_{nit}}{N_{in}} = \frac{TKN_{\,in} - N_{solids\,out} - TKN_{\,out}}{TKN_{\,in}}$$

$$\%DEN = \frac{N_{den}}{N_{nit}} = \frac{N_{nit} - NO_{x\,out}}{N_{nit}} = \frac{N_{nit} - NO_{2\,out} - NO_{3\,out}}{N_{nit}}$$

where:

$TKN_{\,in}$ = TKN loaded (gN d^{-1})

$N_{solids\ out}$ = N (gN d^{-1}) in the solids extracted (with the effluent and with sludge wastage) assuming 12% N content in the VSS (gN d^{-1})

TKN_{out} =TKN discharged (gN d^{-1})

$NO_{2\ out}$ = nitrite discharged (gN d^{-1})

$NO_{3\ out}$ = nitrate discarged (gN d^{-1})

Nnit = $TKN_{in} - N_{solids\ out} - TKN_{out}$

Nden =$N_{nit} - NO_{x\ out}$

4.3 Experimental periods

The experimentation was divided into six different periods characterized either by different influent characteristics (substrates fed to the digester) or different operating parameters (HRT, SRT or temperature) as summarized in table 4.4.

The main differences in the operating parameters were the following:

- low SRT (3-5 d) in the first two period and high SRT (20-30 d) in periods 3 to 6;
- process temperature 30°C during the first 5 periods and decreased to 25°C in period 6;
- dissolved oxygen concentration during aeration increased at 0.75-1.0 mgO_2 L^{-1} during period 5 and 6.

HRT was maintained between 2-3 days, decreased to 4 days during period 2 and 3.Influent characteristics during the periods varied considerably as already discussed, mainly in terms of COD load and N load. As a consequence the COD/N ratios varied from 1.3±0.3 of period 1 up to 3.8±1.6 in period 5.

The substrates fed to the anaerobic digesters varied also during the experimentation. In Table 4.5 the fractions (as volatile solid) of co-digested substrates in the different periods are summarized. Piggery manure accounted for the 80% of the influent volatile solids only during period 1, while in the following periods it remained between 50% and 60% on average. Period 2, 5 and 6 are characterized by higher poultry manure contribution. This is just an indication because the dilution effect due to the digester HRT (that was averagely between 19 and 21d) has to be taken into account.

Table 4.4 – Influent characteristics and operational parameters during each experimental period

Parameter	Period 1 I start-up	Period 2 Influent variation	Period 3 II start up	Period 4 Steady state	Period 5 Influent variation	Period 6 Lower temperature
Experimental days	0-28	29-78	79-105	106-134	135-168	169-204
T (°C)	30	30	30	30	30	25
DO (mgO_2 L^{-1})	0.5	0.5	0.5	0.5	0.75-1-0	0.75-1.0
influent flow rate (L d^{-1})	248±64	175±38	195±71	320±0	320±0	271±41
NLR (gN $L_{react}^{-1}d^{-1}$)	0.50±0.12	0.34±0.06	0.31±0.08	0.44±0.03	0.44±0.10	0.52±0.04
HRT (d)	2-3	3-4	2-4	2	2	2-3
SRT (d)	4-5	3-4	20-40	20-25	25-30	18-25
influent NH_4^+ (mgN L^{-1})	1361±72	1333±165	1003±55	900±63	937±230	1342±168
influent COD (mgCOD L^{-1})	1940±464	2354±543	2031±277	1993±486	3281±1495	3023±1443
influent COD/N (gCOD $gTKN^{-1}$)	1.3±0.3	1.6±0.4	1.8±0.2	2.0±0.5	3.8±1.6	2.1±0.9
influent TSS (g L^{-1})	0.35±0.19	0.57±0.56	0.4±0.13	0.52±0.34	0.52±0.10	0.3±0.12
Acetate added (mgCOD L^{-1})*	1567±750	1572±1276	392±352	1246±738	1012±157	1567±750

(*) calculated as the mass of external COD loaded per day/influent flow rate

Table 4.5 – Substrates fed to the anaerobic digesters during the different periods as % of the influent volatile solids

Substrates	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6
Piggery manure (%VS)	77±8	51±10	62±11	57±7	59±4	60±2
Poultry manure (%VS)	13±4	24±9	-	3±7	23±2	22±1
Energy crops* (%VS)	10±5	25±14	38±11	40±8	18±4	18±2

*includes the contribution of maize and wheat

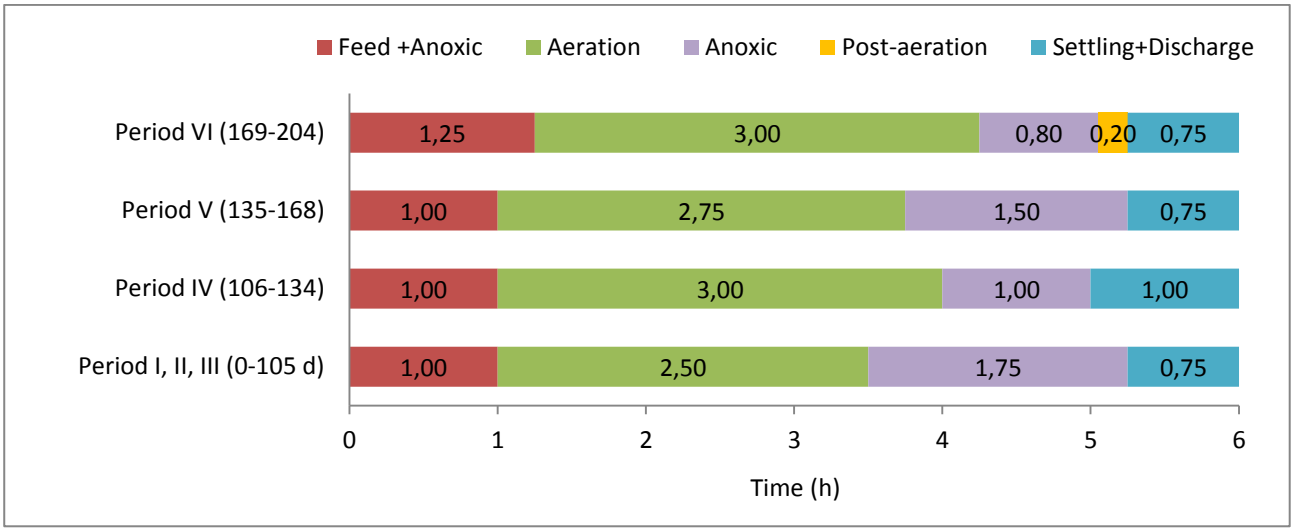


Figure 4.3 – Scheme of the cycle phases during the different experimental periods

During the entire experimentation, the cycle began with an anoxic phase during which filling took place, too. In this way, nitrate and nitrite remaining from the previous cycle were partially or totally removed, proportionally to the biodegradable carbon content in the influent. After the initial anoxic phase, an aeration phase took place for 2.5 to 3 hours, depending on the experimental period. DO concentration was kept around 0.5 $mgO_2\ L^{-1}$ and increased at 0.75-1.0 $mgO_2\ L^{-1}$ in periods 5 and 6 (manually controlled). Then, a further anoxic phase took place, to perform post-denitrification by adding external carbon (sodium acetate). In the last experimental period, a 10-minutes post-aeration phase was also included to release nitrogen bubbles trapped in the sludge flocs and avoid sludge floatation. Finally, the settling phase took place for 30 minutes, followed by effluent discharge. In Figure 4.3 the scheme of the cycle phases during the different experimental periods are reported.

4.4 Results

4.4.1 Solid concentrations

Total solid concentrations in the influent and in the effluent are reported in Figure 4.4, while in Figure 4.5 mixed liquor suspended solids are shown.

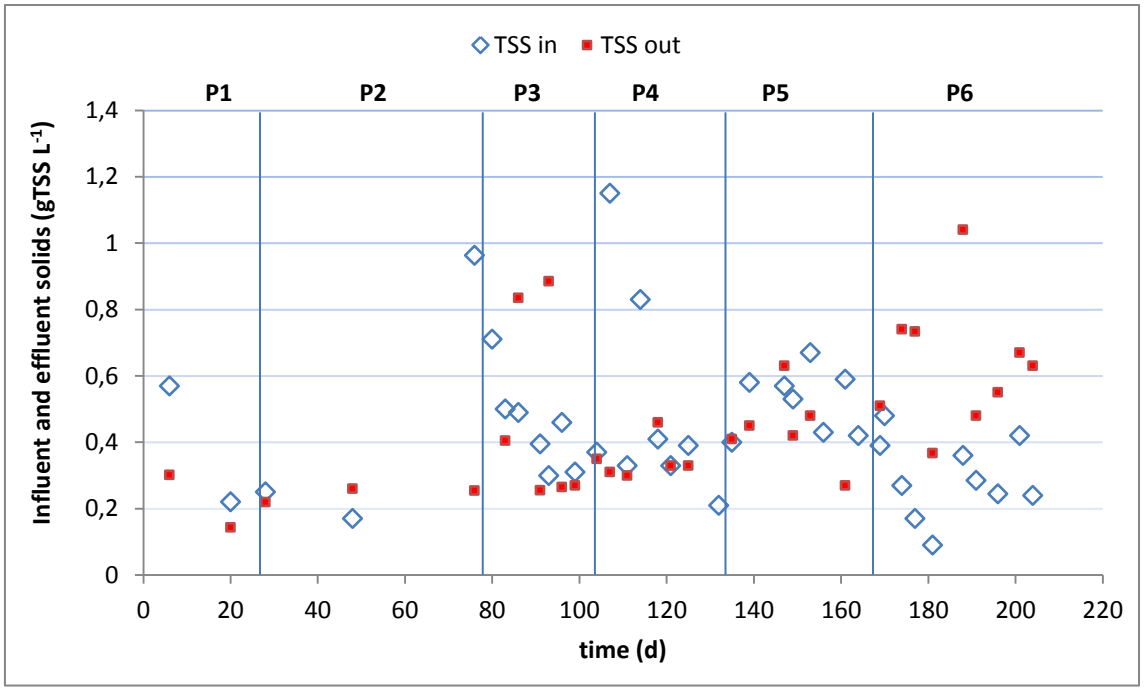


Figure 4.4 - Total suspended solid concentrations in the influent and in the effluent during the experimentation

Influent solid concentrations varied remarkably during the whole experimentation due to the variability in the centrifuge solid/liquid separation efficiency, with occasional very high values, such as those experienced during periods 3 and 4.

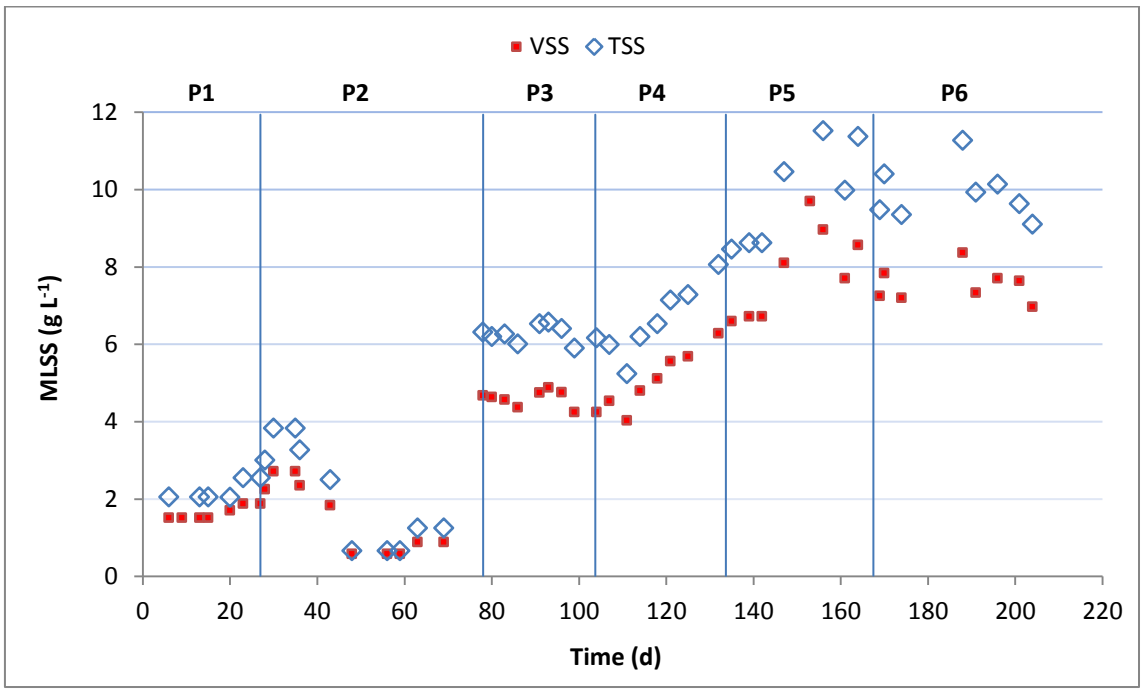


Figure 4.5 - Total and volatile suspended solid concentrations in the reactor during the experimentation

Nevertheless, the average TSS influent concentration remained almost constant around 0.4 gTSS L^{-1} during the different periods. As for the effluent solids, they varied around 0.3 gTSS L^{-1} during periods 1-4, while they increased slightly during period 5 and 6 because of the higher biomass concentration in the reactor. Occasional higher values are possibly due to sludge flotation during the settling phase caused by the release of N_2 gas bubbles produced during the post-denitrification phase.

The Sludge Volume Index (SVI) has been low during all the experimentation ranging between 50 and 80 mL $gTSS^{-1}$, confirming good settling properties of the sludge.

The MLTSS and MLVSS concentrations varied as a consequence of the influent organic and nitrogen load and of the SRT. During periods 1 and 2, the MLVSS remained low (1.6 g L^{-1} in period 2) in accordance with the low SRT (3-4 d) which was selected to facilitate NOB wash-out. However, the SRT was found to be too low to support a stable nitritation. Therefore, at the beginning of period 3 the SBR was re-inoculated with 300 L of concentrated activated sludge (8 gTSS L^{-1}) that made the MLVSS concentration achieving 6.3 gMLTSS L^{-1}. Later on, the substantial increase in the SRT caused a coherent increase in the MLVSS concentration. The higher MLVSS values measured during phase 5-6 are to be related to the increased organic load.

4.4.2 Effluent concentrations and removal efficiencies

The SBR influent and effluent concentrations of NH_4^+-N, NO_2^--N and NO_3^--N are shown in Figure 4.6. High ammonium concentrations were mainly registered during period 2, during which influent and effluent ammonia concentrations almost coincided. During this period, the SRT was too low to support a stable nitritation. The ammonia oxidisers washout could be facilitated by the concurrent change in the digester feed with an increase in the poultry manure fraction.

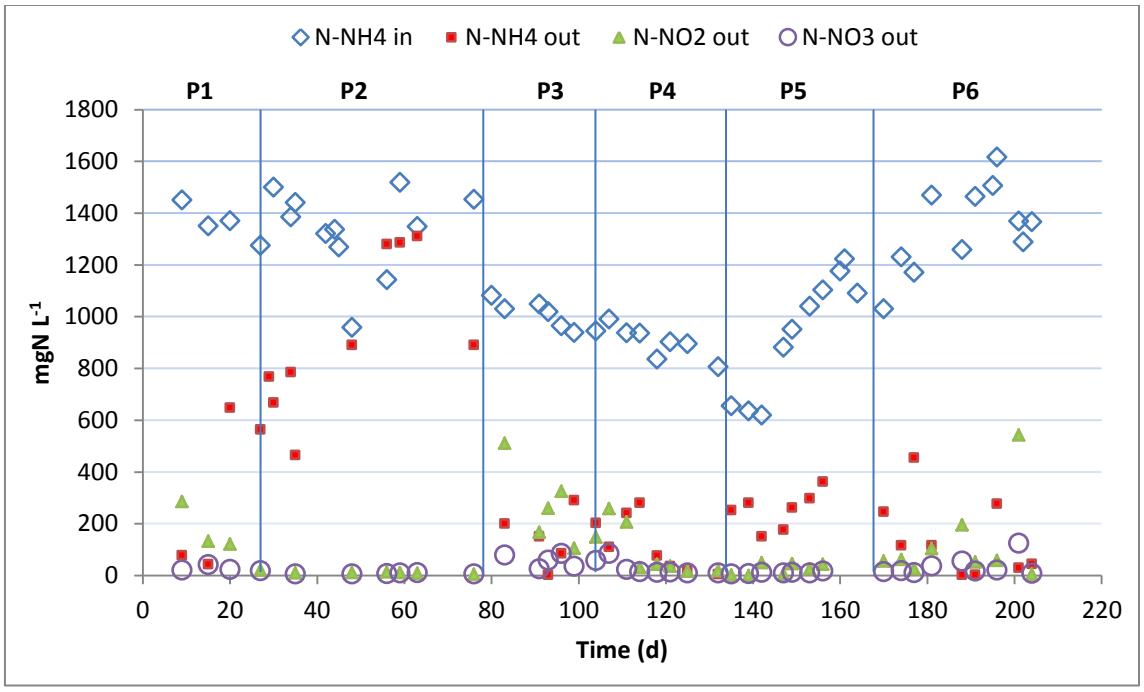


Figure 4.6 - Influent and effluent concentrations of NH_4^+-N, NO_2^--N and NO_3^--N

The nitrogen loading and removal rates are plotted in Figure 4.7. The NLR was kept between 0.4 and 0.6 kgN m^{-3}_{react} d^{-1} with NRR between 0.17 and 0.51 kgN m^{-3}_{react} d^{-1}, with the exception of period 2 when biomass washout took place, as commented before.

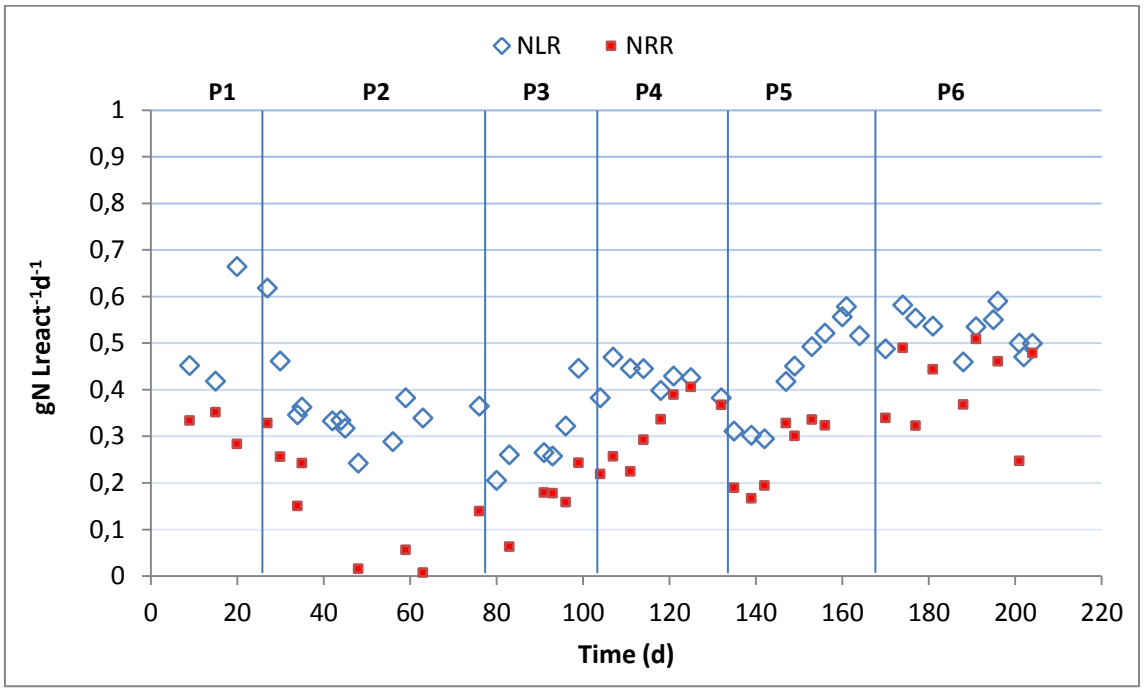


Figure 4.7 - Nitrogen Loading Rate (NLR) and Nitrogen Removal Rate (NRR) during the experimentation

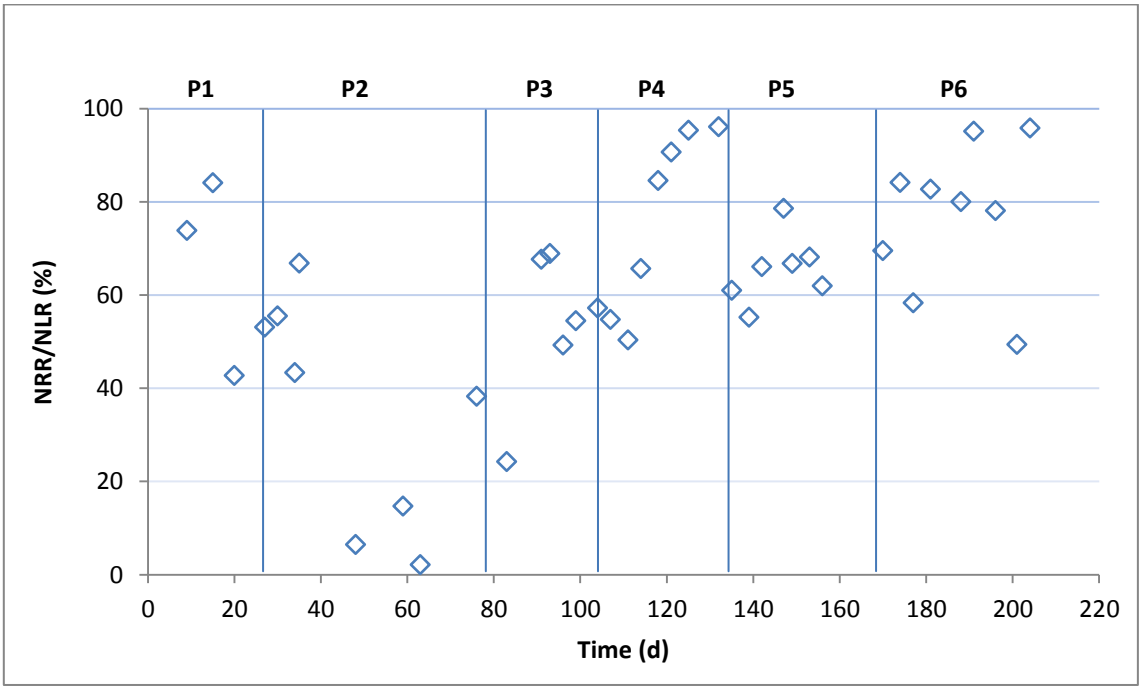


Figure 4.8 –Soluble N removal efficiency during the experimentation

The N removal efficiency, calculated as the ratio between the NLR and the NRR, is reported in Figure 4.8. It remained between 50% and 80% during period 1 and then drop during period 2. During period 3, after biomass re-inoculation, the nitrogen removal efficiency improved and remained around 60%. During phase 4, the SBR operation was optimised and the N removal efficiency grew up to 95% at the end of this period. On the contrary, the N removal efficiency dropped to around 60% at the beginning of period 5, when the COD/N in the influent increased suddenly up to 5-6. During this period the anaerobic digester was overloaded and poultry manure feeding was restarted. In this period, the fraction of nitrifying biomass over the total biomass was reasonably lower. Nevertheless, the high SRT value allowed the SBR to maintain a stable AOB population and to face the variation in the influent quality.

In period 6, despite the high variability in terms of influent characteristics and the lower operational temperature, the average N removal efficiency was higher than 80%. At day 201 no external C-source was added and then nitrogen removal efficiency fell down to 50% because post-denitrification could not take place. Later, when external carbon dosage was restored, the nitrogen removal efficiency raised again to 95%.

Influent and effluent COD concentrations are reported in Figure 4.9. During period 1 to 4 the influent COD concentrations were in the range 1500-2400 mgCOD L^{-1} while in periods 5 and 6 it increased (around 3000 mgCOD L^{-1}) and it was also more variable. During these last 2 periods, the biodegradable COD residuing after the anaerobic digestion stage and removed by the SBR was extremely high. During period 6, peaks of COD concentrations up to 6000 mgCOD L^{-1} were in fact related to technical problems in the digesters (e.g. low temperature, foam production).

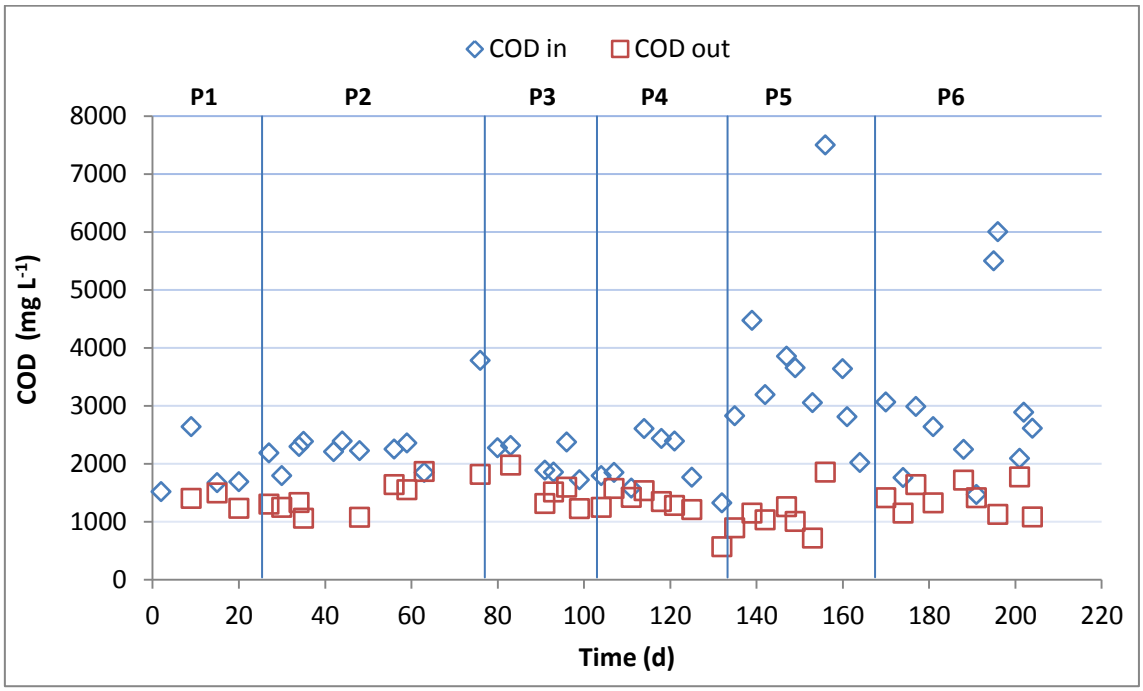


Figure 4.9 - Influent and effluent COD concentrations

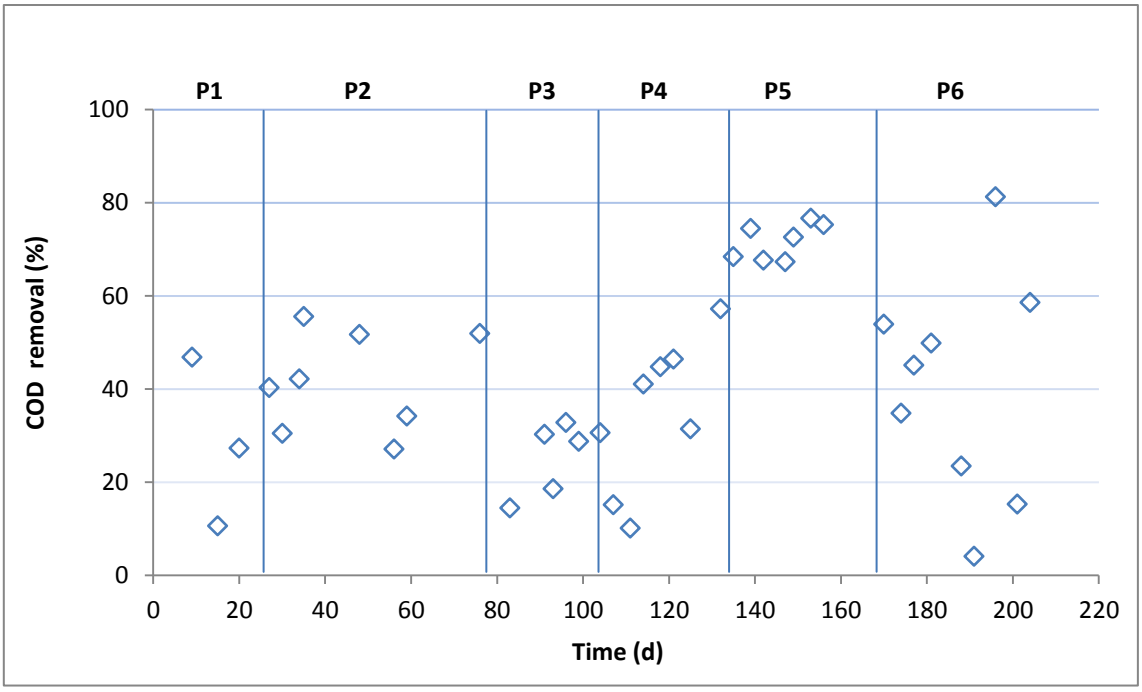


Figure 4.10 - COD removal efficiency during the experimentation

The COD removal efficiency during the experimentation, not including the external COD dosage, is depicted in Figure 4.10. It is worth noticing that the COD removal in the reactor changed from 10% to up to 80% confirming the remarkable variability of the influent quality. Moreover, periods with an increase in COD removal are characterized by a decrease in the N removal and vice versa.

4.4.3 Assessment of nitrification process

The average nitrification efficiency, during the whole experimentation was 76% (period 2 excluuded). Nitrification efficiency trend during the whole experimentation is reported in Figure Figure 4.11.

The composition of the nitrogen compound (ammonium, nitrite, nitrate) across the phases of each cycle was measured once or twice per week. These data allowed the nitrification rates and the nitritation efficiency to be quantified. In Figure 4.12 the specific ammonium removal rates are depicted. During period 1, the specific ammonium removal rates were higher than in other periods (27 $mgNH_4^+$-N $gVSS^{-1}$ h^{-1}) mainly because of the lower SRT resulting in a higher active biomass fraction in the MLVSS. During period 3 to 6, the observed ammonium removal rates ranged between 2 and 8 $mgNH_4^+$-N $gVSS^{-1}$ h^{-1}. These lower rates are partly due to higher SRT in the SBR (and, hence, to a lower fraction of active biomass), and partly related to oxygen limitation, as described later.

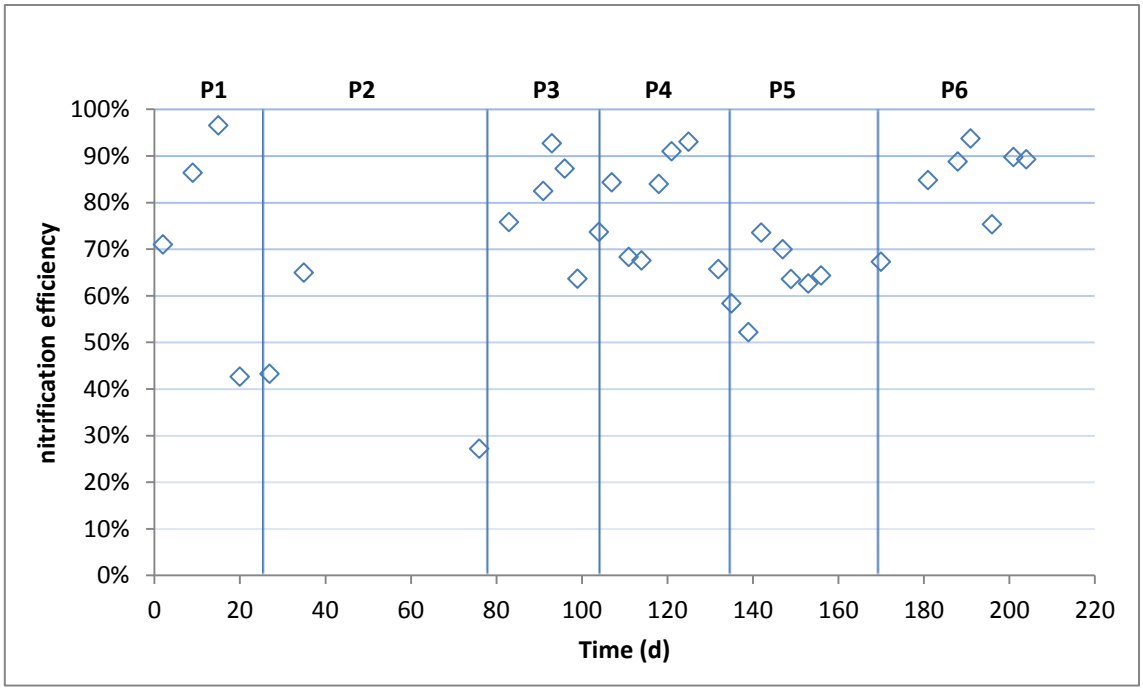


Figure 4.11 – Nitrification efficiency during the experimentation

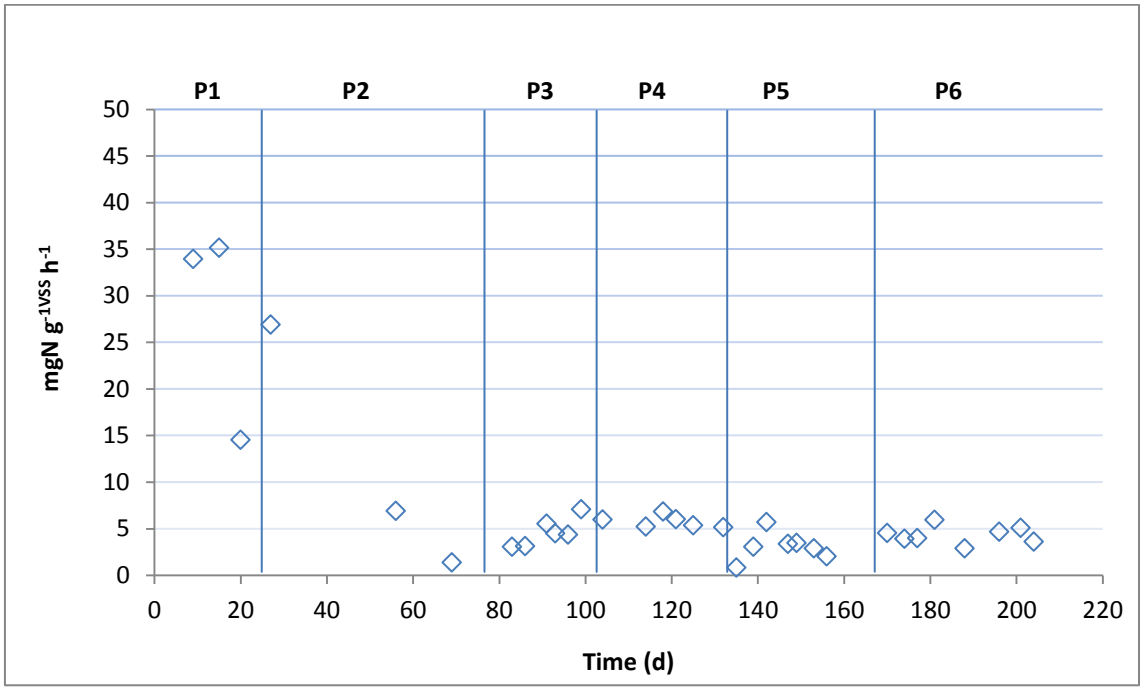


Figure 4.12 – Specific ammonium removal rates

During the aeration phase the following processes may occur besides nitrification and COD oxidation:

- simultaneous ammonia stripping;
- simultaneous denitrification, which might occur inside the flocs or in poorly aerated areas of the reactor.

By comparing the ammoniacal nitrogen removed and the NOx-N produced across the aeration phase (Figure 4.13), it is possible to quantify the relevance of the sum of these two effects. The difference between the 2 rates was on average between 10 and 15%. Period 2 was not considered for this estimation, because the ammonium consumption and nitrite production were low and therefore difficult to estimate.

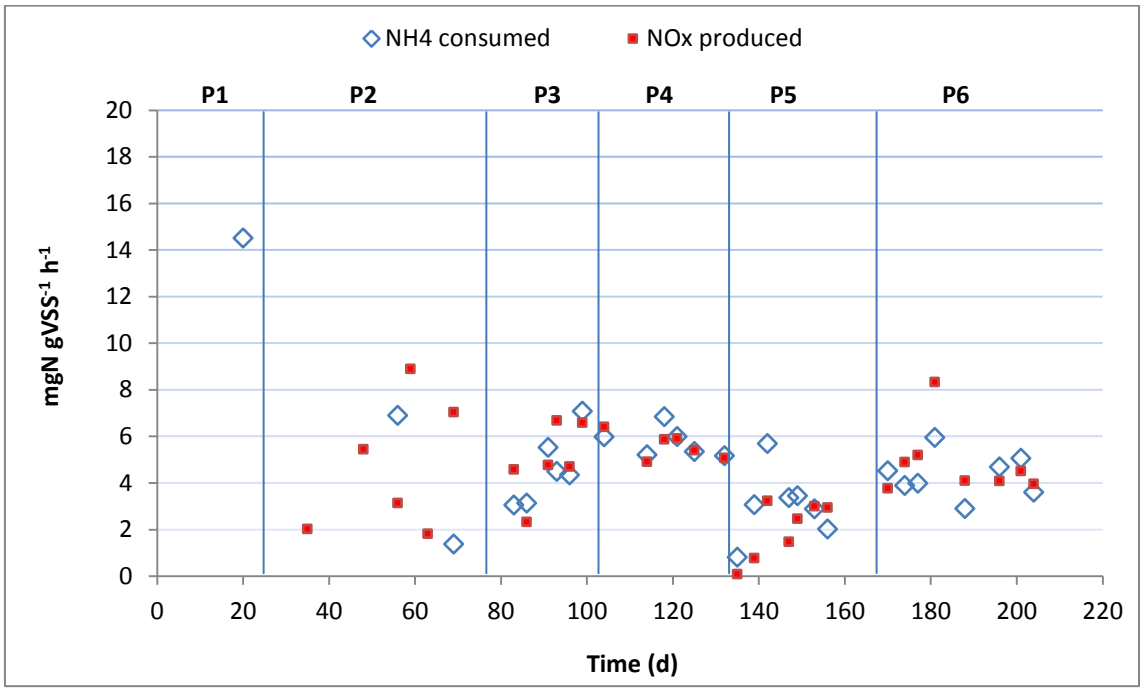


Figure 4.13 - Comparison between the NH_4^+-N removed and the NO_x (NO_2^--N + NO_3^--N) produced in aeration phase

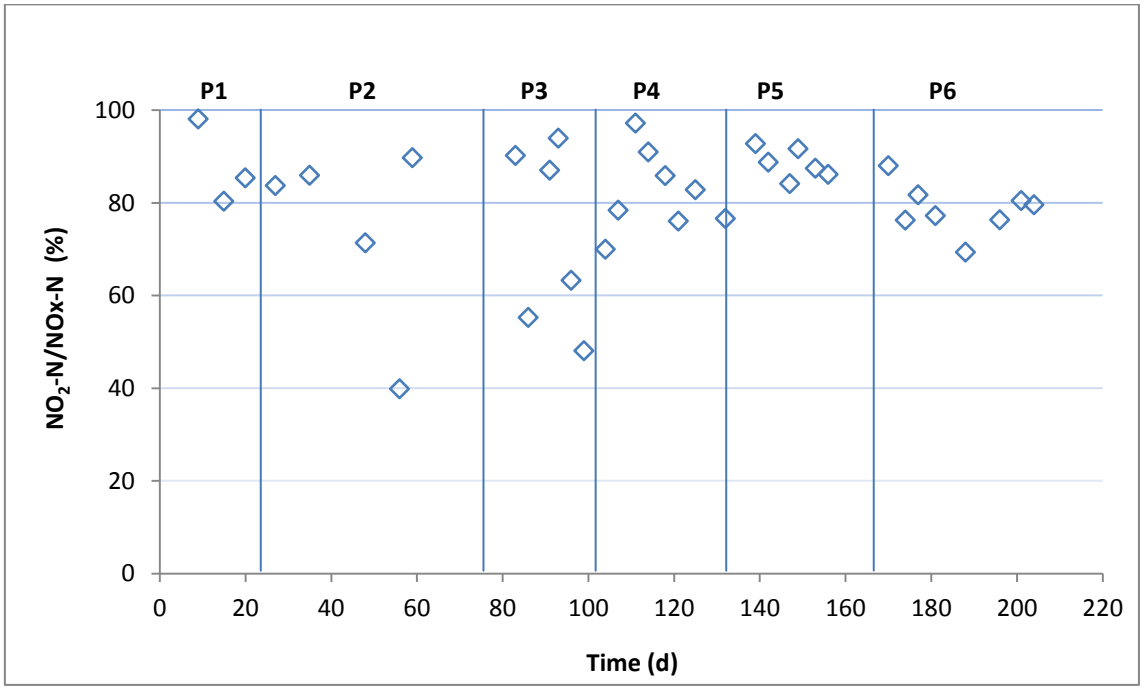


Figure 4.14 - Fraction of NO_2^--N produced at the end of the aeration phase over NO_x (NO_2^--N + NO_3^--N)

As reported in Figure 4.14, the fraction of nitrite over the NOx produced during the aeration phase was higher than 80%, already at the beginning of the experimentation. This phenomenon probably arise from:

- the microbial quality of the inoculum (as confirmed in par 4.4.8), which was already poor in NOB;
- the quick inactivation of NOB bacteria because of the low DO concentration (0.5 mg L^{-1}), the higher temperature (30°C) and the high free ammonia (FA) concentrations, present in the pilot plant in period 1 (further details in par 4.4.5).

In period 3, when the reactor was re-inoculated, it took approximately 30 days to achieve a stable nitritation process. This longer transient phase was probably due to the higher SRT in the reactor. In period 6, at 25°C, the fraction of NO_2/NOx decreased slightly with respect to the previous period.

4.4.4 Denitrification process

During the aoxic phase NOx were reduced to N_2. This process is here referred to as denitrification although the prevalent process was denitritation nitrite accounting for 80-90% of the overall NOx. Dosage of external carbon source was necessary to achieve complete denitrification, as internal biodegradable carbon was not enough. A concentrated solution of sodium acetate has been added at the beginning of the second anoxic phase.
In the first anoxic phase of each cycle, biodegradable influent COD has been used to denitrify the residual NO_2^--N coming from the previous cycle.
As reported in

Figure 4.15, the ratio between the COD removed (CODrem) and the nitrogen denitrified (N_{den}) across a cycle varied notably. In

Figure 4.15, the stoichiometric ratios expected are also reported. These ratios are estimated assuming 80% of nitrite and 20% of nitrate produced in nitrification and calculating the average ratio from the 2 contributions. The COD/N ratios are:

$$\text{for nitrite: } \frac{COD}{N_{den}} = \frac{1.71}{1-Y_{obs}}$$

$$\text{for nitrate: } \frac{COD}{N_{den}} = \frac{2.86}{1-Y_{obs}}$$

and

$$Y_{obs} = \frac{Y}{(1+b' \times SRT)}$$

where:

$$b' = b \times [1 - (1 - f) \times Y]$$

Y = biomass yield = 0.63 (Henze et al., 2000);

b= heterotrophic decay (calculated from Henze et al. 2000). In the first 5 periods (30°C) assumed 1.76 d^{-1} and in period 6 (25°C) assumed 1.07 d^{-1}, considering an Arrhenius constant θ=1.11 (Van Hulle, 2005);

f= 0.1 (inert fraction, from Henze et al., 2000);

SRT assumed as 2 days in the first 2 periods and 13 days in period 3 to 6 (anoxic SRT).

The resulting estimated COD/N (considering both nitrite and nitrate contribution) are 2.6 gCOD gN^{-1} in period 1-2 and 2.1 gCOD gN^{-1} in period 3 to 6 (the different decay does not influence significantly the result).

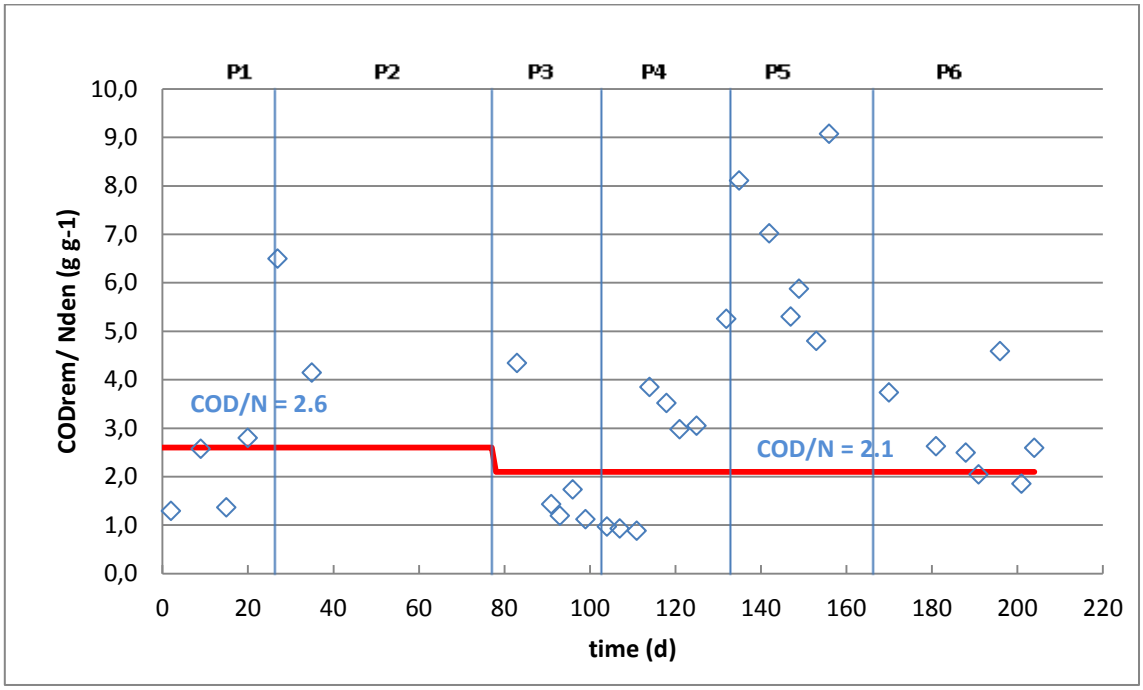


Figure 4.15 - Ratio COD removed over nitrogen denitrified during the experimentation

Optimization of the denitrification process could not be performed during the experimentation. However, some considerations can be drawn.

During period 3, the observed CODrem/Nden ratio was below the stoichiometric minimum expected. In that period, denitrification was performed only with internal carbon in the first anoxic phase. So, all the available bCOD was used for denitrification and not consumed in the aeration phase. Moreover, the SRT in this phase was higher (no sludge wastage) and probably a higher fraction of COD used in denitrification came from biomass decay and hydrolysis. Thus the CODrem/Nden ratio observed was close to the pure catabolic need for nitrite reduction (i.e. 1.71).

In the periods with low CODrem/Nden, incomplete denitrification may occur and high N_2O emissions can be expected (e.g.: Kampschreur et al., 2009). Some preliminary results on N_2O emissions are reported in paragraph 4.4.7.

On the contrary, during periods characterized by high denitrification efficiency, no residual NO_x are present in the first anoxic phase and all the residual internal carbon of the wastewater was oxidized in the aeration phase. Thus, CODrem/Nden resulted higher than the stoichiometric need. For example, during period 5 the high CODrem/Nden ratio would have allowed a more efficient use of the internal carbon for denitrification, such as by fractionating the influent load in multiple anoxic phases with a step-feed strategy. In this way, biodegradable COD would have not been wasted in the aeration phase. Unfortunately, it was not possible to perform the cycle in this way because of technical limitations on the PLC controller.

On average, during periods 4, 5 and 6, the fraction of N denitrified in the first anoxic phase (without any external carbon addition) has been around 50% of the total N denitrified. These values come from the weekly analyses across the different phases of each cycle and by comparing data taken from the first and the second anoxic phase.

On average, denitrification efficiency was 84%. Denitrification efficiency during the entire experimental period is shown in Figure 4.16 and has been calculated by means of N mass balance.

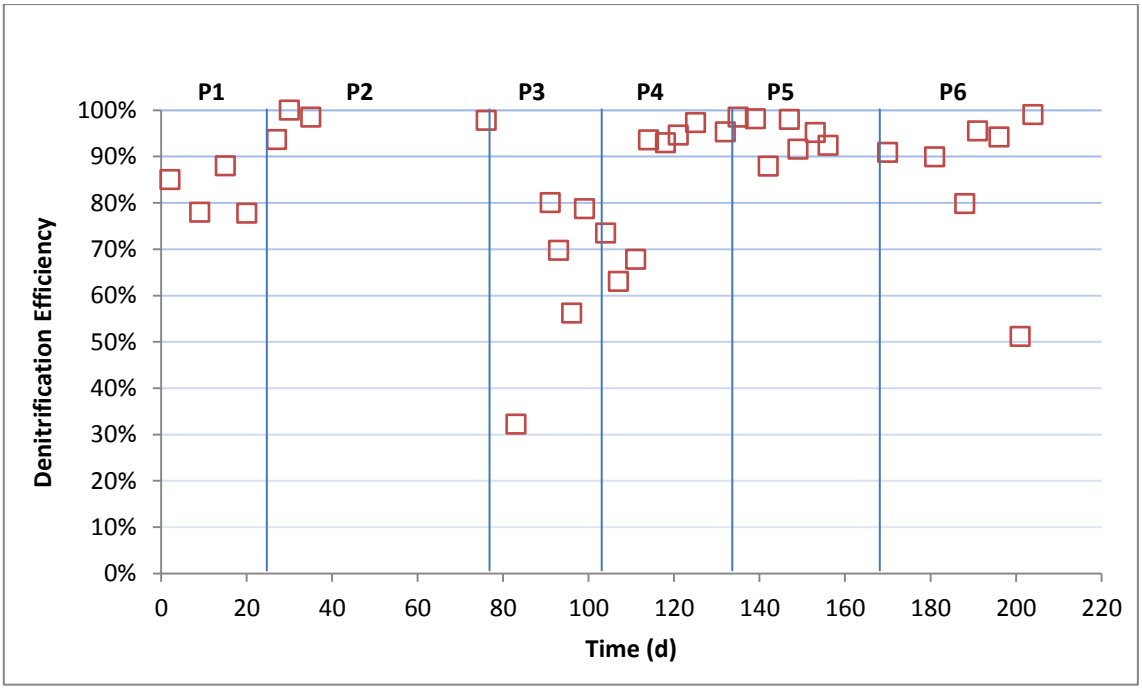


Figure 4.16 – Denitrification efficiency during all the experimental periods

4.4.5 Respirometric tests

The nitrification activity was measured by laboratory batch tests at 20°C by means of an automatic titrator (MARTINA, as described in par. 3.2.1 and in Artiga et al., 2005) with no DO limitation (DO-stat mode at DO = 6.5 $mgO_2\ L^{-1}$ and pH-stat mode at pH 8). These tests confirmed that the NOB activity in the biomass was not detectable.

Specific nitrification rates that have been measured in the pilot plant under real working conditions have been compared with the maximum specific activity values that have been

measured in the laboratory tests (par 3.2.1). To take into account the difference in temperature and in the oxygen concentration between the two conditions, the following equation has been used:

$$r_{real} = r_{lab} \times \vartheta^{(T_{react} - T_{lab})} \times \frac{DO_{react}}{DO_{react} + kO_a} \times \frac{DO_{lab} + kO_a}{DO_{lab}}$$

Where:

r_{lab}, T_{lab} and DO_{lab} are the specific activity, T and DO during the titrimetric tests carried out in the laboratory on biomass taken from the pilot SBR;

r_{real} is the resulting estimated specific activity under real operating conditions;

T_{react} and DO_{react} are T and DO values measured in the pilot SBR during the corresponding experimental period.

ϑ is the temperature correction factor for temperature = 1.099 (Van Hulle, 2005)

kO_a is the affinity constant for oxygen of AOB = 0.74 mg L^{-1} (Jubany, 2007)[1]

The comparison between the estimated values of specific nitrification activity (r_{real}) and the actual specific nitrification rates (mgNH_4^+-N gVSS^{-1} h^{-1}) measured in the SBR are shown in Figure 4.17.

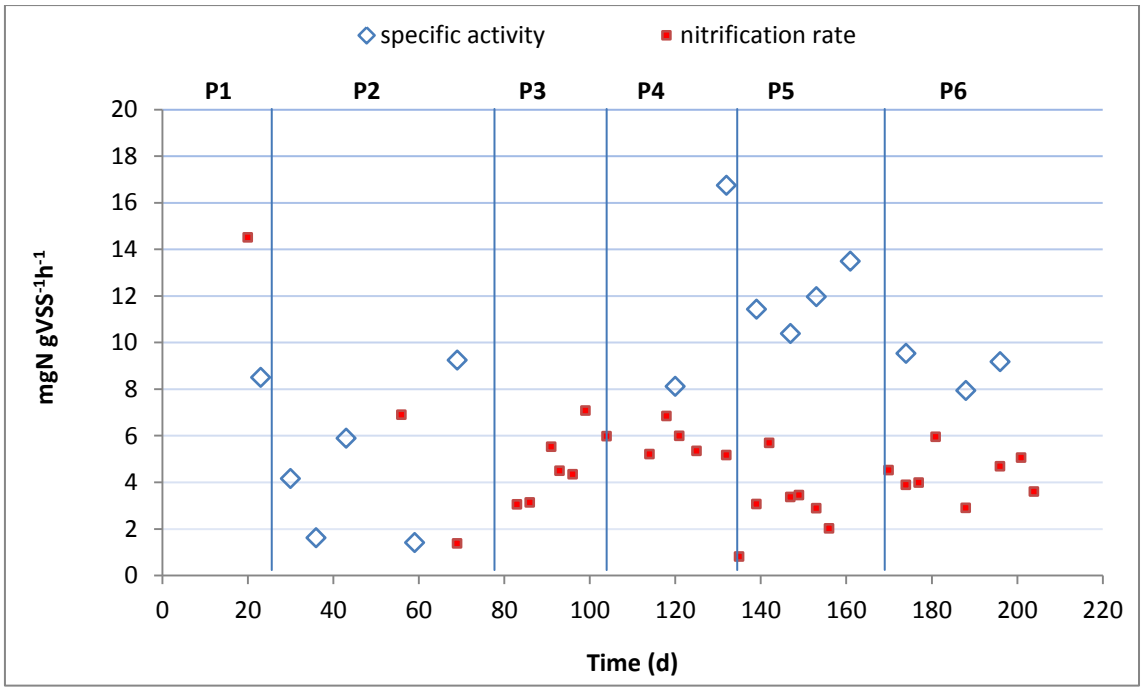


Figure 4.17 - Comparison between specific nitrification activities measured by titration and specific nitrification rates in the aeration phase

A large difference between specific activity and specific nitrification rates can be observed during periods 5 and 6. During those periods the biomass samples taken from the SBR have been diluted 1:3 with tap water before the respirometric test.

[1] This value is among the highest found in the literature. This choice has been made to take into account the possible diffusional resistances that can be found in high MLSS concentration. More comments on this are given later.

The presence of a positive effects of the dilution with tap water has been investigated by performing a test in parallel: one sample diluted 1:3 with tap-water and the second sample diluted 1:3 with the effluent of the pilot plant. The test revealed a 30% decrease in the activity when the sample was diluted with the effluent compared to the one diluted with tap water. This reduction on the activity can be related not only to inhibition due to FA and FNA which were present in the real wastewater, but it may also be ascribed to other compounds (e.g. metals or antibiotics), which may have been present in the wastewater.

To go in deeper details about these aspects, the activity tests have been performed again in parallel by keeping the same initial ammonium and nitrite concentration in both samples (125 $mgNH_4^+$-N L^{-1} and 56 $mgNO_2^-$-N L^{-1} in tap water and real effluent). The resulting specific activities were 10.6 and 9.6 mgN $gVSS^{-1}$ h^{-1} respectively for the test diluted with tap water and with effluent, showing that compounds different form FA and FNA could account for an overall activity reduction of about 10%.

The difference between specific activity measurements and nitrification rates in the reactor was higher when a higher BOD_5/N ratio in the influent and high biomass concentration in the SBR occurred at the same time. As biomass concentration in the SBR in the last two periods was around 9 and 10 gTSS L^{-1}, and oxygen concentration was kept below 1 mgO_2 L^{-1}, part of the difference might have been caused by diffusion limitations of oxygen inside the flocs. In other words, oxygen consumption by heterotrophs located in the outer part of the flocs may reduce the oxygen concentration available for nitrifiers located in the inner parts. As reported by Blackburne et al. (2008), the apparent Ko values are affected by mass transfer limitations and are different from the pure biological Ko values of AOB.

This difference between the Ko measured for AOB in the absence of heterotrophic activity as it is the case during lab-tests and the actual value in presence of concurrent heterotrophic activity have been confirmed by performing a multiple respirometric test on a sludge sample taken from the reactor at day 204.

A series of respirometric tests have been performed by:

- saturating the mixed liquor with oxygen,
- measuring the DO depletion during time,
- correlating the OUR (Oxygen Uptake Rate) value to the corresponding DO concentration.

After the endogenous OUR has been measured as the baseline, the following substrates have been added in series, corresponding to the following three experimental runs:

- ***experimental run 1***: ammonium (50 $mgNH_4^+$-N L^{-1}) to measure the AOB activity; this dosage allowed to keep ammonium at concentration above 5 $mgNH_4^+$-N L^{-1} all through the duration of the test;
- ***experimental run 2***: acetate (500 mgCOD L^{-1}) to measure AOB activity in presence of heterotrophic activity,
- ***experimental run 3***: ATU (10 mg L^{-1}) to inhibit AOBs and measure the heterotrophic respiration only.

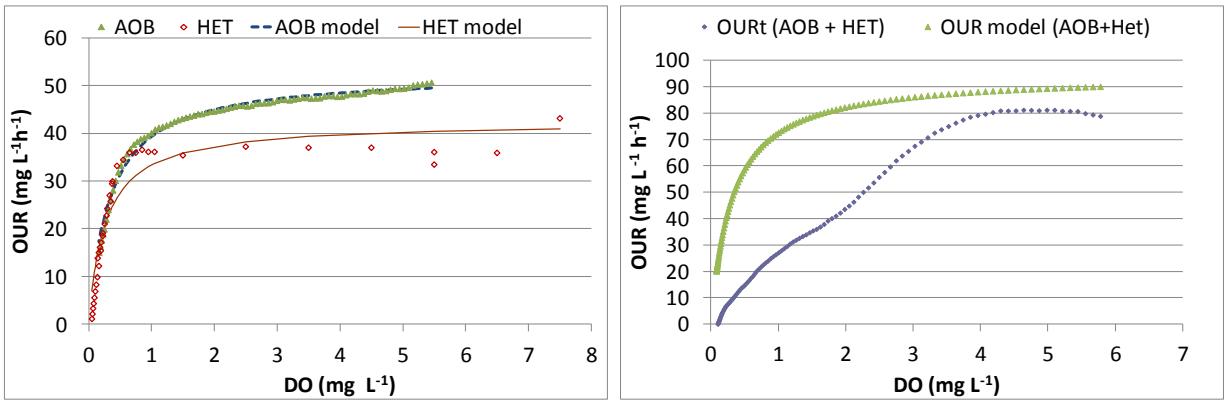


Figure 4.18 – Results of the respirometric tests with AOB and heterotrophs considered separately (from experimental runs 1 and 3, left) and simultaneously (from experimental run 2, right). The green line in the right graph is the theoretical OUR when AOB and heterotrophs are simultaneously active

Figure 4.18-left shows the results of the respirometric tests (OUR versus DO curves for AOB and heterotrophs, measured separately, from experimental runs 1 and 3). Data have been fitted with the conventional Monod model. The least-square error method allowed to estimate the best fitting values for Ko_{AOB} = 0.23 mgO_2 L^{-1} and Ko_{HET} = 0.27 mgO_2 L^{-1}.

Figure 4.18-right shows the results of the respirometric tests (OUR-DO curves for AOB and heterotrophs, measured simultaneously, from experimental run 2). The real OUR vs DO curve does not match at all the theoretical curve, which has been obtained by simply considering the simultaneous occurrence of AOB and heterotrophs respiration as previously estimated.

By making the assumption that the heterotrophic biomass is less affected by oxygen limitation, it is possible to subtract its contribution from the total OUR vs DO curve and to estimate the OUR vs DO curve of AOB in the presence of heterotrophic activity. Figure 4.19 shows the result. One can see that the conventional Monod model doesn't describe the shape of the OUR vs DO curve of AOB, which is more similar to an S-shaped curve with equation:

$$OUR = OUR_{max} \frac{1}{1-(DO/a)^b}$$

with OUR_{max} = 53 mgO_2 L^{-1} h^{-1}, as derived from the AOB-model fitting in experimental run 1.

The least-square error method has provided the following best-fit parameters:

a=2.63 mgO_2 L^{-1} and b= 3.19, R^2=0.99.

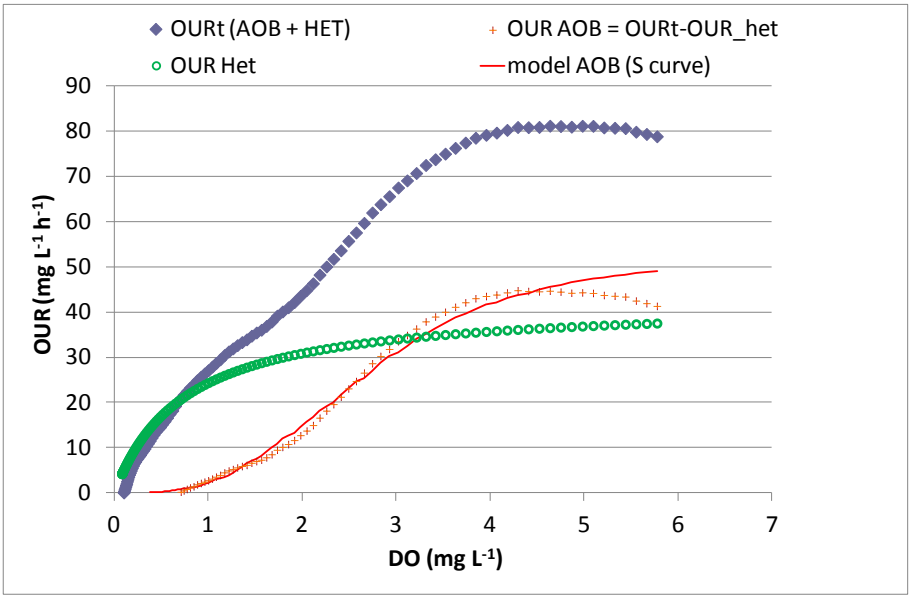


Figure 4.19 – Results of the respirometric test performed with simultaneous AOB and HET activity and estimation of the AOB curve compared to a best-fit S-shaped curve

The S-shaped curve may be the result of diffusional resistances of the DO in the floc, which are most evident at low concentration. As a matter of fact, a higher fraction of heterotrophic biomass than AOB is present in the outer part of the floc, due to the faster growth of heterotrophs than AOB at high BOD_5/N ratios, while AOB remain confined in the inner part of the floc. Similar considerations have been reported by Harremoës 1982, in the case of biofilm growth and an experimental confirmation by FISH analysis will be reported later.
This would explain the difference between the nitrification rates in the pilot SBR at low oxygen concentration and the maximum AOB activity measured during the batch respirometric tests. This is particularly evident during periods 5 and 6 which are characterized by high COD/N and BOD_5/N ratios in the influent.

Free ammonia (FA) and free nitrous acid (FNA) inhibition

The calculation to estimate FA (NH_3-N) and FNA (HNO_2-N) concentrations have been performed with the following equations (Anthonisen, 1976):

$$FA(mgNL^{-1}) = \frac{TAN}{1 + (\frac{10^{-pH}}{K_e^{NH}})}$$

$$FNA(mgNL^{-1}) = \frac{TNO_2}{1 + (\frac{K_e^{NO}}{10^{-pH}})}$$

where:

$$K_e^{NH} = e^{-6344/(273+T)}$$

$K_e^{NO} = e^{-2300/(273+T)}$

TAN=NH_4+NH_3, TNO_2=NO_2+HNO_2

If ammonium and nitrite concentrations, pH and temperature inside the reactor at the beginning and at the end of the aeration phase are known, it is possible to estimate FA and FNA concentrations. Results are reported in Figure 4.20 and Figure 4.21.

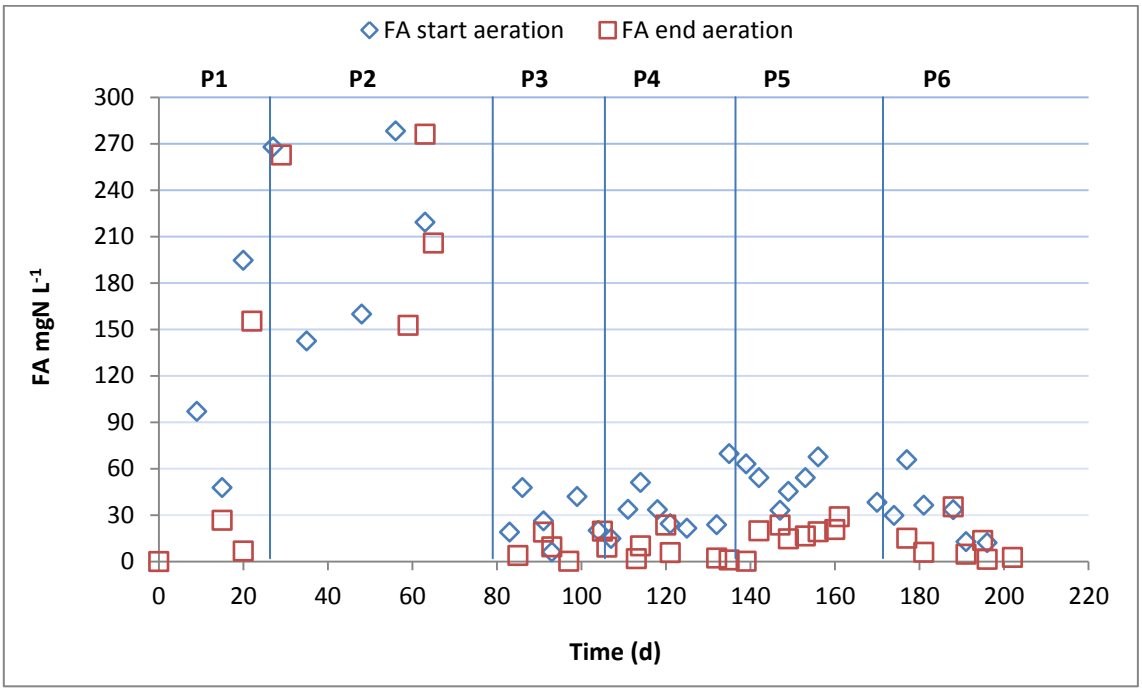


Figure 4.20 – Free ammonia (FA) concentrations at the beginning and the end of the aeration phase

Excluding the period between day 20 and 80, when N removal efficiency was very low and FA concentrations were in the range 120-300 mgNH_3-N L^{-1}, FA concentration at the beginning of the nitrification phase was in the range 20-70 mgNH_3-N L^{-1} and between 0 and 38 mgNH_3-N L^{-1} at the end of the aeration phase.

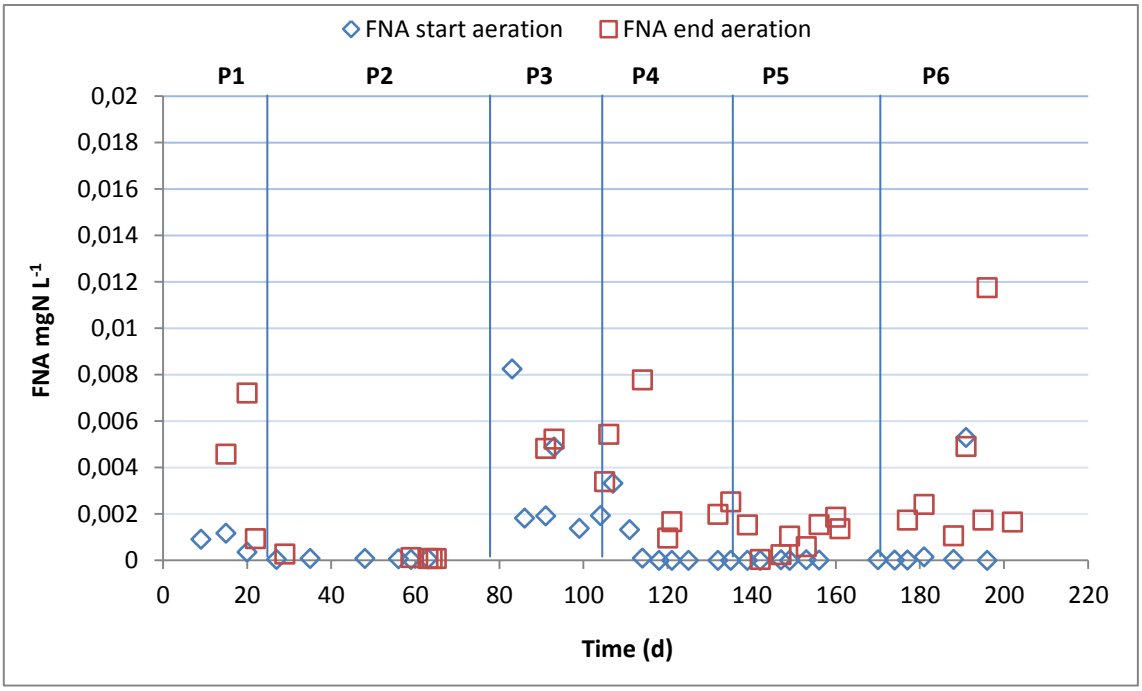


Figure 4.21 – Free nitrous acid (FNA) concentrations at the beginning and the end of the aeration phase

Apart from period 3, the concentration of FNA at the beginning of the aeration phase was negligible, mainly because of high pH (8.4). At the end of the aeration phase the FNA concentration rose up to 0.002 mgHNO_2-N L^{-1} on average. Titrimetric batch tests were run to measure the decrease of AOB activity at different FA and FNA concentrations. In Table 4.6 the results of a preliminary inhibition test (TEST A1) at increasing NH_3-N concentrations on a sample of $DENO_2$ sludge are reported. A second test (TEST A2) was run, reaching FA concentrations of up to 221 mgNH_3-N L^{-1}, by working at higher pH and temperature.

Table 4.6 - Specific AOB activity tests at different FA concentrations

FA ($mgNH_3$-N L^{-1})	Specific AOB activity ($mgN\ gVSS^{-1}h^{-1}$)	Activity reduction (%)
TEST A1 (T = 20°C, pH = 8, DO = 6.5 mg L^{-1})		
2.1	8.70	0%
3.0	8.60	1.1%
5.3	8.45	2.9%
9.9	8.36	3.9%
17.5	8.40	3.4%
TEST A2 (T = 25°C, pH = 8.5, DO = 6.5 mg L^{-1})		
2	13.70	0.0%
14	13.30	2.9%
27	13.19	3.7%
50	11.37	17.0%
105	8.74	36.2%
166	6.61	51.7%
221	4.53	66.9%

In Figure 4.22, the activity reduction (as % inhibition) due to increasing FA concentration is reported with reference to the two tests. Three different models were compared by fitting inhibition versus concentration data with the least-square errors method: 1) extended non competitive inhibition model (R^2= 0.995), 2) non competitive reversible inhibition model (R^2= 0.964) and 3) exponential model (R^2= 0.986).

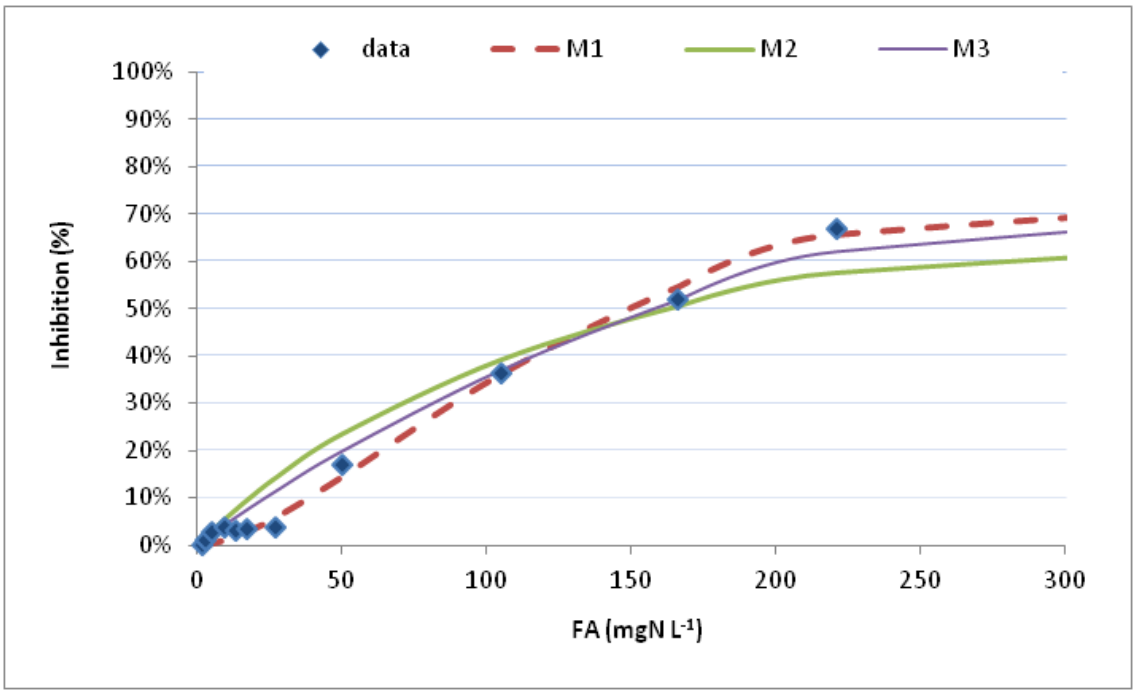


Figure 4.22 - Inhibition values at different FA concentrations and 3 different models

Results of the fitting procedure are summarized in Table 4.7. It is interesting to notice that model 1 and model 2 are similar and the latter can be considered a sub-case of model 1.
Several authors (Carrera, 2001; Magrí et al., 2007; Pambrun et al., 2006) assumed the Haldane kinetics as the most suitable model to describe FA inhibition of AOB, since it takes into account the double effect of FA as substrate and inhibitor. However, in this experimentation a non-competitive reversible inhibition (NOCRI) model has been chosen because the Haldane model becomes mathematically equal to the NOCRI when substrate is not limiting (i.e. well above the affinity constant). In this case, the best fit value for kI_{FA} with NOCRI model is 163±23 $mgNH_3$-N L^{-1}.

Table 4.7 - Results of different models fitting for Free Ammonia (FA) inhibition data set

Model	Type	Equation (% inhibition)	Parameters	R^2
M1	Extended non competitive inhibition (S-shaped)	$1-\frac{1}{1+\left(\frac{FA}{kIa}\right)^{kIb}}=1-\frac{1}{1+\frac{FA^{kIb}}{kIa'}}$ $IC_{50}=kIa$ =*148*±5.5 $mgNH_3$-N L^{-1}	kIa=*148*±5.5 $mgNH_3$-N L^{-1} kIb=1.64±0.11	0.995
M2	Non-competitive reversible inhibition	$1-\frac{kI_{FA}}{FA+kI_{FA}}=1-\frac{1}{1+\frac{FA^1}{kI_{FA}}}$ $IC_{50}=kI_{FA}$ = 163±23 $mgNH_3$-N L^{-1}	kI_{FA} =163±23 $mgNH_3$-N L^{-1}	0.964
M3	Exponential	$1-exp^{(-FA\times kIa)}$ $IC_{50}=-\frac{\ln(0.5)}{kIa}$=158±8.6 $mgNH_3$-N L^{-1}	kIa=0.0044±0.000236 $mgNH_3$-N^{-1} L	0.986

However, the best fitting curve for the measured data-set is the S-shaped curve, which corresponds to the extended non-competitive inhibition model (Kroiss et al., 1992) and is described by the following equation:

$$I(\%)=100\times\left(1-\frac{1}{1+\left(\frac{FA}{kIa}\right)^{kIb}}\right)$$

where I% represents the inhibition response (I% = 100 - residual activity), kI_a and kI_b are fitting parameters. Note that parameter kI_a is the model estimation of the IC_{50} (50% inhibition concentration) value and in our experimentation equals 148±5 $mgNH_3$-N L^{-1}. This behaviour may be explained by diffusion of free ammonia through thick flocs. At low concentrations diffusion may reduce free ammonia concentration in the inner layers of the floc, thus mitigating its inhibition effect. To check the validity of this hypothesis further research is needed, but this is beyond the scope of this thesis.
In Table 4.7 the IC_{50} values for the other fitting models are also reported and all resulted very close to each other.

Results of specific AOB activity (mgN $gVSS^{-1}$ h^{-1}) versus increasing FNA concentration are reported in Table 4.8. These tests have been carried out under similar experimental conditions as for tests A1 and A2, but with increasing concentration of nitrite instead of ammonium.

Table 4.8 - Specific AOB activity at different FNA concentrations

FNA (mg HNO_2-N L^{-1})	Specific AOB activity (mgN $gVSS^{-1}h^{-1}$)	Activity reduction (%)
B1 (T = 20°C, pH = 8, DO = 6.5 mg L^{-1})		
0.000	8.83	0%
0.001	8.66	0.5%
0.002	8.20	5.7%
0.004	8.04	7.6%
0.007	7.85	9.8%
0.012	7.80	10.3%
B2 (T = 20°C, pH = 7, DO = 6.5 mg L^{-1})		
0.000	6.12	0.0%
0.026	5.78	5.6%
0.051	4.5	26.5%
0.103	3.46	43.5%
0.205	2.98	51.3%

A second batch test has been run at higher FNA concentrations by dosing 100, 200, 400, 800 mgNO_2^--N L^{-1} at pH 7 and 20°C obtaining inhibitions up to 50% (B2 in Table 4.8).

The activity reduction (as % inhibition) in tests B1 and B2 have been fitted with the same models by means of the least-square errors method (Figure 4.23): extended non competitive inhibition model (S-shaped curve) (R^2= 0.940), exponential curve (R^2= 0.928) and non competitive reversible inhibition model (R^2= 0.943).

The non competitive reversible inhibition model resulted the best fitting, and the best fitting kI_{FNA} is 0.16±0.02 mgHNO_2-N L^{-1}. Many authors considered this model suitable to describe FNA inhibition over AOB (Hellinga et al., 1998; Wett and Rauch, 2003; Van Hulle et al., 2004, Pambrum et al., 2006).

Table 4.9 - Results of different models fitting for Free Nitrous Ammonia (FNA) inhibition data set

Model	Type	Equation (% inhibition)	Parameters	R^2
M1	Extended non competitive inhibition (S-shaped)	$1-\frac{1}{1+\left(\frac{FNA}{kIa}\right)^{kIb}}=1-\frac{1}{1+\frac{FNA^{kIb}}{kIa'}}$ $IC_{50}=kIa$=0.177±0.029 mgHNO$_2$-N L^{-1}	kIa=0.177±0.029 mgHNO$_2$-N L^{-1} kIb=0.86±0.12	0.940
M2	Non competitive reversible inhibition	$1-\frac{kI_{FNA}}{FNA+kI_{FNA}}$ $IC_{50}=kI_{FNA}$ 0.16±0.02 mgHNO$_2$-N L^{-1}	kI_{FNA} =0.16±0.02 mgHNO$_2$-N L^{-1}	0.943
M3	Exponential	$1-exp^{(-FNA\times kIa)}$ $IC_{50}=-\frac{\ln(0.5)}{kIa}$ =0.156±0.016 mgHNO$_2$-N L^{-1}	kIa=4.45±0.51 mgHNO$_2$-N^{-1} L	0.928

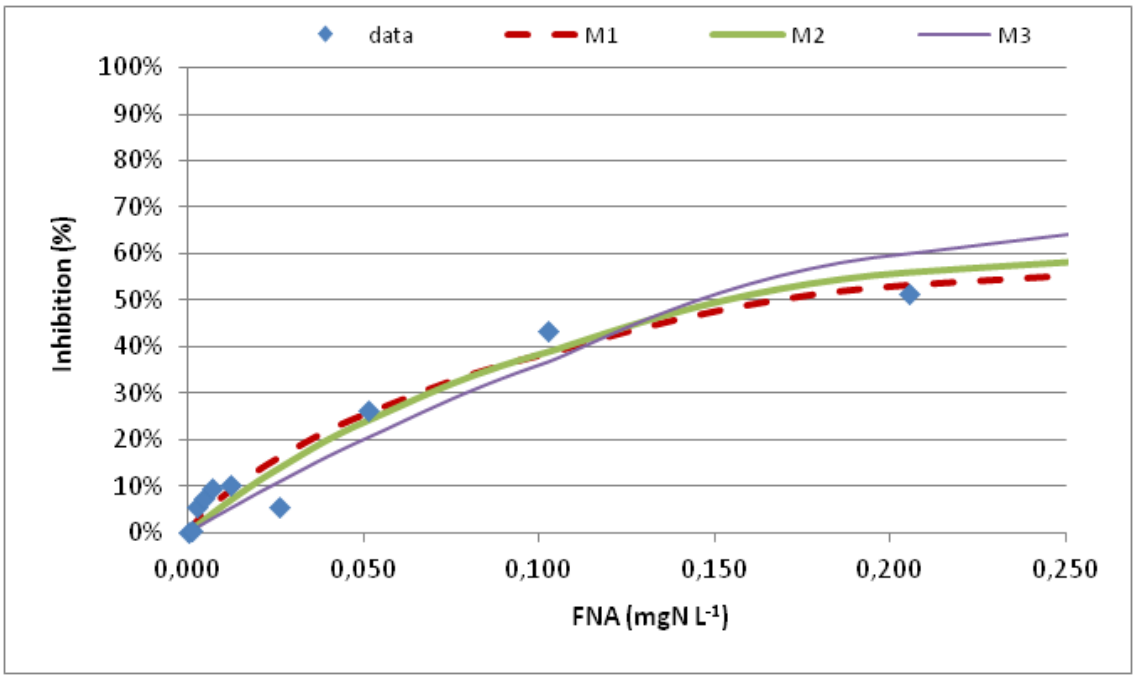


Figure 4.23 – Inhibition values at different FNA concentrations and 3 different models

In the pilot SBR, FNA concentration has always been lower than 0.01 mgHNO$_2$-N L^{-1}, so that we can consider that only FA inhibition could affect the ammonium oxidation rate. However, during the last experimental period, at the end of the aeration phase FNA was present at an average concentration of 0.002 mgHNO$_2$-N L^{-1}, corresponding to no more than 5% activity reduction (test B1), or even less, according to test B2.

Concentration of FNA up to 0.15 mgHNO_2-N L^{-1} are expected in a partial nitritation reactor (without denitrification) operating at 30°C, due to lower pH reached and higher nitrite concentrations.
The following equation can be used to estimate the overall inhibition on the nitrification rate due to both FA and FNA:

$$v_{obs} = v_{max} \times \frac{1}{1 + \left(\frac{FA}{kIa}\right)^{kIb}} \times \frac{kI_{FNA}}{FNA + kI_{FNA}}$$

Considering the FA and FNA concentrations across the nitrification phase (Figure 4.20 and Figure 4.21) it is possible to estimate the overall AOB inhibition (1-v_{obs}/v_{max}) during the experimentation. During periods 3 to 6 the inihibition resulted 10±6% at the beginning of the aeration phase and 5±5% at the end of the aeration phase, thus always lower than 20%. While, at the end of period 1 and in period 2 the estimated overall inhibition reached levels of 70-80%. The main AOB inhibition factor during the experimentation was always free ammonia.
Assuming the non competitive inhibition model, it is possible to make a comparison with other results reported in the literature (Table 4.10). The highly scattered values found in literature indicate that the biomass acclimation and composition must be considered to choose appropriate values for these parameters.

Table 4.10 - Comparison between obtained and some literature values for kI_{FNA} and kI_{FA}

Reference	kI_{FA} (mgNH_3-N L^{-1})	kI_{FNA} (mgHNO_2-N L^{-1})
This study	163	0.16
Pambrun et al., 2006	241	0.03
Ganigué et al., 2007	605	0.5
Wiesmann, 1994 (pure culture)	656	0.87
Jubany, 2007 (acclimated)	93	0.45
Jubany, 2007 (not acclimated)	7	0.06
Magrí et al., 2007	45.8	0.24

4.4.6 Cycle profiles

A detailed cycle monitoring test has been performed during period 6 (T = 25°C), at day 196, by taking samples at 15-30-minute intervals and by measuring ammonium, nitrite, nitrate and COD concentrations (Figure 4.24). During the first 37 minutes, 60L of influent (1616 mgNH_4^+-N L^{-1}) have been loaded. At the beginning of the second anoxic phase acetate has been dosed, corresponding to an increase of 88mgCOD L^{-1} in the mixed liquor.

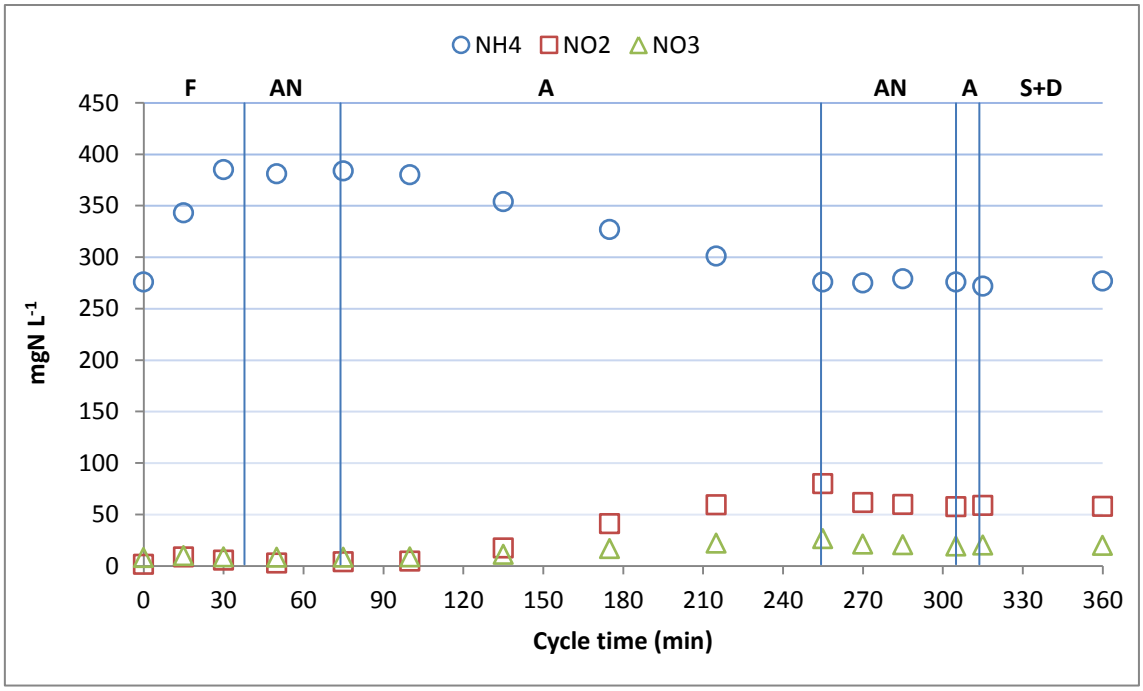


Figure 4.24 –Soluble N compounds concentrations during a cycle (period 6, day 196): F: filling phase – AN: anoxic phase – A: aeration phase – S+D: sedimentation and discharge

The N consumption/production rates during the aeration phase were analysed in details. In Figure 4.25 the concentrations of NH_4^+-N, NO_2^--N, NO_3^--N are depicted. During the first 25 minutes, consumption of COD is high, while nitrification activity is low. The nitrification rate in the first 25 minutes is 4 times lower than the rate measured afterward. This confirms what we observed in the respirometric batch tests: the nitrification activity in the presence of heterotrophic activity is reduced, probably due to oxygen limitation under high COD/N conditions which favour heterotrophs over autotrophs.

The calculated NH_4^+-N consumption rate (after the first 25 minutes) is 40 mgN L^{-1} h^{-1} corresponding to an activity of 5.2 mgN $gVSS^{-1}$ h^{-1}, the related NOx-N production rate is 37 mgN L^{-1} h^{-1} corresponding to an activity of 4.7 mgN $gVSS^{-1}$ h^{-1}. The difference (4%) is of the same magnitude of the analytical error; however, it can also be ascribed to some ammonia stripping and/or simultaneous denitrification or bacterial uptake for growth. If the overall average rate is measured by the difference in NOx-N between the starting time (75') and the end (265'), the resulting value would be 10% lower than the actual denitrification rate.

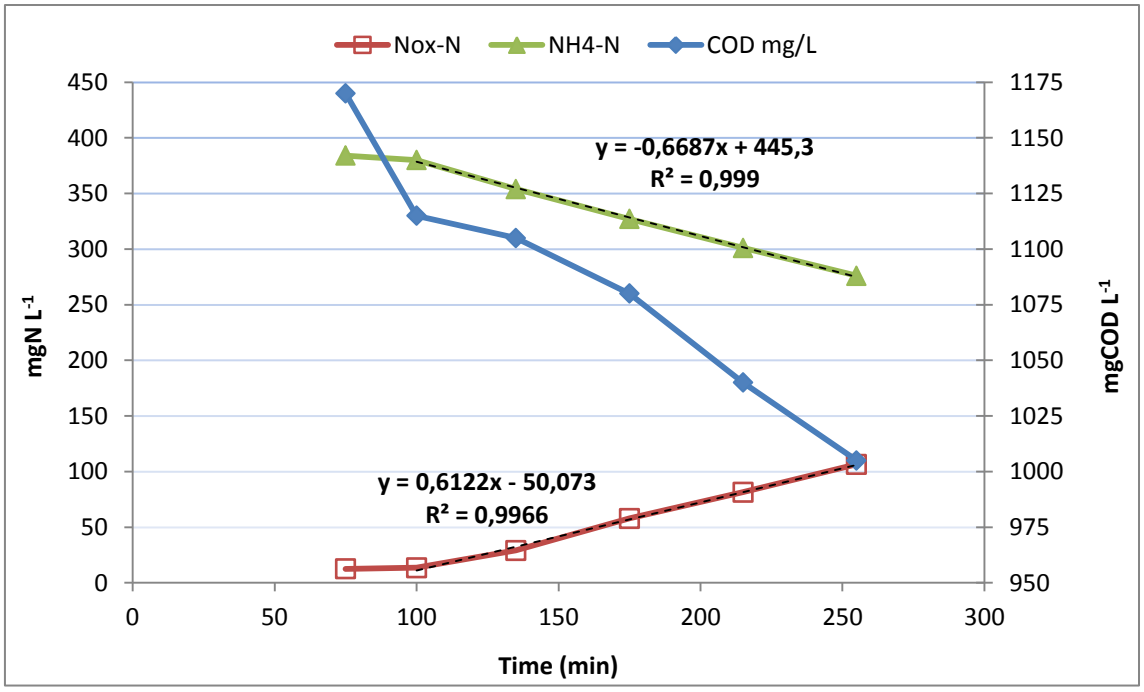


Figure 4.25 – NH_4^+-N, NO_x-N (NO_2^--N + NO_3^--N), sCOD concentrations during the aeration phase

During the second anoxic phase, 23 mgNO_2^--N L^{-1} and 7 mgNO_3^--N L^{-1} are denitrified with 88 mgCOD L^{-1} of acetate dosed. The resulting COD/N ratio is 3.0. Denitrification during this phase was not complete because of bCOD limitation.

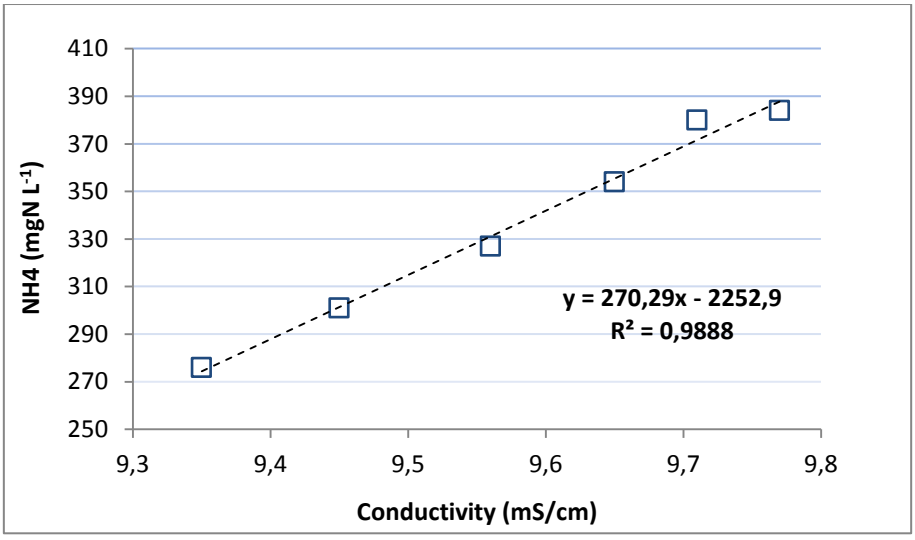


Figure 4.26 – Linear relationship between ammonium concentration and conductivity (at 22°C) during the nitrification phase

In Figure 4.26 the relationship between the ammonium concentrations and the conductivity measured during the nitrification phase are reported. The linear regression curve fits data very well (R^2=0.988). As already reported by Fux (2003), Gut et al. (2006) and Joss et al. (2009), conductivity could be a useful parameter when ammonium concentration is a direct or indirect indicator of bioreactor performance (for example in an anammox reactor). In the usual range of

ammonia concentration in water and wastewater (< 1 mol L^{-1}). The conductivity increases linearly with ammonia (Shcherbakov et al., 2009). Theoretically, 100 mgNH_4^+-N L^{-1} correspond to 0.53 mS cm^{-1} at 25 °C (CRC Handbook, 1995, cited by Fux, 2003). Fux (2003) reports an increase of 0.74 mS cm^{-1} for an increase of 100 mg L^{-1} of ammoniacal nitrogen and Joss et al. (2009) 0.79±1.1 mS cm^{-1} for the same increase of ammoniacal nitrogen concentration. In our test (Figure 3.26), the increase is lower, only 0.37 mS cm^{-1} for an increase of 100 mg L^{-1} of ammoniacal nitrogen. This difference can be due to the fact that our wastewater has more than the double background salinity of the average values in urban digester supernatants. Therefore, periodic calibration should be performed to obtain information about ammonium concentration in each specific treated wastewater characterized by different background salinity as suggested also by Joss et al. (2009).

4.4.7 N_2O emissions

Preliminary off-gas emission measurements were performed during the first 60 minutes of the aeration phase (5 samples every 10-15 min). Two cycles have been analysed at day 181 (test 1) and at day 196 (test 2). The concentrations obtained are reported in
Figure 4.27. All samples were analyzed with micro-GC. For test 1, samples at minutes 30 and 55 were measured also with FTIR spectroscopy and the values reported in the figure are the mean of the two instrument results (1148±68 ppm and 314±22 ppm).

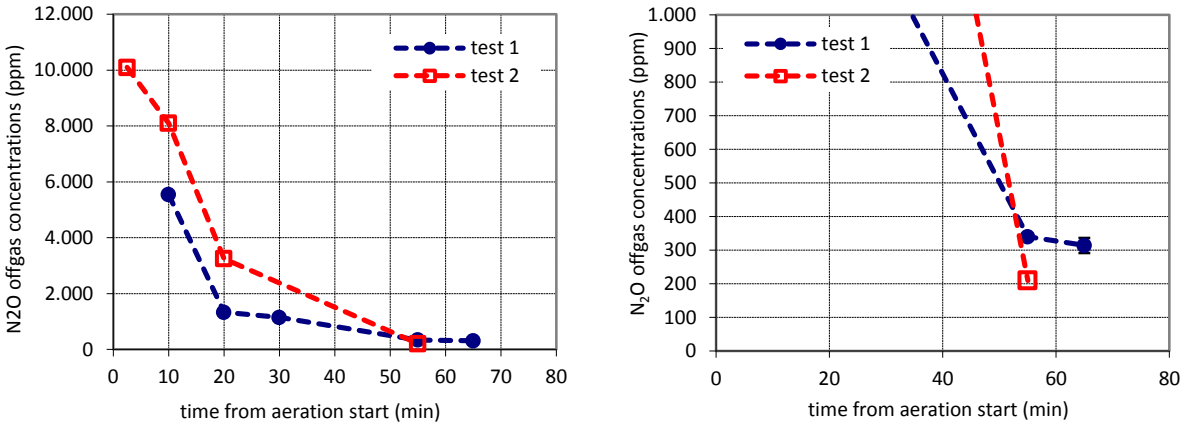


Figure 4.27 – Nitrous oxide emissions measured during the aeration phase in 2 different cycles (left) and detail on the last sample (right)

The N_2O mass was mainly emitted in the first 20 minutes of aeration and it may be related to a stripping phenomenon of the N_2O accumulated in the liquid during the anoxic phase. In fact, a low bCOD/N ratio, is known to increase N_2O emission during denitrification, as reported by many authors (e.g.: Itokawa et al., 2001). By comparing the two tests (Table 4.11), it results that higher N_2O emissions are related to test 2 when the COD/N ratio during the first anoxic phase was 1.3, while in test 1 the COD/N ratio was 1.6. Considering the whole cycle (including acetate addition), the COD/N ratios result 2.6 and 1.8 for test 1 and test 2, respectively. The COD/N ratios are here

assessed by calculating the COD removed over the nitrogen removed. N_2O concentrations after 55 minutes from the beginning of the aeration resulted to be 340 ppm and 210 ppm for test 1 and test 2, respectively. Even if the contribution of nitrification seems to be lower in this case, it is still significant and higher in test 1. The nitrite concentration during nitrification reached higher values (180 $mgNO_2^-$-N L^{-1}) in test 1. Moreover, during test 1 the DO concentration during aeration was 0.7 mgO_2 $L^{-1}$and 0.9 mgO_2 L^{-1} during test 2. Other authors claim that conditions favoring N_2O emission during nitrification are high nitrite concentrations and oxygen limitation, which can be caused by high organic loading rates in combination with limited aeration capacity, but can also occur due to mass transfer limitations in dense granules or large sludge flocs, as in our case (Kampschreur et al., 2009).

Table 4.11 – Results of two N_2O sampling test during the first hour of aeration and reactor condition

	Parameter	Test 1	Test 2
	Day	181	196
	NLR (gN L_{react}^{-1} d^{-1})	0.53	0.55
	NRR (gN L_{react}^{-1} d^{-1})	0.43	0.46
	N_2O emitted (%N load)	14	20
	COD/N*	2.6	1.8
1st anoxic phase	**COD/N**	1.6	1.3
	NO_2 (mgN L^{-1})	87-20	58-3
	NH_4 (mgN L^{-1})	230	384
	DO (mgO_2 L^{-1})	0	0
Aeration phase	**NO_2 (mgN L^{-1})**	21-140	4-80
	NH_4 (mgN L^{-1})	232-128	384-276
	DO (mgO_2 L^{-1})	0.7	0.9

*Ratio between the COD removed and the N denitrified considering the whole cycle

In Table 4.11, the main experimental conditions during the two sampling campaigns are reported. The range indicated for NO_2^--N and NH_4^+-N concentrations are the values at the beginning and at the end of each phase. The overall N_2O emission was estimated by integrating the measured N_2O values during time and by multiplying this value by the aeration flow-rate (5 m^3 h^{-1}). In these calculations the assumption is made that N_2O emissions during the non-aerated phases are negligible. Moreover, the N_2O emitted during the last part of the aeration period and during the 10 minutes of post-aeration have not been considered. The N_2O emitted corresponded to 14% and 20% of the treated nitrogen load for test 1 and 2 respectively.

Kishida et al. (2004) studied N_2O emissions from piggery wastewater treatment in two SBRs (nitrification-denitrification mode). They found a total N_2O emission for the denitrification stage of

1.71% of the treated nitrogen while working with influent BOD_5/TN of 4.5 that increased to 17.7% with BOD_5/TN in the influent of 2.6. In our case the average estimated BOD_5/N of the influent during period 6 was 0.8±0.4 and, by considering the acetate added, the ratio becomes 2.0±0.3.
More tests on N_2O emissions are needed to verify if increasing COD/N ratio in denitrification by external carbon addition would reduce the N_2O emissions.
To better understand the mechanism of N_2O production and confirm our hypothesis, dissolved N_2O concentrations analyses during the whole cycle should be performed allowing to set out a complete mass balance.

4.4.8 Microbiological results

In Figure 4.28, an optical microscope picture of a biomass sample taken from the reactor at day 200 is reported. In the image, it is possible to see flocs of different size, most of them ranging from 0.5 to 2 mm.

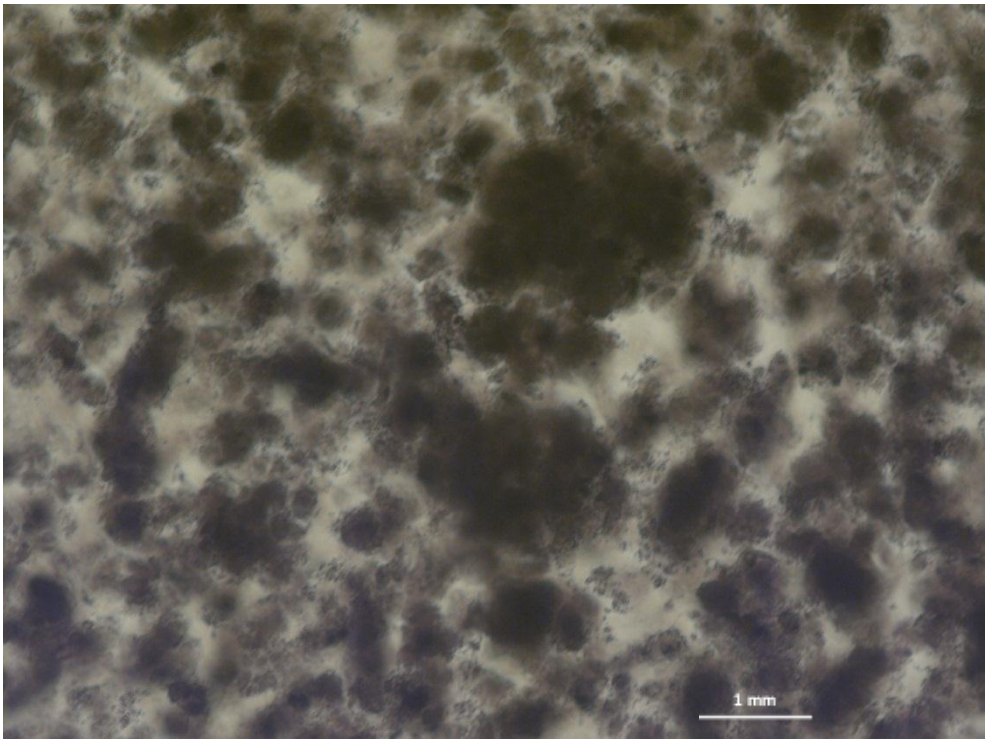


Figure 4.28 - Optical microscope picture of the flocs at day 200 (reference scale 1mm)

FISH analyses confirmed that the inoculum was already rich in AOB clusters and few NOB bacteria (belonging to the genus *Nitrobacter*) have been detected. In all the subsequent samples, the presence of NOB population was scarce or in negligible quantity confirming the success in maintain a stable nitritation process.
InTable 4.12 FISH images collected in different days are shown. Observations on the same sample compared the results obtained from the probe NSO190 for AOB-β-Proteobacteria with the results obtained from the probes EUB338I+EUB338II+EUB338III, which can detect most bacteria. AOB-β-Proteobacteria includes *Nitrosomonas* and *Nitrosospira.*

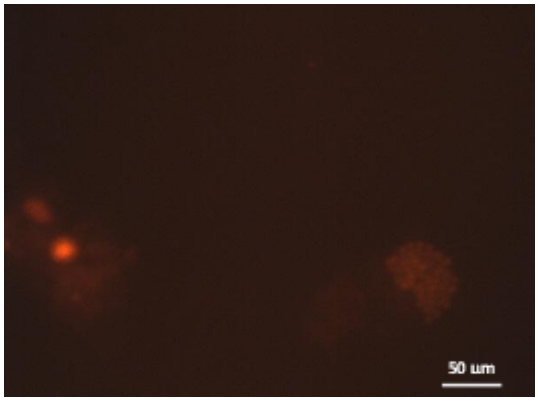
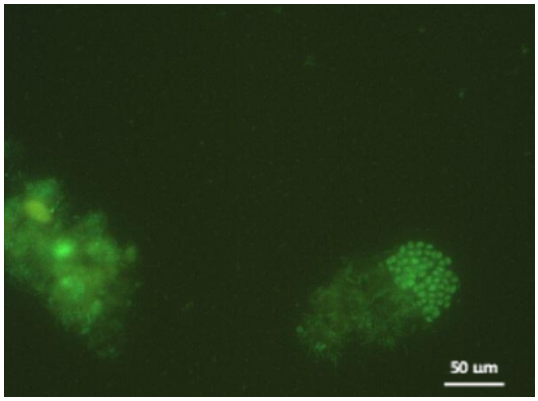
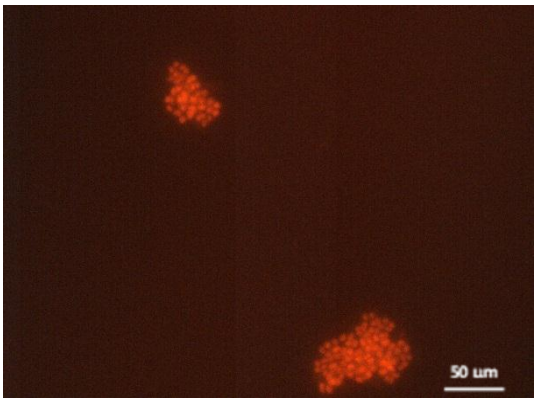
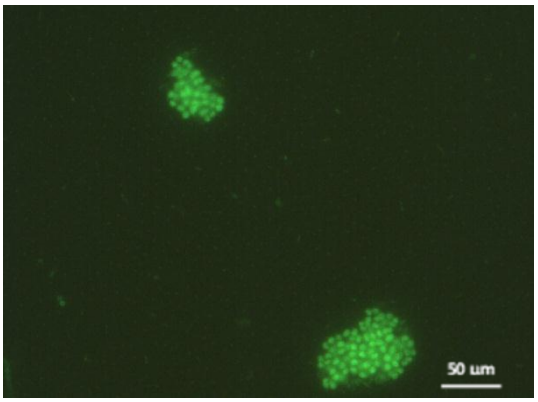
Observations made with probes Nsm156 and Nsv443 revealed the dominant presence of *Nitrosomonas* genus in all the analyzed samples.

As already commented before, the inoculum was rich in AOB bacteria, as the first two pictures show clearly.

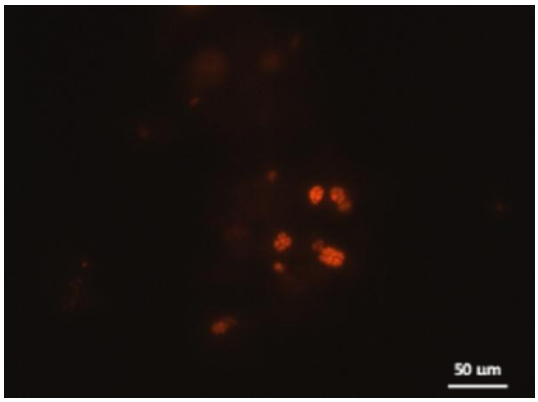
At day 67, when the efficiency of the reactor was poor because of low biomass concentration, the AOB population was still active and accounted for an important fraction of the total active bacteria.

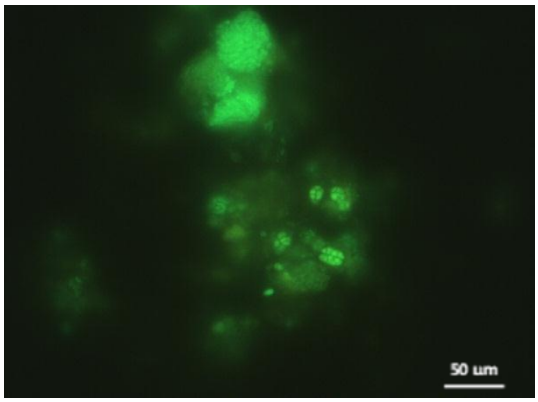
At day 77 the reactor has been re-inoculated and the AOB fraction decreased because the AOB-rich sludge was blended with conventional activated sludge. At day 162 (period 5) and 175 (period 6) AOB bacteria were still present and active but their clusters were in the inner part of larger flocs mostly made by other bacteria. This further observation supports the hypothesis that AOB suffered by oxygen limitations, and heterotrophic biomass in the external part of the floc was active and growing fast, due to the high biodegradable organic carbon load.

Table 4.12 – FISH images at different experimental time comparing AOB bacteria with most of bacteria (ref. scale 50µm)

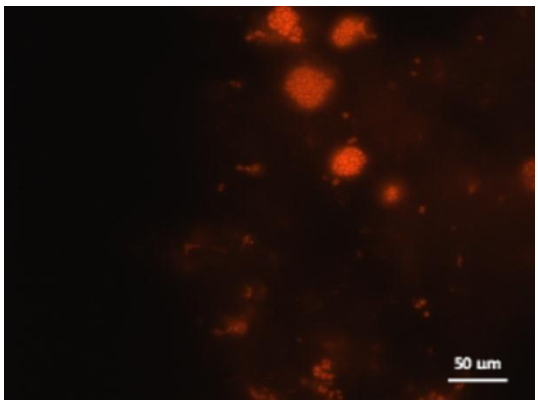
Day	AOB-β-Proteobacteria (probe NSO190)	Most Eubacteria
0 (INOCULUM)		
0 (INOCULUM)		

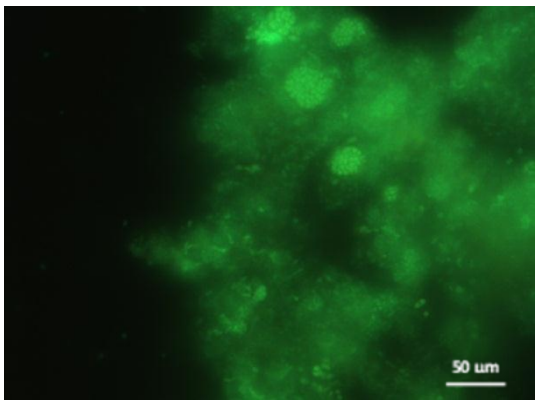
21
(PERIOD 1)



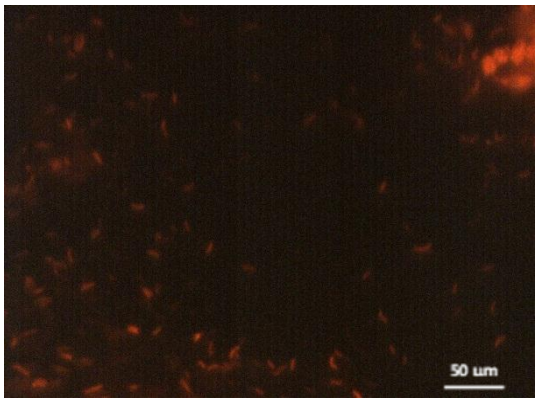


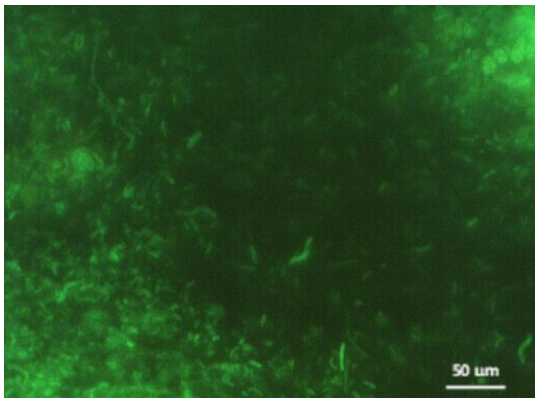
67
(PERIOD 2)



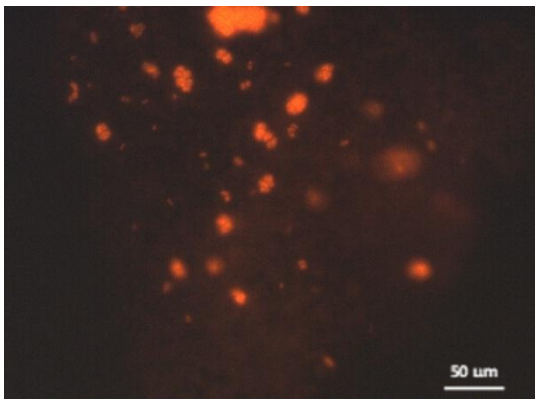


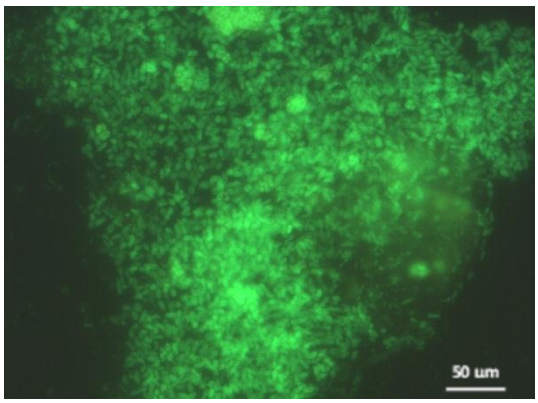
77
(PERIOD 2)



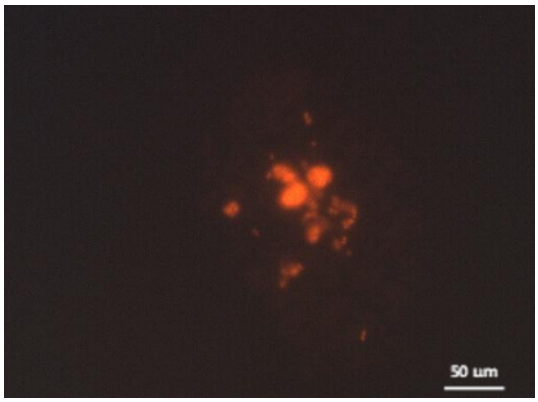


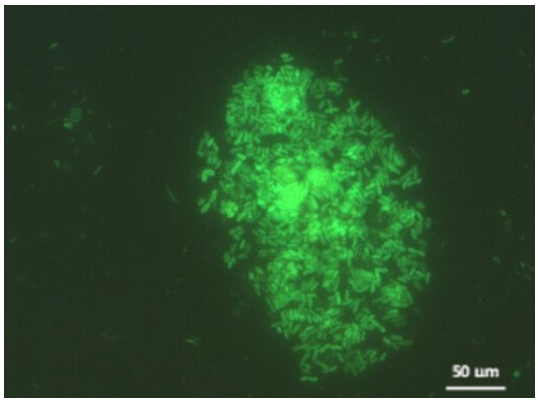
162
(PERIOD 5)





175
(PERIOD 6)





4.5 Discussion and conclusions

In Table 4.13, main results obtained in the different periods of the experimentation are summarized.

Table 4.13 – Summary of main results during the different experimental periods

Parameter	Period 1	Period 2	Period 3	Period 4	Period 5	Period 6
Experimental days	0-28	29-78	79-105	106-134	135-168	169-204
SVI (mL $gTSS^{-1}$)	39±15	19±12	99±49	81±8	56±17	84±28
MLTSS (gTSS L^{-1})	2.3±0.4	2.2±1.4	6.3±0.2	6.6±0.9	9.9±1.3	9.7±0.5
MLVSS (gVSS L^{-1})	1.7±0.3	1.6±1.0	4.6±0.2	5.1±0.8	7.6±1.0	7.4±0.3
effluent NH_4^+ (mgN L^{-1})	333±317	915±287	155±101	109±110	254±72	143±154
effluent NO_2^- (mgN L^{-1})	140±109	9±3	252±150	87±101	24±22	122±167
effluent NO_3^- (mgN L^{-1})	26±11	7±2	57±23	24±27	10±4	34±37
effluent COD (mgCOD L^{-1})	1358±117	1448±317	1478±285	1275±338	1129±364	1403±260
% solubleN rem	63±19	32±25	54±16	77±19	66±7	80±12
% COD rem	31±16	42±11	26±7	35±17	67±18	41±24
Specific AOB activity (mgN $gVSS^{-1}h^{-1}$)	n.d.	4.0±3.1	n.d.	12.4±6.1	11.8±1.3	9.8±2.0
Nitrification rate (mgN $gVSS^{-1}h^{-1}$)	27.6±9.5	4.4±2.3	4.9±1.3	7.0±2.3	3.0±0.9	4.3±0.9
NO_2/NOx produced (%)	87±8	-	73±18	86±3	90±3	79±5

Period 1: start-up of the pilot SBR. In the first phase the SRT was maintained low, around 4-5 days to avoid NOB growth. As a matter of fact, we observed a rapid inhibition of the NOB biomass as 90% nitrogen was converted to nitrite in the oxidation phase after one week from the start-up. FISH analyses confirmed also that the inoculum was poor in NOB. Then the load in the SBR has been increased and the HRT was decreased from 3 to 2 days. The average nitrogen removal

efficiency was 63±19% The specific nitrification rates at the beginning of this period were high (27.6±9.5 mgN $gVSS^{-1}h^{-1}$) due to the initial high fraction of active biomass and the low SRT. Moreover, the influent COD/N (lower than the following periods) limited the heterotrophic growth.

Period 2: In this period a biomass washout was registered; biomass concentration dropped down to 0.7 gVSS L^{-1}. Moreover, probably a concurrent AOB inhibition happened due to the increase in the feeding of poultry manure in the digesters. Nitrogen removal efficiency drop and consequently concentrations of FA up to 280 mgNH_3-N L^{-1}accumulated in the reactor contributing to inhibit the AOB population.

Period 3: This was a transition period, as the reactor was re-inoculated and operated without any wastage of excess biomass in order to increase the SRT and the biomass concentration in the SBR. In this period, denitrification was performed just on the internal carbon and with almost no external carbon dosage. As COD/N ratio was lower than the stoichiometric ratio requested for denitrification, only 54% N removal was achieved, on average. Nitrification performed well, as 80% of the influent nitrogen was oxidized during the aerobic phase, of which 70% was converted into NO_2–N and 30% into NO_3–N, confirming that NOB population could grow because of the higher SRT.

Period 4: The influent composition was stable and no poultry manure was fed in the digester and in this period the best DENO2 performance was achieved. The average NH_4-N concentration in the influent was 900±63 mgN L^{-1} and the COD/N ratio 2.0±0.5. The SRT was kept around 20 days and an average load around 0.44 gN L^{-1}_{react} d^{-1}. N removal was 77±19% reaching 95% at the end of the period. The nitritation efficiency (NO_2/NO_x) increased to 86% on average.

Period 5: The influent changed because of different substrates fed to the digester (poultry manure), and, in addition, temperature in the digester varied and the process lost in efficiency. As a consequence of the incomplete digestion the liquid fraction of the digestate had higher COD/N and BOD_5/COD ratios. The average DO concentration in the reactor was increased to 0.75 mg/L but the nitrification process was still limited by oxygen availability. Nevertheless, thanks to the high SRT, the process coped with the new operational conditions and the N removal efficiency dropped by 10%, only.

Period 6: During this period, the digester worked under variable conditions of efficiency. The COD/N of the DENO2 influent was highly variable. The main operational variation was the working temperature of the SBR that have been decreased to 25°C. The N removal efficiency was 80±12% with peaks at 96% with a constant NLR around 0.5 gN L^{-1}_{react} d^{-1}. The nitritation efficiency (NO_2/NO_x) decreased from the previews period from 90% to 80%.

Piggery wastewater treatment with conventional biological process in SBR configuration is not uncommon (Magrí et al., 2007). The main issue when applying conventional nitrification-

denitrification process to digestate treatment is the low C/N ratio in the influent, causing the need of external carbon dosage to support denitrification. Though for simplicity of supply in this study acetate was used, an economic alternative could be the dosage of fresh pig slurry as carbon-source as reported by some lab-scale experimentations. For instance, Obaja et al. (2005) obtained 99% nitrogen removal efficiency in a lab-scale SBR with diluted digested piggery waste. Deng et al. (2007) confirmed the feasibility of adding raw wastewater in a full-scale SBR treating piggery digestate. Eum and Choi (2002) proposed nitrification via nitrite pathway working with diluted piggery waste (not digested) in a lab-scale configuration, obtaining 80-90% N removal efficiency with applied NLR of 0.13-0.53 gN $L^{-1}_{reactor}$ d^{-1}. Dosta et al. (2008) proposed a coagulation/flocculation step on the liquid fraction of anaerobically digested piggery wastewater before COD and nitrogen removal in a SBR working in nitritation-denitritation mode. In lab-scale tests with diluted wastewater, they obtained more than 98% total nitrogen removal. Inhibition of NOB was achieved by high free ammonia concentrations and by keeping dissolved oxygen concentration below 1 mgO_2 L^{-1}.

To our knowledge, there is no literature available about pilot-scale continuous experimentations treating piggery digestate without dilution or pretreatment in a nitritation-denitritation mode. Moreover, in our case we have treated the liquid fraction of a digested mixture of piggery primary sludge, poultry manure and other fractions of agro-wastes. Full scale applications could provide a significant reduction of the operational costs of digestate disposal. The $DENO_2$ process in SBR configuration confirmed to be a technically feasible option to treat liquid fraction of agricultural digestate despite the high influent variability. In the case of the piggery farm that hosted this experimentation, the nitrogen flow that is presently recycled back to the main stream WWTP, account for approximately 260 kgN d^{-1}, which corresponds to 34% of the total N treated by the WWTP. After more than 200 days of experimentation, with a pilot–DENO2 SBR treating the liquid fraction of agricultural digestate, the following conclusions can be drawn:

- the influent characteristics were highly variable because of seasonal variation in the piggery waste production, variation in the co-substrates fed to the digester in addition to the piggery wastewater (maize, wheat, poultry manure) and unstable anaerobic digestion efficiency;
- FISH analyses confirmed that the inoculum taken from the activated sludge basin of the main wastewater treatment plant was already rich in AOB biomass; in the SBR, NOB were almost always absent, while the heterotrophic fraction increased during time in accordance with the increase in the influent COD/N ratio;
- a stable nitritation efficiency has been maintained: in fact, the NO_2/NOx ratio at the end of the aeration phase remained between 80 and 90%, even at 25°C, at an SRT of 25 d and at an oxygen concentration of 0.75-1 mg L^{-1} DO;
- working at high SRT (20-25d) allowed to cope with influent variability (COD/N) reaching N-NH_4 removal from 60-70% up to 90-95%;
- 50% of the C-source for denitrification was on average already available in the influent, so a step-feed option for future implementation is suggested to exploit the internal biodegradable organic carbon;

- During the last two periods the measured nitrification rate in the reactor was lower than the maximum estimated AOB activity with respirometric tests. This because of oxygen limitation in case of simultaneous heterotrophic activity. The shape of the OUR curve for different DO concentrations for AOB in case of concurrent heterotrophic activity fitted well with a S-shaped curve. This oxygen limitation is probably due to diffusion mechanisms in the flocs. FISH analyses confirm the presence of heterotrophic biomass in the external part of the flocs and AOB biomass in the inner part, especially during period 5 and 6;
- A reduction of only 10% of the nitritation activity was registered in a respirometric test run with the wastewater treated compared to a blank with tap water, suggesting that the digestate liquid fraction did not have significant inhibitory effects on AOB activity;
- Free ammonia (FA) and Free Nitrous Acid (FNA) inhibition on AOB were assessed with DO-stat and pH-stat respirometric tests at increasing ammonium and nitrite concentrations. The best-fitting curve for FA inhibition resulted to be the S-shaped (extended non-competitive inhibition model) and k*I* is the model estimation of the IC_{50} value and in our experimentation resulted 148±5 $mgNH_3\text{-}N\ L^{-1}$. For FNA case, the best fitting curve was the conventional non competitive inhibition model and kI_{FNA} was 0.16±0.02 $mgHNO_2\text{-}N\ L^{-1}$. According to these data, during period 3 to period 6, AOB activity reduction due to FA and FNA inhibition was averagely 8±4% and always lower than 20%. The main inhibition factor during the experimentation resulted free ammonia;
- N_2O emissions accounting for the 14-20% of the N treated have been detected in preliminary tests (sampling along the first 60 minutes of the aeration phase) and may be caused by the low C/N during the pre-denitrification phase and can hopefully reduced by increasing the external carbon dosage. Should those high emissions of N_2O be confirmed, the environmental feasibility of this process in this configuration may be questionable.

5 ANAMMOX ENRICHMENT FROM CONVENTIONAL SLUDGE SAMPLES

The aim of this chapter was to verify the efficacy of a simple, low-tech fed-batch procedure for anammox enrichment coupled with activity measures. Results of an experimental campaign on 6 sludge samples collected from Italian wastewater treatment plants are here presented. Three of the samples were later mixed and used to inoculate a SBR reactor fed with synthetic wastewater.

5.1 Simple fed-batch procedure for Anammox enrichment

5.1.1 Inocula

The origin of the sludge samples (named S1 …S6) used as enrichment inocula is the following:
- S1: suspended anaerobic sludge from the sludge digester of a large municipal WWTP (750,000 p.e.), located in Monza (Lombardy);
- S2: Granular anaerobic sludge from a UASB (Up Flow Anaerobic Sludge Blanket) reactor treating the effluent from a yeast production factory, located in Lombardy;
- S3: activated sludge from the denitrification basin of a WWTP treating the supernatant from the anaerobic digestion of manure from a 20,000-swine farm located in Lombardy.
- S4: suspended anaerobic sludge from a sludge digester of a large municipal WWTP (600,000 p.e.), located in S. Colombano (Tuscany);
- S5: activated sludge from the denitrification basin of the S. Colombano WWTP;
- S6: activated sludge from the denitrification basin of a medium-size municipal WWTP (70,000 p.e.), located in Pistoia (Tuscany).

5.1.2 Mineral medium (Synthetic wastewater)

The mineral medium used during the experimentation in chapters 5-6-7 was prepared as summarized in Table 5.1 and it is similar to other commonly used medium suggested in the literature to grow anammox bacteria (e.g. Van de Graaf et al. , 1996 and Lopez et al., 2008).

Table 5.1 Mineral medium composition

Mineral medium	
$NaHCO_3$ (g L^{-1})	1.05
KH_2PO_4 (g L^{-1})	0.0625
$CaCl_2*2H_2O$ (g L^{-1})	0.3
$MgSO_4*7H_2O$ (g L^{-1})	0.2
HCl 1N (mL L^{-1})	2
solution I (mL L^{-1})	1.25
solution II (mL L^{-1})	1.25

solution I	
EDTA (g L^{-1})	5
$FeSO_4$ (g L^{-1})	5
solution II	
EDTA (g L^{-1})	15
$ZnSO_4*7H_2O$ (g L^{-1})	0.43
$CoCl_2*6H_2O$ (g L^{-1})	0.24
$MnCl_2*4H_2O$ (g L^{-1})	0.99
$CuSO_4*5H_2O$ (g L^{-1})	0.25
$NaMoO_4*2H_2O$ (g L^{-1})	0.22
$NiCl_2*6H_2O$ (g L^{-1})	0.19
$NaSeO_3*10H_2O$ (g L^{-1})	0.16
H_3BO_4 (g L^{-1})	0.014

5.1.3 Enrichment procedure

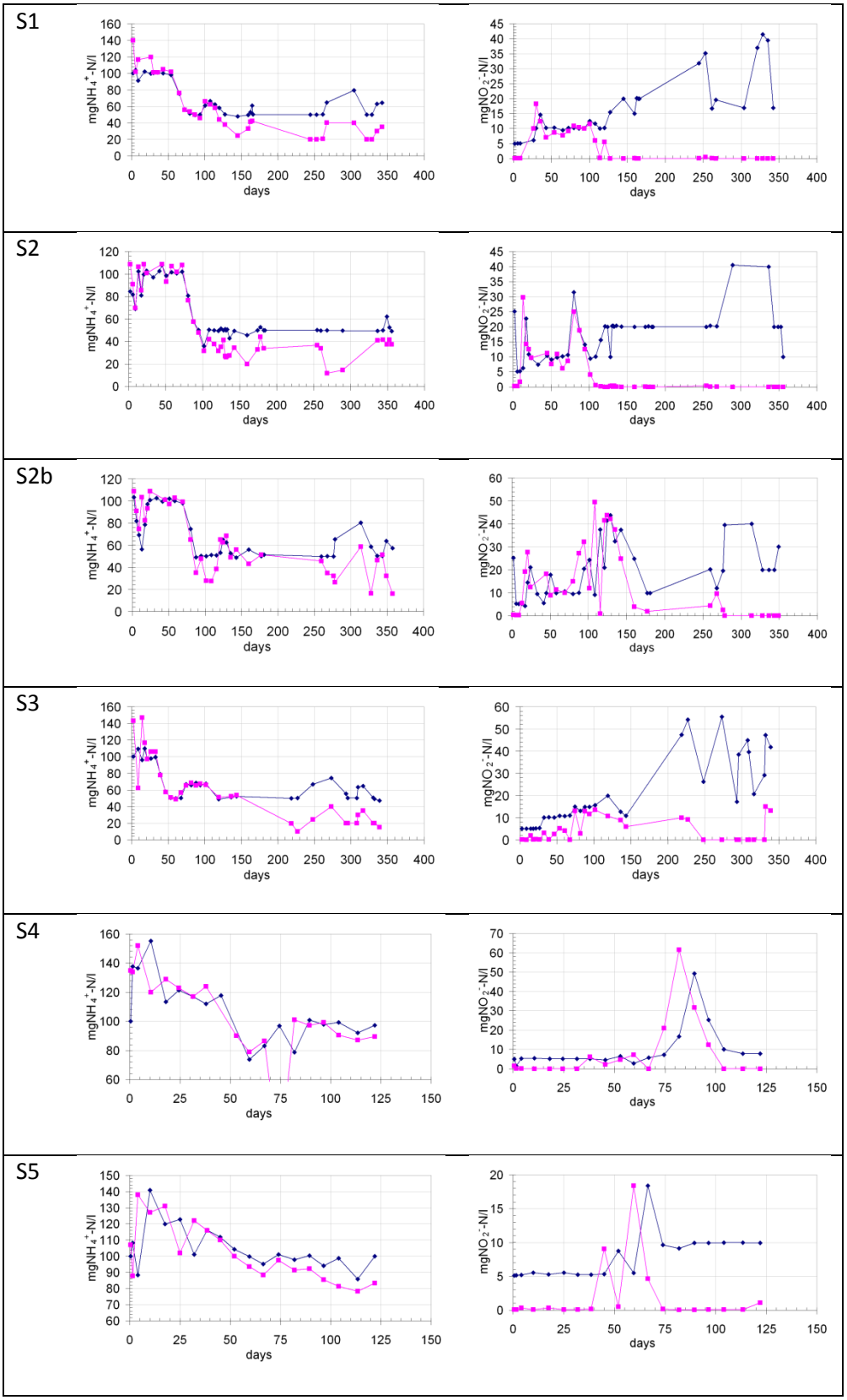
From each sludge sample, approximately 4 gTSS were drawn, settled and re-suspended in the mineral medium containing micro and macro-nutrients (Table 5.1) to a final volume of 1L. Each 1L-sample was poured into 1140 mL glass bottle (see par. 3.2.3). Before sealing, the sludge suspension was sparged with N_2 gas for 15 min. The main opening was closed by a screw cap with sealing gasket during the first part of the experimentation, while later this opening was equipped with a digital manometer for overpressure data logging. An overall of 8 samples were prepared, since S2 and S5 were started in double (S2 and S2b, S5 and S5b) and differentiated by the fact that, into samples S2b and S5b, 5 mg L^{-1} of hydrazine were dosed at each feeding during the first 50 days.

Figure 5.1 - Bottles for the enrichment placed in the incubator.

All bottles were kept in a thermostat (35-37°C), magnetically mixed and operated in a fed-batch mode. At defined time intervals (normally every 2 to 7 days), some supernatant was extracted and replaced by deoxygenated mineral medium so that a pre-defined experimental HRT was maintained (from day 1÷30: HRT = 14 d; from day 31÷90: HRT = 28 d); from day 90 onward, the HRT was increased to 112 d and sampling of supernatant and dosage of mineral medium were performed with a syringe through the rubber septa to prevent oxygen intrusions which might have occurred by opening the bottle. No sludge wastage was performed, but some sludge was removed with supernatant withdrawal; the resulting SRT was higher than 160 d. At each cycle, ammonium, nitrate and nitrite concentrations were measured and adjusted to their desired values by addition of concentrated stoke solutions. Finally, pH was also corrected to 7.5-7.7 and oxygen removed by N_2 flushing. During the first 50 days, ammonium and nitrate concentrations in each bottle were adjusted to 100 mgN L^{-1}. After 50 d, in S1, S2 and S3, ammonium and nitrate concentrations in each bottle were reduced by dilution with the mineral medium to values around 50 mgN L^{-1}. Nitrite concentration was adjusted at the beginning of each batch cycle to remain between 5 and 20 mgN L^{-1}. Higher concentrations were tested once a stable anammox activity was observed.
Anammox activity was periodically monitored by measuring the rate of N_2 production. A detailed description of this procedure can be found in par 3.2.2.

5.1.4 Batch cycles results

In Figure 5.2, ammonium and nitrite trends for the six sludge samples are presented.



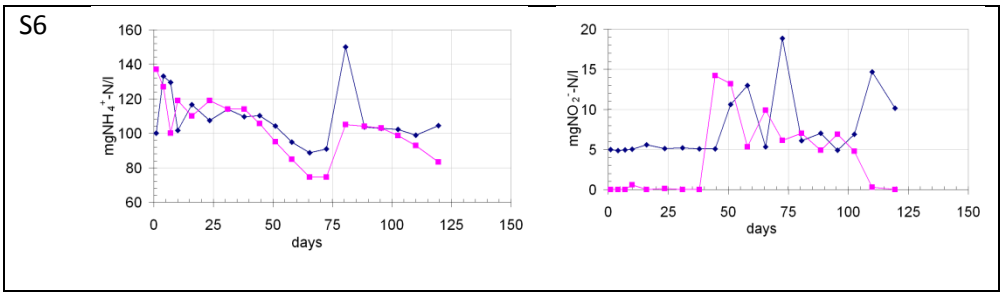


Figure 5.2 - Ammonium and nitrite trends during the anammox enrichment for the tested samples. Data series refers to the initial (diamonds) and final (squares) concentrations across each fed-batch cycle.

For most samples, typical patterns for the monitored parameters were observed. Initially, ammonium concentration at the end of each batch-cycle was normally similar or slightly higher than the initial value due to ammonium release by sludge hydrolysis. At the same time, denitrification of the solubilized organic matter took place, leading to nitrate consumption and to pH increase. As an intermediate of denitrification, nitrites built up occasionally. Later on, after a period of 20 to 60 days, depending on the sample, denitrification slowed down considerably as long as biodegradable organic matter was consumed, and concentrations of all nitrogen species showed limited variations across each cycle. Temporary increases in nitrite concentration accompanied by ammonium and pH decreases were observed and were likely due to the occurrence of nitrification that may have been supported by oxygen leakage. Finally, a slight and constant reduction of both ammonium and nitrite concentration was measured suggesting the occurrence of a measurable anaerobic ammonium oxidation. The molar ratio between nitrite and ammonium consumption stabilized during time around the expected stoichiometric ratio of 1.32 (NO_2^-/NH_4^+) suggesting that a prevailing anaerobic ammonium oxidation was finally reached, as reported in Figure 5.3 for sample S2.

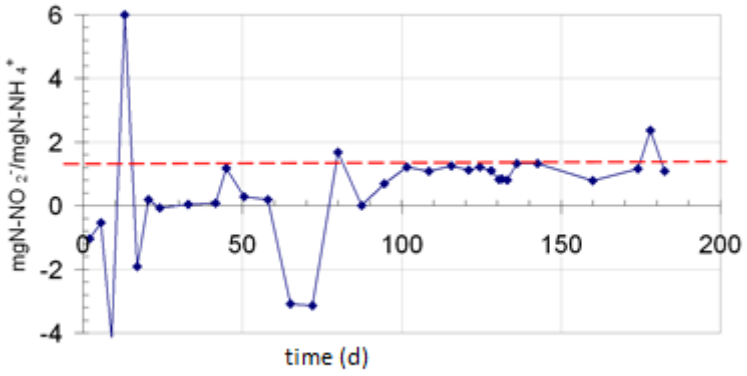


Figure 5.3 – Nitrite over ammonium removal ratio across each cycle in sample S2 (red line= stoichiometric value : 1.32)

The length of the lag-phase that preceded the show up of a clear anammox activity, i.e. before a concomitant ammonium and nitrite reduction was observed, varied among the tested sludge samples. Table 5.2 compares the lengths of this lag-phase with those reported in previous attempts (Dapena-Mora et al., 2004; Chamchoi & Nitisoravut, 2006; Dexian et al., 2007; Lopez et al., 2008; Tang et al., 2010). In all samples but S3, the first clear anammox activity had been

registered after about 100 days. Sample S3 showed the longest lag phase, with almost no ammonium reduction for the first 143 days. However, after a feeding break of about 70 days (from day 143 to day 218) a clear activity was finally evidenced. This sample was the only one coming from a treatment plant fed on piggery wastewater. This evidence may suggest that swine manure probably contains compounds, such as antibiotics or heavy metals, that limit the growth of anammox bacteria, reducing their cell density in the sludge sample used as inoculum.
Previous experiences reported lag times that were, in some cases, shorter although never below 50-60 days. The longer lag phase that was experienced in the present experimentation may be attributed to the sub-optimal cultivation conditions (e.g. variable pH and nitrate concentration, temporary oxygen leakages) that the proposed fed-batch set-up would offered when compared with chemostats or sequencing batch systems. However, set-up simplicity has its own advantages, the main one being the feasibility to work with several inocula in parallel.

Table 5.2 - Length of the lag-phase before a clear anammox activity is displayed.

Inoculum	Length of the lag-phase (d)	Reactor	Author
Municipal Activated Sludge	60	SBR	Dapena-Mora et al., 2004
Municipal Activated Sludge	98	SBR	Third et al., 2005
Different conventional sludges	120	SBR	Chamchoi & Nitisoravut, 2006
Methanogenic anaerobic sludge	54	SBR	Dexian et al., 2007
Mix different sludges	60	SBR	Lopez et al., 2008
Anaerobic granular sludge	83	UASB	Tang et al., 2010
S1: Suspended anaerobic sludge from the sludge digester	108	Semi batch	This experimentation
S2: Granular anaerobic sludge from a UASB	102		
S2b: Granular anaerobic sludge from a UASB (+hydrazine)	270		
S3: Activated sludge from the denitrification basin from a plant treating digestate from swine manure	220		
S4: Suspended anaerobic sludge from a sludge digester	110		
S5: Activated sludge from the denitrification basin	75		
S5b: Activated sludge from the denitrification basin (+hydrazine)	n.d.		
S6: Activated sludge from the denitrification basin	110		

In sample S2b and S5b, the effect of adding low concentrations of hydrazine was tested. The rational behind this idea was to verify whether this compound could help selecting the anammox

microorganisms, within those present in the sludge inoculum, by exerting a selective inhibition of heterotrophic bacteria. Being hydrazine a highly reactive compound it is quoted to be toxic for activated sludge at concentrations around 1 mg L^{-1} (WHO, 1991). On the other hand, anammox are known to store hydrazine within their cell and hydrazine spikes are known to help restoring anammox activity during nitrite accumulation (Strous et al., 1999, Third et al., 2005). However, experimental results from this enrichment campaign did not support this hypothesis. As shown in Figure 5.2, a stable ammonium reduction in sample S2b (with hydrazine) was observed later than in sample S2 (without hydrazine), although this may also be caused by a leakage of oxygen that may have occurred around day 110 as suggested by the concomitant decrease in ammonium and increase in nitrite concentration. Similarly, no clear evidence of anammox activity could be observed in sample S5b (data not shown) after 125 days of enrichment.
FISH analyses made on sample S1, S2, S2b and S3 (30 days after the first anammox activity signals), confirmed the presence of anammox bacteria. All the anammox bacteria found in the tested samples belong to Candidatus Brocadia anammoxidans. In sample S1 few cluster belonging to *Candidatus Kuenenia Stuttgartiensis* were also identified.

5.1.5 Activity test results

Activity measurements were started as soon as ammonium reduction was observed. These measurements allowed to verify the time required for completion of nitrite reduction, as indicated by a sharp decrease in the cumulated gas production curve (Figure 5.4). An increase in the gas production rate during the cultivation time was observed, indicating an increase in anammox activity. Moreover, peculiar behaviors, such as a remarkably long lag-phases in gas production after a prolonged endogenous period (due to the summer break) could be easily detected, thus assisting in load adjustment to fit actual biomass degradation capacity.

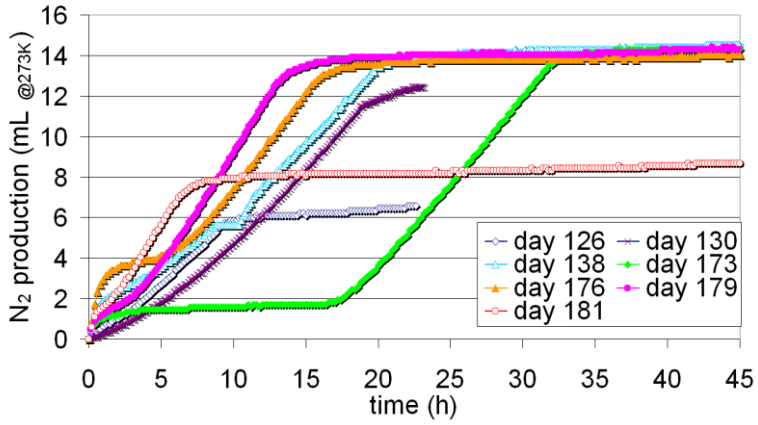


Figure 5.4 - Cumulated gas production curves for S2 sludge at various enrichment time (day 126→181) in response to the addition of 10 to 20 mgNO_2^--N. The lag phase observed at day 173 is likely due to the 18 days endogenous period for summer break.

In Table 5.3, the values of the anammox activity that was measured approximately 2-3 weeks after the first appearance of the anammox activity (suggested by ammonium and nitrite simultaneous consumption), are summarized. These values were calculated from the maximum slope of the cumulated N_2 gas production in time. It is worth noting that all activities were of the same order of magnitude in all samples and around 17-70 $mgNH_4^+$-N $L^{-1}week^{-1}$. Such a removal capacity is quite surprising since it should have led to an earlier measurable disappearance of ammonium in the fed-batch reactors, considering the slow growth rate of these microorganisms. A possible explanation of this quite sudden appearance of the anammox metabolism could be that, as suggested by Strous et al. (1999), the anammox cells are fully active when their cell concentration is higher than 10^{10}-10^{11} cells mL^{-1}. This may lead to the fact that once this minimum quorum is approached, anammox activity rises suddenly and faster than what expected from their duplication time.

Table 5.3 - Anammox activity as calculated from N_2 production rates in batch manometric tests.

Sample	Enrichment time (d)	Anammox activity ($mgNH_4^+$-N $L^{-1}h^{-1}$)
S1	132	0.13
S2	126	0.42
S4	243	0.10
S5	108	0.25
S6	125	0.10

An attempt can therefore be made to assess the Anammox cell density in the fed-batch reactors from the Anammox activity measurements. This calculation can be performed by assuming that:
(i) the maximum ammonium removal rate from literature is around 45 nmol min^{-1} (mg protein)$^{-1}$ (Strous et al., 1998),
(ii) the Anammox cell volume is that one of a sphere of 0.8 μm of diameter (Van Niftrik et al. 2008),
(iii) the protein cell density in the Anammox cell is 0.6 g_{prot}/ $g_{biomass}$ (derived from Strous et al., 1998),
(iv) the settled sludge volume was around 300mL for all samples.
According to these hypotheses, the Anammox cell density at the time reported in table 2 was in the range $0.6 \div 2.3 \cdot 10^9$ cells mL_{sludge}^{-1} for all sludge samples. By further considering that the spatial distribution of anammox microorganisms within the sludge aggregates was not homogeneous, the calculated cell density is in accordance with that one suggested by Strous et al. (1999).

5.2 SBR enrichment

5.2.1 Start-up and operational parameters

Samples S1-S2-S3 after the fed-batch enrichment were mixed and used to inoculate the SBR described in par. 3.1.2. The sludge was diluted with mineral medium (see Table 5.1) up to 5L, reaching an initial solids concentration of 0.56 gTSS L^{-1} and 0.30 gVSS L^{-1}. The SBR main parameters are summarized in Table 5.4.

In the first period, the SBR was operated with a 24 hours cycle including 18 hours of slow feeding with reaction, 4.5 hours of reaction, 30 min settling and 5 min of discharge. N_2 gas was purged into the system during the discharge phase and the reactor was always kept in pressure (1.05-1.1 bar) with a backpressure valve to avoid oxygen penetration. The pH was controlled automatically below 8.0 with dosage of 1M HCl.

After day 104, the cycle duration was shortened from 18 to 12 hours, the settling time was gradually reduced to 20 and 15 minutes and the HRT was decreased to 3 days. During period 2, a mixture 95% N_2 + 5% CO_2 was used as purging gas.

Table 5.4 Main parameters for the SBR during the enrichment experimentation.

Parameter	Period 1 (days 0-104)	Period 2 (days 105-127)
Temperature (°C)	35-36	35-36
Cycle duration (h)	24	12
Feeding phase duration(h)	18	9
Reaction phase duration (h)	4.5	2.5
Settling and discharge phase duration (min)	25-35	15-25
pH control (below 8)	HCl automatic dosage	95% N_2 +5% CO_2flushing
Minimum Volume (L)	5	5
Maximum Volume (L)	6	6
HRT (d)	5.5-6.6	2.7-3.6
SRT (d)	100-200	100-200
NLR (gN L^{-1} d^{-1})	0.02-0.16	0.10-0.19
Influent concentrations (NH_4^++NO_2^-) (mgN/L)	100-1060	600-680
Influent NO_2^-/NH_4^+ molar ratio	0.9-1.0	0.75-1.16

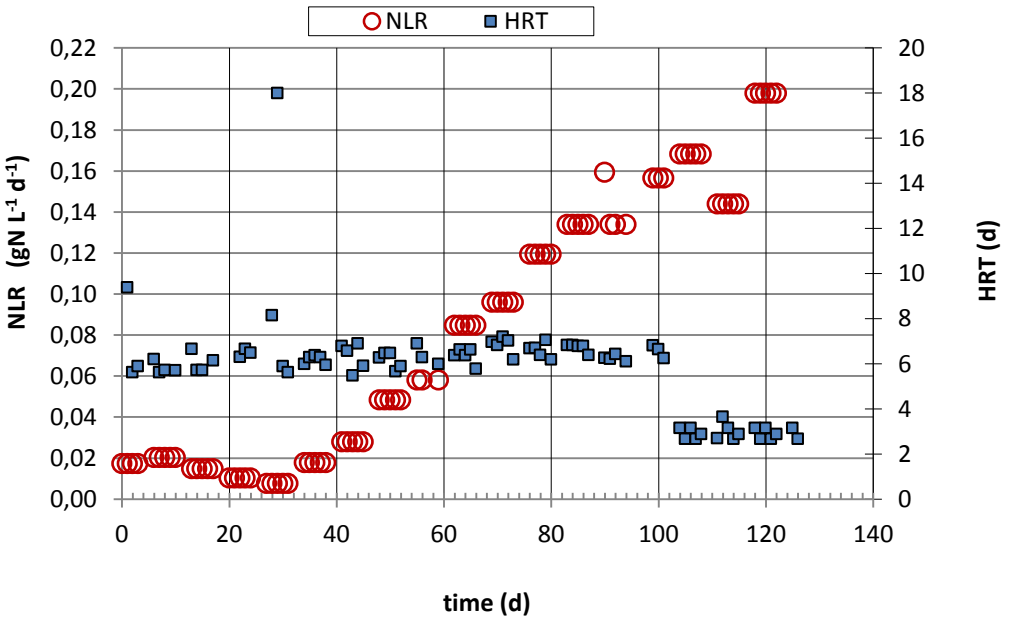


Figure 5.5 – HRT and NLR (weekly average) applied during the SBR enrichment experimentation.

The applied nitrogen loading rates (NLR) and hydraulic retention time (HRT) are reported in Figure 5.5. During the first 30 days, the NLR was kept low (0.01-0.02 gN L^{-1} d^{-1}). Later, up to day 104, the NLR was increased by increasing the ammonium and nitrite influent concentrations from 100 to 1060 mg L^{-1} of total nitrogen. In the last period, the NLR was increased by reducing the HRT to 3 days on average.

The SBR was monitored by measuring:

-ammonium , nitrite and nitrate effluent concentrations 3 times per week;

-effluent solids (TSS, VSS) concentrations once per week;

-mixed liquor suspended solids concentrations (MLTSS and MLVSS) every 2-3 weeks.

5.2.2 SBR enrichment results

In Figure 5.6 the ammonium, nitrite, nitrate effluent concentrations are reported and compared with the influent ammonium and nitrite concentrations. During the first 20 days, anammox activity was very low and more than half of the ammonium and nitrite fed kept accumulating in the reactor. Influent concentrations were 55 mgN L^{-1} for both ammonium and nitrite, while the effluent was characterized by concentrations of 34±11 mgNH_4^+-N L^{-1} and 30±18 mgNO_2^--N L^{-1}. This lag-phase could be related to adaptation of the biomass to the new working conditions. Later, the ammonium and nitrite concentrations in the effluent decreased and remained stable at concentrations of 5±8 mgNH_4^+-N L^{-1} and 4±8 mgNO_2^--N L^{-1} respectively, while the nitrogen content of the influent was continuously increased up to 500 mgN L^{-1}. Nitrate effluent concentrations between day 30 and day 60 rose from around 40 mgNO_3^--N L^{-1} to around 100 mgNO_3^--N L^{-1} confirming the intensification of the anammox activity in the reactor.

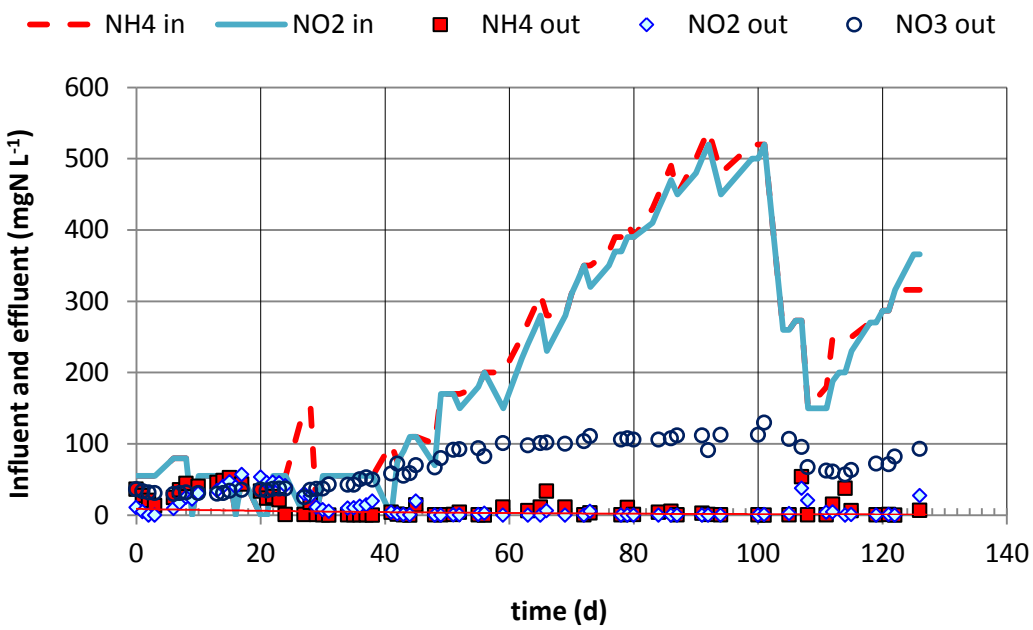


Figure 5.6 – Influent and effluent soluble nitrogen species during the SBR enrichment experimentation.

At day 104, the HRT was reduced from 6 to 3 days in order to reduce the influent nitrogen concentrations and the cycle changed in a 12h cycle (see table 4.3), the NLR was temporary reduced to 0.11 gN $L^{-1}d^{-1}$ and later progressively increased until the end of the experimentation.

In Figure 5.7, the nitrogen loading rates and nitrogen removal rates are reported. After the first 40 days of low activity, the NRR increased 10 times in 80 days, reaching 0.22 gN $L^{-1}d^{-1}$. Total soluble N removal efficiency after day 40 was on average 82%±7% while ammonium and nitrite removal were 96±5%.

The values of NLR and NRR reached are comparable with those reported in similar experimentations. As an example, Lopez et al. (2008), in an anammox enrichment experimentation performed in a SBR, could increased the NLR from 0.02 to 0.25 gN $L^{-1}d^{-1}$ in 146 days.

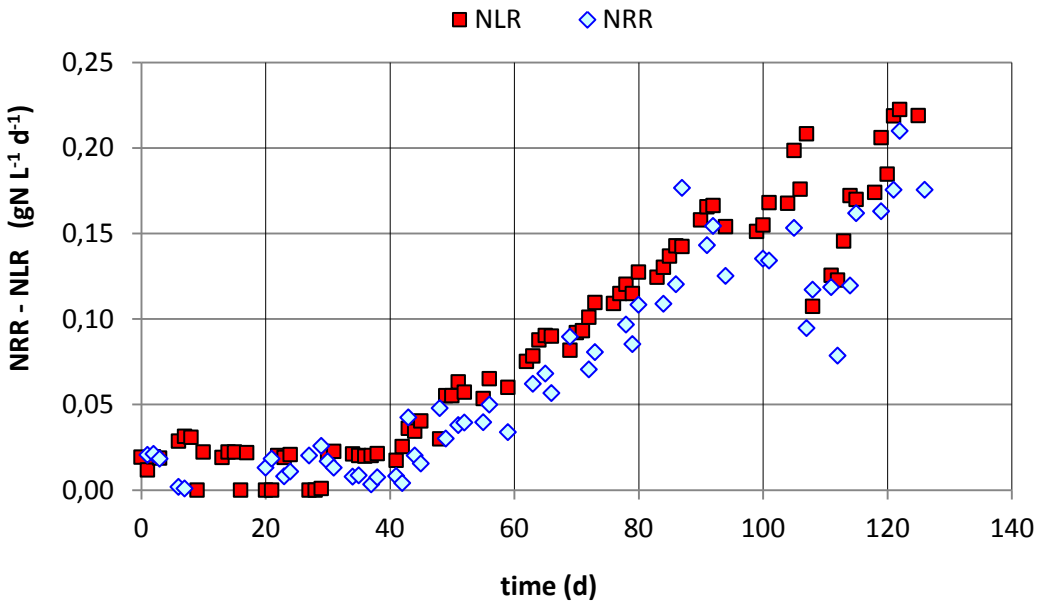


Figure 5.7 - Nitrogen Loading Rates (NLR) and Nitrogen Removal Rates (NRR) during the SBR enrichment.

In Figure 5.8, the experimental molar ratios of NO_2^--N removed over NH_4^+-N removed ($\Delta NO_2^-/\Delta NH_4^+$) and NO_3^--N produced over NH_4^+-N removed ($\Delta NO_3^-/\Delta NH_4^+$) are reported and compared to the stoichiometric ones from Strous et al. (1998). The $\Delta NO_2^-/\Delta NH_4^+$ ratio in the first part of the experimentation was variable, because of the low anammox activity. In the last 60 days the $\Delta NO_2^-/\Delta NH_4^+$ ration remained stable around 1 (0.98 ± 0.09). This value is lower than the anammox stoichiometric value of 1.32 and could be related to a presence of AOB bacteria which converted part of the ammonium to nitrite (possible oxygen leakage). In the last 60 days, the $\Delta NO_3^-/\Delta NH_4^+$ ratio was on average 0.29 ± 0.12 similar to 0.26, i.e. the stoichiometric value indicated by Strous et al. (1999).

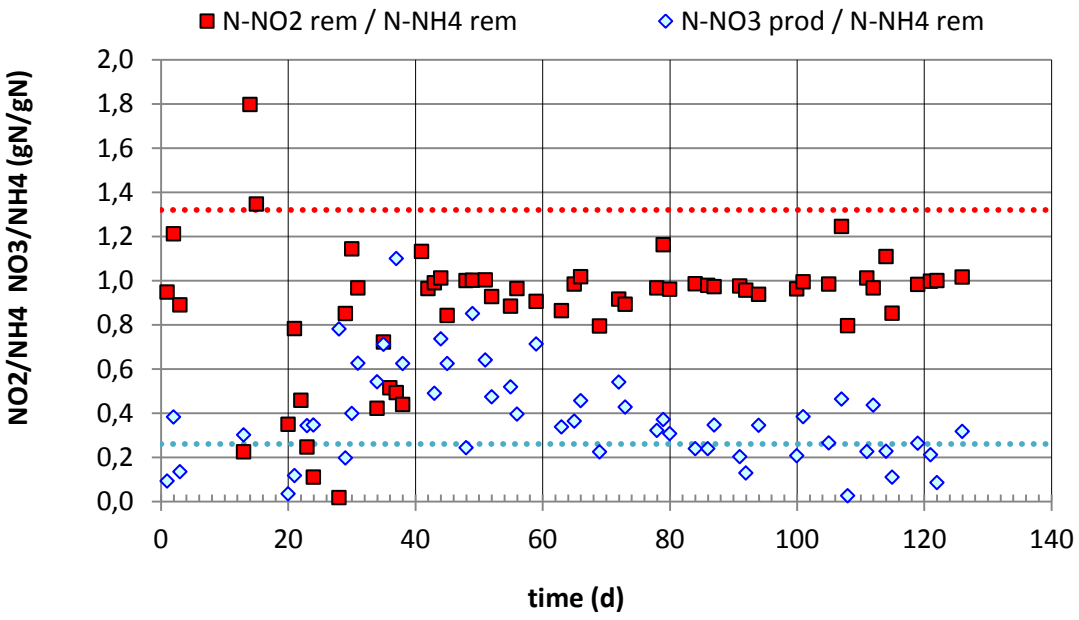


Figure 5.8 - Ratios of N-NO_2 removed and N-NO_3 produced over N-NH_4 removed compared to the stoichiometric values during the SBR enrichment.

The solids concentration in the effluent were quite stable around 15±5 mg TSS L^{-1} and 11±4 mg VSS L^{-1} during the whole experimentation even though the settling phase was reduced from 30 to 15 minutes. This confirms the good settling properties of the suspended sludge.

The total solids concentration of the SBR mixed liquor, from the beginning to the end of the experimentation, decreased from 0.56 to 0.45 gTSS L^{-1} while the volatile solids concentration slightly increased from 0.30 to 0.35 gVSS L^{-1}. From the beginning to the end of the experimentation the VSS/TSS fraction in the reactor increased from 54% to 73%.

By knowing the volatile solids concentration and the NRR in the reactor, the actual specific anammox activity in the SBR can be estimated as:

$$SAA(gN\ L^{-1}d^{-1}) = \frac{NRR(gN\ L^{-1}d^{-1})}{VSS\ (gVSS\ L^{-1})}$$

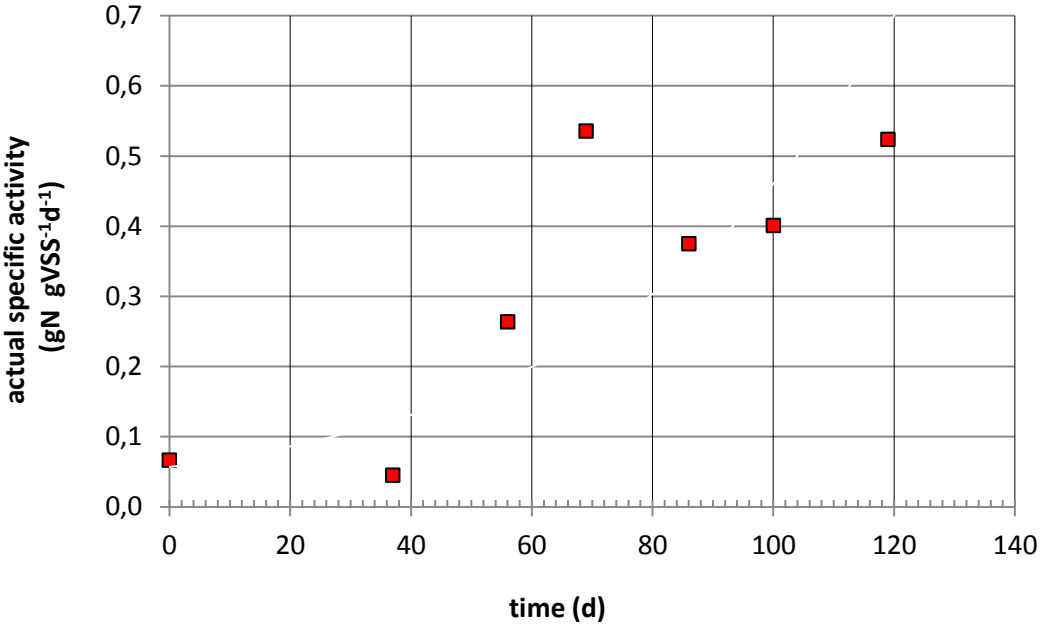


Figure 5.9 – Actual specific anammox activity in the SBR during the experimentation.

From the beginning to the end of the experimentation, the actual specific anammox activity increased from 0.045 to 0.52 gN $gVSS^{-1}$ d^{-1} (Figure 5.9). This more than 10-fold augmentation confirms that the fraction of anammox in the biomass increased in time. Anyway, a concurrent reactivation phenomenon of already existing bacteria could have also taken place.

By considering the observed 10-fold NRR increase in 80 days and by making the reasonable assumption that it was mainly related to anammox biomass growth, the resulting doubling time was 24 days. This is lower than the maximum duplication time of anammox biomass (e.g. 6 – 8 days, Van der Star et al. 2008) because growth was here limited by the NLR applied.

5.3 Conclusions

The following main conclusions can be drawn from the first part of the experimental campaign (enrichment of anammox biomass from Italian sludge samples):

- as for the procedure: the very simple, low-tech fed-batch procedure was successful in growing anammox biomass; hydrazine addition did not favor anammox development; the manometric activity measurements proved to be a powerful monitoring tool, since the full consumption of the limiting substrate (nitrite) was evidenced by the change in the overpressure growth curve, thus assisting in load definition and in preventing dangerous nitrite accumulation;
- as for the anammox biomass enrichment: all tested inocula could develop anammox biomass, independently from the type of wastewater treated and from the treatment scheme of the wastewater treatment plants were the inocula were taken; this suggests that anammox bacteria are likely to be present in most Italian wastewater treatment plants;

- the anammox activity achieved after about 110-130 days was within 0.007 and 0.021 gN L^{-1} d^{-1} and appeared to be sufficient to inoculate a continuously operated anammox reactor.

The cultivation in continuous mode with the SBR reactor of three of the enriched samples allowed to increase the anammox removal capacity of the biomass. After a lag phase of 30 days, the NRR increased proportionally to the nitrogen loading rates applie, rising from 0.013 gN L^{-1} d^{-1} of day 36 to 0.22 gN L^{-1} d^{-1} of day 126. The actual specific anammox activity in the reactor increased 10 times in 80 days and at the end of the experimentation it was 0.52 gN $gVSS^{-1}$ d^{-1}. This positive results confirmed that SBR is a good option to enrich anammox biomass.

6 ANAMMOX SBR TO TREAT THE LIQUID FRACTION OF AGRICULTURAL DIGESTATE

Aim of this chapter was to verify the applicability of the anammox process to the treatment of the liquid fraction of an agricultural digestate after partial nitritiation at different dilutions levels.

In this chapter, an experimental study run on the same a 7L SBR previously used for anammox enrichment (details in par 3.1.2) is presented. The SBR was inoculated with granular anammox During the first period, the reactor was fed with a synthetic wastewater; then, increasing fractions (from 10% up to 100% v/v) of real wastewater were blended to the synthetic wastewater. The response of the anammox biomass is discussed.

6.1 Influent characteristics

The main aim of this experimentation was to test the capability of the anammox biomass to get adapted to a specific type of real wastewater. This wastewater was the effluent of the nitritation-denitritation pilot plant described in chapter 4 and no pretreatments were applied before feeding it to the anammox SBR. Batch samples of the DENO2 reactor were collected, transferred to the lab and stored at 4°C and used to feed the anammox lab-scale SBR for up to 3 months.
Details on the three batch samples of real wastewater composition used in the experimentation are reported in Table 6.1. Sample 1 was used as feeding for most of the experimentation. Samples 2 and 3 were fed at the end of the experimentation. All batches were characterized by a quite high pH (8.0-8.4), a conductivity between 7.8 and 9.4 (mS cm^{-1}) and moderate solid content (160-258 mgTSS L^{-1}). No particularly high metal concentrations have been detected. TSS and COD concentrations progressively increased in sample 2 and 3. As a matter of fact, when sample 2 and 3 were taken, the digester efficiency in the farm was poor, the HRT was lower and a higher fraction of poultry manure was fed as co-substrate. The TSS content in sample 3 was exceptionally high, probably because it was withdrawn from the bottom of the storage tank.

Table 6.1 – Real waste-water characteristics.

Sample		Sample 1	Sample 2	Sample 3
Feeding period (days)		44-153	154-167	168-170
pH (pH units)		8.0-8.4		
Alkalinity ($mgCaCO_3\ L^{-1}$)		2200	2228	2700
conductivity ($mS\ cm^{-1}$)		n.d.	7.8	9.4
Nitrogen compound	NH_4^+-N/N_{org} (%)	83±3		
	NH_4 ($mgNH_4^+$-N L^{-1})	22.2	28	0
	NO_2 ($mgNO_2^-$-N L^{-1})	404	403	182
	NO_3 ($mgNO_3^-$-N L^{-1})	79	0	5
Total COD ($mg\ L^{-1}$)		1380	2210	3850
Soluble COD ($mg\ L^{-1}$)		1038	1598	2629
$sBOD_5$/sCOD (%)		n.d.	12	8
$sBOD_{20}$/sCOD (%)		n.d.	19	27
TSS ($mg\ L^{-1}$)		160	258	1100
Metals	Al ($mg\ L^{-1}$)	< 0.2	0.2	0.2
	Sb ($mg\ L^{-1}$)	< 0.02	< 0.02	< 0.02
	As ($mg\ L^{-1}$)	< 0.02	< 0.02	< 0.02
	Ba ($mg\ L^{-1}$)	< 0.02	< 0.02	< 0.02
	B ($mg\ L^{-1}$)	0.4	1.13	1.22
	Cd ($mg\ L^{-1}$)	< 0.003	< 0.003	< 0.003
	Cr tot ($mg\ L^{-1}$)	< 0.02	< 0.02	< 0.02
	Fe ($mg\ L^{-1}$)	0.19	0.27	0.25
	Mn ($mg\ L^{-1}$)	0.04	0.14	0.07
	Hg ($mg\ L^{-1}$)	< 0.001	< 0.001	< 0.001
	Mb ($mg\ L^{-1}$)	< 0.02	< 0.02	< 0.02
	Ni ($mg\ L^{-1}$)	< 0.02	0.05	0.05
	Pb ($mg\ L^{-1}$)	< 0.01	< 0.01	< 0.01
	Cu ($mg\ L^{-1}$)	0.02	< 0.02	0.02
	Se ($mg\ L^{-1}$)	< 0.005	0.006	0.005
	Sn ($mg\ L^{-1}$)	< 0.02	< 0.02	< 0.02
	Zn ($mg\ L^{-1}$)	0.1	0.13	0.15

6.2 Reactor operation

6.2.1 Inoculum and start-up

The SBR was inoculated with granular sludge from the upper part of the lower compartment of the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, the Netherlands (van der Star et al., 2007). The reactor contains granular anammox sludge and treats reject water after partial nitritation in a SHARON reactor. When the inoculum was sampled, the reactor was operated at the design volumetric load of 7.1 kg N m^{-3} d^{-1} (van der Star et al., 2007).

The granular inoculum was stored at 4°C for 2 months before starting this experimentation. The granular biomass was diluted with mineral medium to reach the initial biomass concentration of 5 gTSS L^{-1} and 2.7 gVSS L^{-1}.

6.2.2 SBR cycle

The reactor operated in a 12 hours cycle (Figure 6.1). Each cycle can be divided in 4 phases. Slow filling of with a peristaltic feeding pump was the first step of the batch process and lasted 9 hours. During the filling phase, the reactor was mixed and the reaction took place. The second was the reaction phase, lasting 2.5 hours, in which the anammox process consumed residual substrates. Then, mixing and heating/cooling were switched off and the sedimentation process started and lasted 5 minutes. Finally, the clarified effluent was discharged and the reactor volume reached its minimum.

Figure 6.1 - Cycle of the anammox SBR

6.2.3 Reactor operational parameters

Operational parameters are reported in Table 6.2. The pH was controlled by the automated addition of a 0.2M HCl solution to keep it below 8.0.

Table 6.2 – Operational parameters of the SBR during the experimentation

Parameter		Value
Volume	L	5-6.8
T	°C	36*
pH	pH units	7.5-8.0
HRT	d	2.5 ±0.5
NLR	gN L^{-1} d^{-1}	0.51 ±0.18
Influent N (NH_4^+ + NO_2^-)	gN L^{-1}	1.31 ±0.24
Influent NO_2^-/NH_4^+ molar ratio	-	1.15 ±0.07

*Between day 0 and 70 – the temperature signal was unreliable: reactor temperature could have ranged between 36 and 38°C.

The SRT was not actively controlled, and depended on the effluent particulate concentration and on the solid extraction for analyses; the average value resulted to be 75 days and varied in the

range 60-150 d, although the reliability of this calculation is hampered by a poorly repeatable solid concentration assessment, as discussed later.

During the first 43 days, the reactor was fed just with mineral medium. The influent ammonium and nitrite concentrations were varied to adjust the NLR while the HRT was kept contant. The resulting nitrogen loading rate was increased from 0.2 to 0.8 gN $L^{-1}d^{-1}$ and then reduced to 0.5 gN $L^{-1}d^{-1}$. On average, the NLR was 0.5±0.2 gN $L^{-1}d^{-1}$.

After 43 days, the feeding was blended with 10% of the real wastewater described in par. 6.1. Then, the fraction of real wastewater was gradually increased to 25%, 40% , 70% and finally 100% (v/v) as described in Table 6.3. The nitrogen loading rate was adjusted by adding proper amount of ammonium and nitrite salts to the influent, with a NO_2^--N:NH_4^+-N of 1:1. The NLR was maintained at 0.6±0.1 gN L^{-1} d^{-1} with a total N concentration in the influent (ammonium plus nitrite) of 1.4 gN L^{-1}.

Table 6.3 – Real waste water fractions fed to the reactor at different experimental periods

Experimental period (d)	Real ww fraction (% v/v)
0-43	0
44-52	10
53-69	lab shut down (biomass at 4°C)
70-84	0
85-90	10
91-106	25
107-116	40
119-162	70
163-169	100
170-172	0

In Figure 6.2, the time trend of the NLR is depicted together with the fraction of real wastewater in the influent. At the end of the experimentation, the total volume of real wastewater treated was 125L.

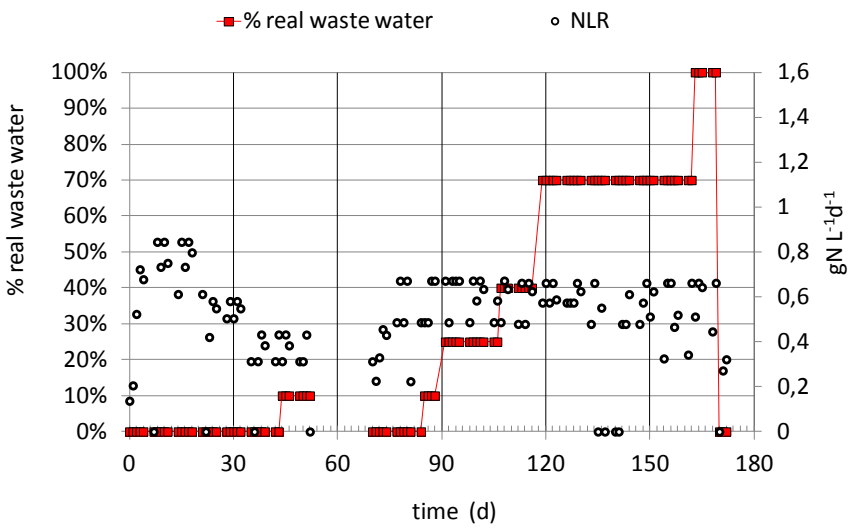


Figure 6.2 - NLR and fraction of real wastewater fed to the reactor during the course of the experimentation.

6.2.4 Process monitoring and calculations

The reactor was monitored by measuring:

- soluble N compounds (ammonium, nitrite, nitrate) in the effluent with a frequency of 3 analyses per week.
- total and volatile solids concentrations in the reactor on a monthly base; however, correct and reliable MLTSS and MLVSS estimation inside the reactor couldn't be performed because of the heterogeneity of the reactor mixed liquor due to the granular nature of the biomass;
- VSS and TSS concentrations in the effluent every 2-3 weeks;
- maximum nitrogen removal rate (NRR_{max}) with batch tests inside the reactor (see below) every 1-2 weeks.

After every change in the influent composition, biomass samples were taken and fixed for FISH analysis (see par. 3.3).

Nitrogen removal rates were calculated according to the dynamic nitrogen mass balance, i.e. by taking into account the accumulation/release of soluble nitrogen inside the reactor:

$$NRRt = \frac{N_{IN} - N_{OUT} + (N_{react,t-1} - N_{react,t})}{\Delta t \times V_{max}}$$

Where:

N_{IN}=mass of N loaded to the reactor in the time-interval,

N_{OUT} = mass of N discharged with the effluent during the time-interval (assuming the average effluent concentration of the time interval),

$N_{react,t-1}$= mass of N in the reactor at the beginning of the time-interval,

$N_{reac,t}$= mass of N remaining in the reactor at the end of the time-interval,

Δt= length of the time interval,

V_{max}= maximum reactor volume.

Similarly, the nitrite and ammonium removal rate and the nitrate production rate were calculated as:

$$\Delta NH_{4,t} = \frac{NH_{4_{IN}} - NH_{4_{OUT}} + (NH_{4_{react,t-1}} - NH_{4_{react,t}})}{\Delta t \times V_{max}}$$

$$\Delta NO_{2,t} = \frac{NO_{2_{IN}} - NO_{2_{OUT}} + (NO_{2_{react,t-1}} - NO_{2_{react,t}})}{\Delta t \times V_{max}}$$

$$\Delta NO_{3,t} = -\frac{NO_{3_{IN}} - NO_{3_{OUT}} + (NO_{3_{react,t-1}} - NO_{3_{react,t}})}{\Delta t \times V_{max}}$$

From these rates it was possible to calculate the $\Delta NO_2^-/\Delta NH_4^+$ and the $\Delta NO_3^-/\Delta NH_4^+$ ratios to make comparisons with the expected stoichiometric values.

Nitrogen removal efficiencies were calculated as the ratio NRR/NLR considering:

- ammonium and nitrite ($NRR_{NH4+NO2}$/ $NLR_{NH4+NO2}$),
- ammonium, nitrite and nitrate ($NRR_{NH4+NO2+NO3}$/ $NLR_{NH4+NO2+NO3}$).

If not specified, the N removal efficiency has to be intended as $NRR_{NH4+NO2}$/ $NLR_{NH4+NO2}$.

Activity batch tests in the reactor have been run every 1-2 weeks to estimate the maximum nitrogen removal rate (NRR_{max}) of the SBR indicating the maximum removal capacity of the biomass present in the reactor. The procedure of the test was as follows:

1. feeding and discharge pumps were stopped, while mixing, heating and pH control were kept active;
2. the initial ammonium and nitrite concentrations were measured;
3. concentrated solutions of NH_4Cl and $NaNO_2$ were added to reach a concentration in the reactor of 50-70 mgN L^{-1} of NH_4^+ and NO_2^-;
4. 3-4 subsequent samples of the reactor suspension were taken every 10-30 minutes (depending on the expected activity);
5. after filtration (0.45 μm), ammonium and nitrite concentrations were measured;
6. for each sample, the total nitrogen concentration was calculated as the sum of ammonium and nitrite.

Total nitrogen concentration data during time were fitted to estimate the nitrogen removal rate; this value was finally divided by the reactor volume to obtain the NRR_{max} (gN L^{-1} d^{-1}).

In Figure 6.3, an example of activity test result is reported. The slope of the linear regression corresponds to the degradation rate of $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NH_4^+ + N\text{-}NO_2^-$ during the activity test. The 95% confidence interval for the estimated value was also computed to quantify its reliability.

The molar ratio $\Delta NO_2^-/\Delta NH_4^+$ can also be calculated to infer possible concurring reactions.

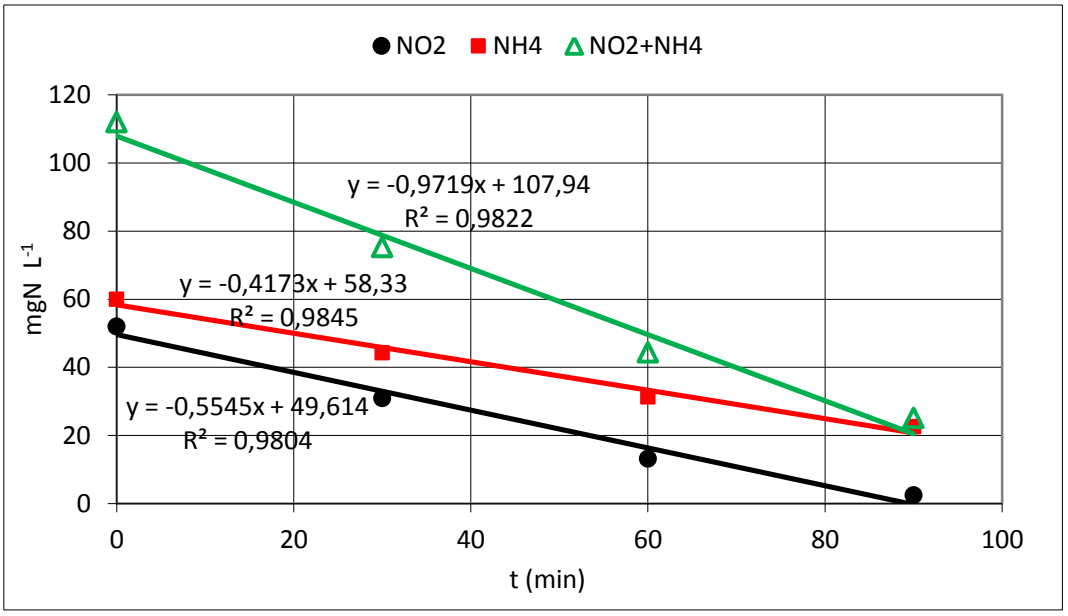


Figure 6.3 – Example of activity test in the reactor to assess maximum N-removal rate (NRR_{max})

In case of $\Delta NO_2^-/\Delta NH_4^+ > 1.32$, a possible concurring denitrification can lead to the overestimation of the anammox activity since nitrite heterotrophic denitrification would contribute to the total nitrogen removal rate. Therefore, the NRR_{max} was calculated using the ammonium removal rate. The following equation, derived from stoichiometric parameters (Strous et al., 1999) was applied:

$$NRR_{max} = NH_4RR_{max} + 1.32 \times NH_4RR_{max}$$

where NH_4RR_{max} is the ammonium removal rate computed by linear regression of ammonium concentration versus time data.

6.3 Results

6.3.1 Manometric batch test

Activity batch tests have been performed with the automatic manometric method described in par.3.2.3 to assess the short-term inhibition effect of the real wastewater (Sample 1 in table 5.1) on non-acclimated granular biomass.

A sample of granular biomass (100 mL) was diluted with mineral medium or real wastewater at different dilutions to a final volume of 1 L. The average TSS concentration in each bottle was 0.5 g L^{-1}. Nitrite and ammonium were both spiked to achieve a concentration of 50 mgN L^{-1} and the production of nitrogen recorded until nitrite was fully used up. Nitrite and ammonium were spiked again after 24 h and 48 h. At the end of the test, the whole granular suspension was dried to reliably assess the volatile solid content to be used to calculate the anammox specific activity. Nitrogen mass balance was computed after each substrate spike by comparing the nitrogen

released into the gas phase with the production expected from anammox stoichiometry. The average difference was 9±6%.

In the first test, three bottles were run in parallel with different percentage of real wastewater, as detailed below:

- bottle 1: 0% real wastewater (mineral medium, only),
- bottle 2: 60% real wastewater,
- bottle 3: 100% real wastewater.

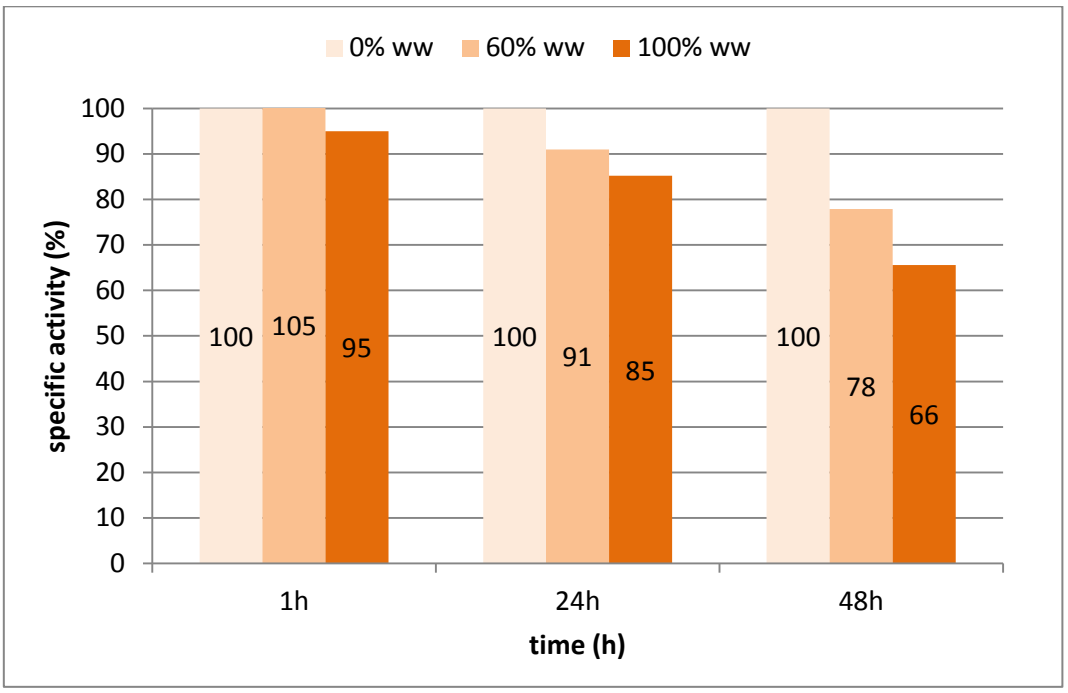


Figure 6.4 –Specific activity batch test with different real wastewater fractions (v/v) at time 0 and after 24h and 48h of exposure.

The SAA in the 3 bottles have been measured 1 hour after the preparation, after 24h and after 48h. In

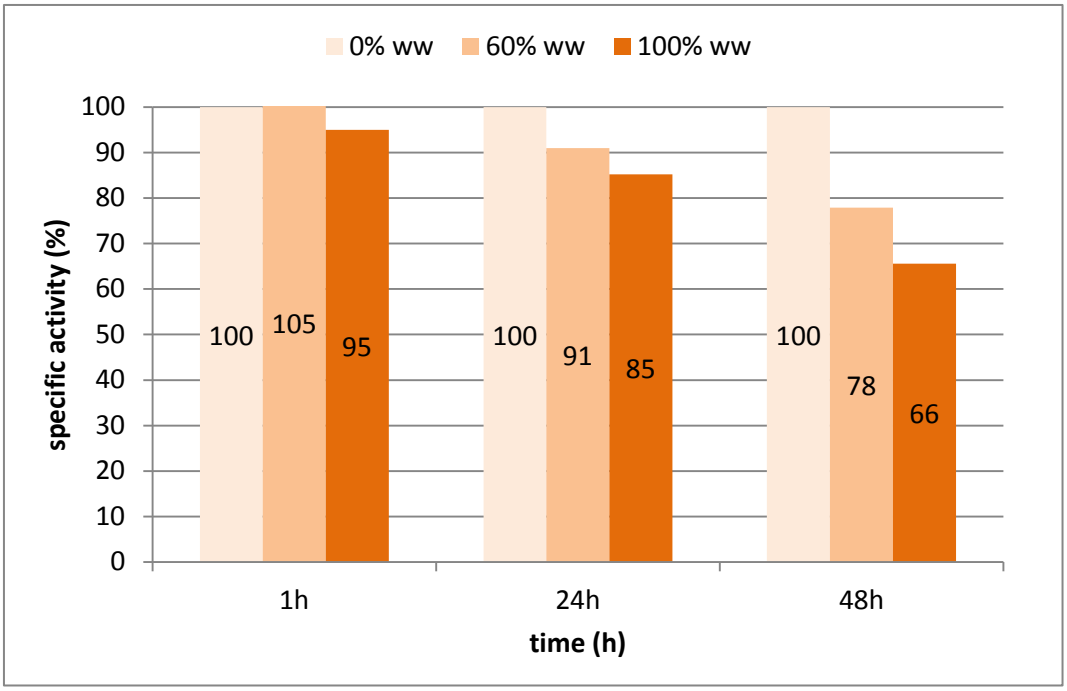


Figure 6.4 the results of the specific activity measured after 24 h and 48 h are reported and espressed as a percentage of the activity measured in bottle 1 with mineral medium only. At the beginning of the test the specific activities in the 3 bottles were comparable (within the method variability). The SAA in bottle 2 was 9% and 22% lower than the SAA in bottle 1 after 24h and 48h respectively. SAA in bottle 3 was 15% and 34% lower than in bottle1 after 24h and 48h respectively. Apparently, a moderate inhibition of the anammox activity took place, that increased with the exposure time.

To better assess the effect of the contact time, a longer test was performed. This second test was performed comparing a bottle with mineral medium with a bottle with 25% of real wastewater. The test was perfomed during 15 days, repeating substrates spikes every 2-6 days and assessing specific anammox activity (SAA). In Figure 6.5, the ratio between the SAA in the bottle with 25% real wastewater and the corresponding SAA in the control bottle with mineral medium are reported. The SAA in the batch test with 25% of real wastewater decreased by 13% after 5 days, by 18% after 9 days and by 25% after 15 days.

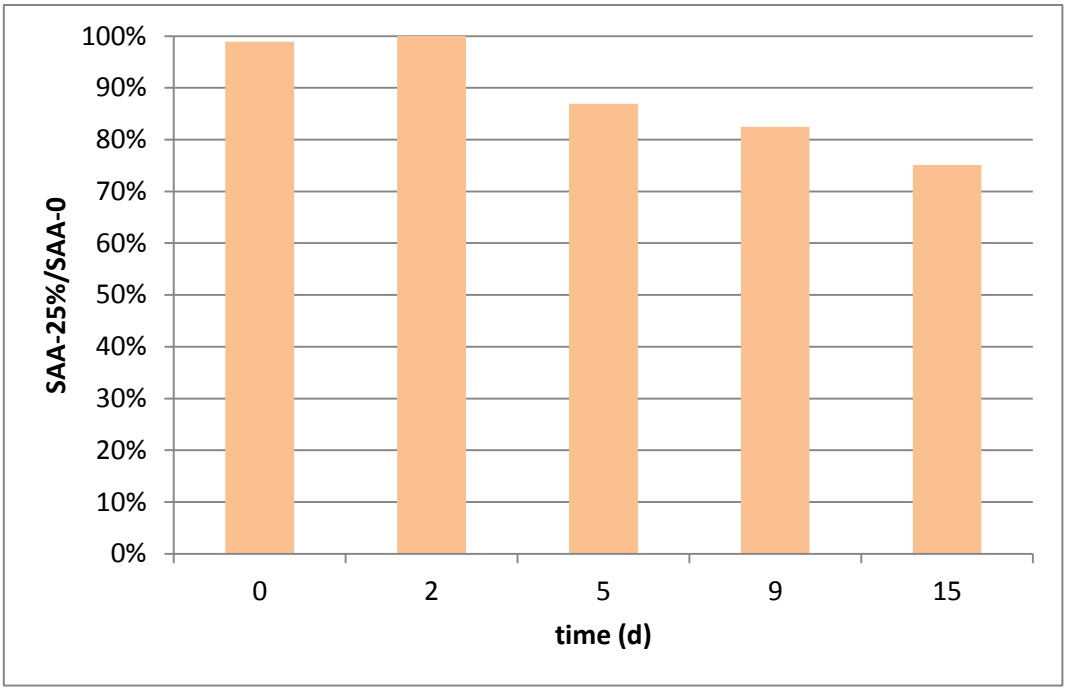


Figure 6.5 – Long term specific anammox activity test with 25% of real wastewater

These resultes suggested a low inhibiting activity effect of the real wastewater on granular anammox biomass if blended at a 1:4 ratio with the mineral medium. Therefore, the short-term inhibition of a non-acclimated anammox granular biomass appears to be moderate.

According to these results, a conservative planning of the real wastewater dosage in the reactor feed was scheduled. Experiments with real wastewater started by feeding a diluted blend of 10% wastewater and 90% mineral medium (table 5.3).

6.3.2 Effluent quality and removal efficiencies

In Figure 6.6, the concentrations of the soluble nitrogen species in the effluent of the SBR are reported. Removal efficiency assessed during the experimentation is reported in Figure 6.7.

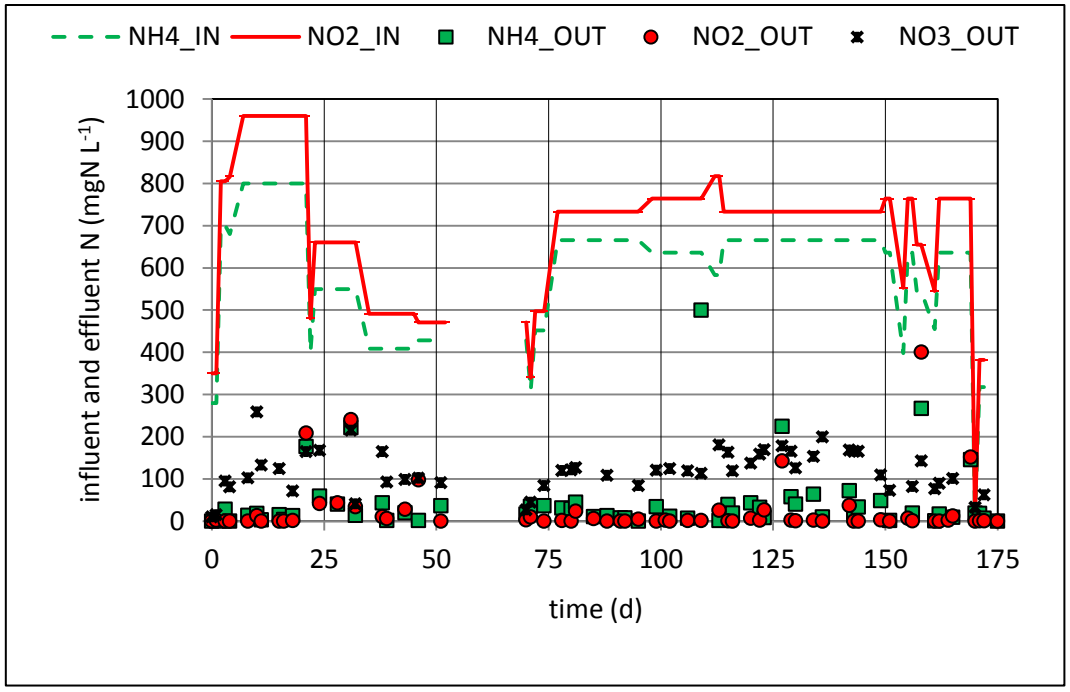


Figure 6.6 - Soluble nitrogen species in the influent and effluent of the SBR.

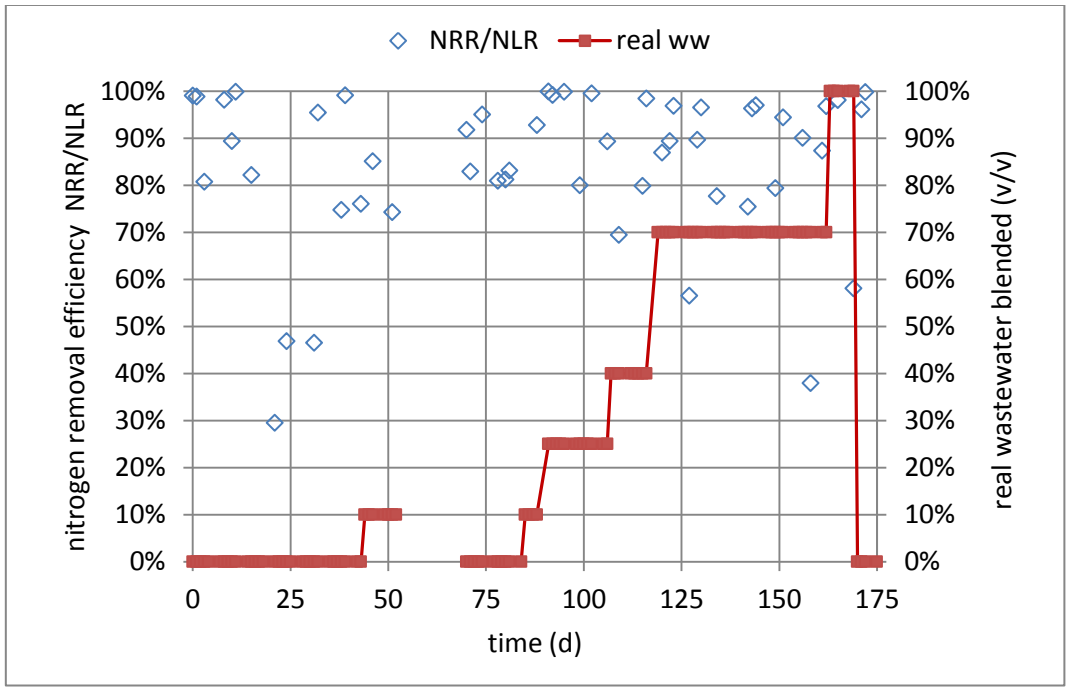


Figure 6.7 - Nitrogen (NH_4^+-N+ NO_2^--N) removal efficiency (NRR/NLR) during the experimentation and % of real wastewater in the feeding blend (wastewater + mineral medium).

During the first 7 days, the total nitrogen concentration in the influent was increased from 630 to 1760 mgN L^{-1} and maintained at this value until day 20, with an average 90% N-removal efficiency. At day 21, the reactor efficiency decreased to 35%, while nitrite in the effluent raised to 241 mgNO_2^--N L^{-1}. Influent concentration was then reduced from 1760 to 1200 mgN L^{-1}, reducing the average weekly NLR from 0.77 to 0.54 gN L^{-1} d^{-1}.

At day 31, after a second nitrite peak, the influent N-concentration was further reduced to 900 mgN L^{-1} and the N-removal efficiency was restored to 93%.

At day 44, the real wastewater blending was started, a 10% (v/v) real wastewater was therefore used to prepare the influent while maintaining the NLR of 0.36 gN L^{-1} d and 80% of average N removal efficiency.

During the summer break (day 52-day 70), the lab was shut down and the biomass was stored at 4°C with excess nitrate (150 mgNO_3^--N L^{-1}) to keep the redox within the anoxic range and avoid sulfide production.

The reactor was re-started with mineral medium with a total nitrogen concentration of 900 mgN L^{-1} (NLR = 0.31 gN L^{-1} d). Since the nitrogen removal efficiency was satisfactory, the NLR was increased within one week to the value of 0.54 gN $L^{-1}d^{-1}$ by increasing the nitrogen concentration to 1400 mgN L^{-1}, and the nitrogen removal efficiency remained in the range 80-95%.

After one week of operation at stable nitrogen removal efficiency (day 85), the blend with 10% real wastewater was fed again to the reactor, with no adverse impact on effluent quality. After 6 days (operational day 91), the wastewater fraction in the blend was increased to 25%, keeping a constant nitrogen concentration in the influent of 1400 gN L^{-1}, and the N removal efficiency remained at very high values (95±8%).

After 16 days, the wastewater fraction in the blend was increased to 40% for 12 days with no remarkable impact on the effluent quality and on the reactor removal efficiency.

At day 119, the wastewater fraction in the blend was increased to 70% and the removal efficiency remained between 79 and 97% up to day 137. On that date, the lab had to be shut down and the reactor was stopped and left for 5 days at room temperature without feeding. Again, excess nitrate was dosed (150 mgNO_3^--N L^{-1}).

At day 142, the reactor was re-started with the same loading rate and the same blending ratio (70% ww and 30% mineral medium); the reactor removal efficiency remained > 90% until day 162.

At day 154, a new batch of real wastewater was used, switching from sample 1 to sample 2 (Table 6.1). Finally, at day 163, 100% wastewater was fed to the reactor. Two days later, a new batch of real wastewater (sample 3, Table 6.1) was used to prepare the reactor feed. At day 170, after 5 days of 100% real wastewater feeding, the removal efficiency dropped, and nitrogen built up to 152 mgNO_2^--N L^{-1} and 146 mgNH_4^+-N L^{-1} in the reactor effluent. Therefore, the NLR was reduced to 0.34 gN L^{-1} d^{-1} and 100% mineral medium was fed to the reactor. This sharp reduction in the nitrogen removal efficiency was confirmed by the reactor activity monitoring (see the next paragraph) and will be discussed later.

During the experimentation some occasional technical problems led to temporary process instability:

- at day 111 a wrong calculation in the preparation of the influent caused a double ammonium load and led to the accumulation of 500 mgNH_4^+-N L^{-1} in the reactor;
- at day 128 technical problems at the acid dosing pump caused an increase in pH up to 8.5 that caused nitrogen accumulation of 225 mgNH_4^+-N L^{-1} and 142 mgNO_2^--N L^{-1};
- during days 158-161 (a week-end) the heating system broke down and the temperature dropped to 15°-18°C; this failure caused a build-up of 267mgNH_4^+-N L^{-1} and 401 mgNO_2^--N L^{-1};

After each process failure, the reactor supernatant was diluted 1:3 or 1:4 with mineral medium to rapidly reduce the nitrite concentration and the feeding was re-stated at the same loading rate. The nitrogen removal efficiency was always rapidly restored indicating that activity rapidly recovered.

Average effluent quality values and removal efficiencies for the different phases at different blending ratio are summarized in Table 6.4. Data related to isolated failures of the reactor were not included in this calculation. As already commented, the effluent quality was good and the removal efficiencies around 90% up to a blending ratio of 70%. While feeding the undiluted real wastewater, ammonium and nitrite accumulated in the effluent and the efficiency dropped down.

Table 6.4 – Summary of ammonium and nitrite effluent concentrations and N removal efficiency during the experimentation

Wastewater fraction in the feed	Effluent NH_4	Effluent NO_2	NH_4+NO_2 removal	N_{tot} removal
%	$mgNH_4^+$-N L^{-1}	$mgNO_2^-$-N L^{-1}	%	%
0 (mineral medium only)	20±17	9±14	89±13	77±19
10	16±15	26±48	88±11	74±11
25	12±12	1±2	95±8	85±10
40	20±19	9±15	89±13	71±10
70	31±23	6±11	90±8	80±9
100	78±97	59±69	78±28	-

In Figure 6.8, the ratio between nitrite and ammonium removal rates and the ratio between the nitrate production rate and the ammonium removal rate are reported and compared to the expected Anammox stoichiometric values ($\Delta NO_2/\Delta NH_4$=1.32 and $\Delta NO_3/\Delta NH_4$=0.26 from Strous et al. 1999).

During the first 150 days, the average $\Delta NO_2/\Delta NH_4$ ratio was 1.22±0.21, while in the last 20 days of operation with undiluted wastewater, it increased up to 1.55±0.24. Similarly, the $\Delta NO_3/\Delta NH_4$ ratio decreased from 0.27±0.19 to 0.18±0.12. Both these trends suggest an increase in the denitrification activity. Since recent results showed that anammox bacteria can use nitrate directly and even outcompete heterotrophs for organic acids in the presence of ammonium (Kartal et al., 2006), the dissimilatory nitrate reduction to ammonium pathway cannot be excluded.

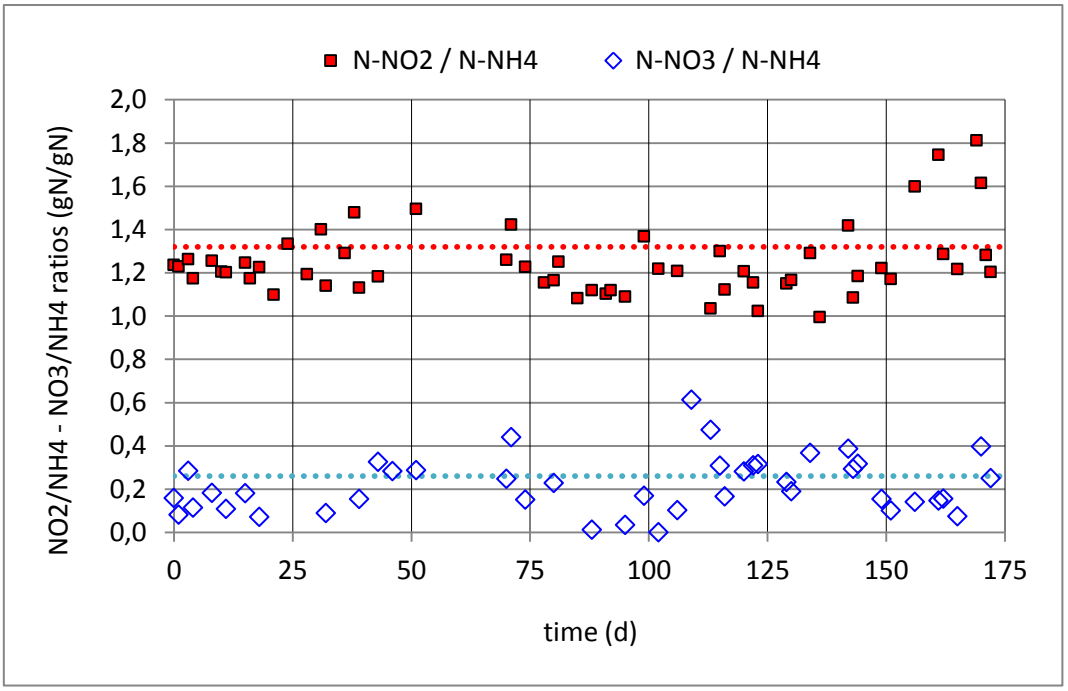


Figure 6.8 - Ratios between the nitrite and ammonium removal rate and the between the nitrate production rate and the ammonium removal during the experimentation.

Table 6.5, the volatile solid concentrations and soluble COD in the influent and effluent are summarized and compared to the fraction of real wastewater in the feed. The load of volatile solids increased proportionally with the fraction of real wastewater treated. During the first part of the experimentation (until day 135), the estimated average sCOD removal in the SBR varied between 5 and 45 mgCOD $L_{react}^{-1}d^{-1}$. This consumption of organic matter might have supported the growth of heterotrophic bacteria. Considering that all the COD served as electron donor for nitrate denitrification, the highest recorded COD removal rate corresponds to a nitrogen removal rate lower than 10 mgN L^{-1} d^{-1}. In our system therefore, the heterotrophic contribution to the total nitrogen removal rate can be considered negligible (at least during the first period of the experimentation).

From day 135 to day 165, three main modifications took place in the SBR operation:
1) the reactor was kept for 4 days at 15°C without feeding;
2) from day 154, much higher COD and TSS were fed with the influent, since it was prepared with a new sample of wastewater (sample 2, table 5.1); and
3) at day 163 the percentage of real wastewater in the feed was increased to 100%.
As a consequence of these modifications the effluent COD increased to 700 mgCOD L^{-1}. Since the reactor was not at steady state and no data were available between day 135 and 165, the actual COD removal in these new conditions could not be computed.

At day 168, a further increase in the influent concentration occurred because the feed was prepared with the last batch of real wastewater (sample 3) which had a much higher COD and TSS

content. The effluent COD and TSS increased accordingly. By computing the dynamic mass balance for the soluble COD in the SBR reactor, the sCOD removal was about 185 mgCOD $L^{-1}d^{-1}$.

Table 6.5 –VSS concentration in the influent and in the effluent at different percentage of real wastewater in the feed.

day	%real wastewater	VSSin (mg L^{-1})	VSSout (mg L^{-1})	sCOD in (mg L^{-1})	sCOD out (mg L^{-1})
2	0	0	37	-	n.d.
44	10	13	14	92	80
105	25	32	17	266	180
120	40	51	31	260	186
135	70	90	87	454	444
165	100	222	n.d.	1598	716
169	100	862	247	2629	1557

6.3.3 Reactor activity monitoring

The reactor performance was tracked by measuring the maximum nitrogen removal rate (NRR_{max}) inside the reactor as described in par 6.2.4. Results are reported in Figure 6.9 and compared to the loading rate applied and the fraction of real wastewater in the influent.

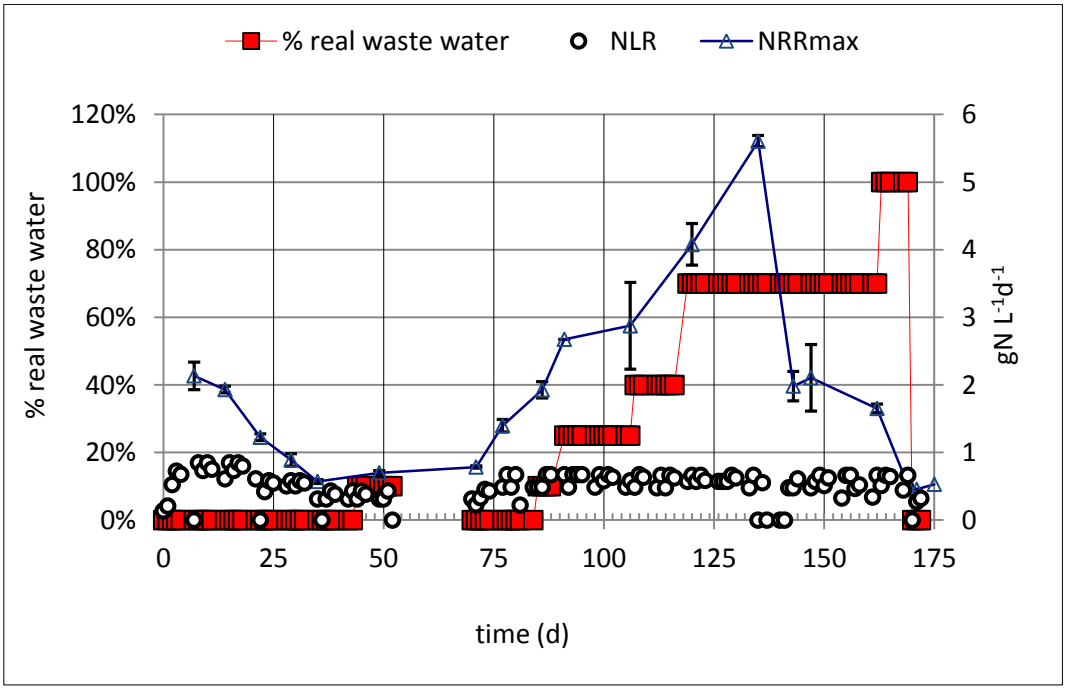


Figure 6.9 - NRR_{max} in the reactor compared with NLR applied and the fraction of real wastewater in the influent

During the first period of SBR operation with mineral medium, the NRR_{max} decreased from 1.9 gN $L^{-1}d^{-1}$ at day 14 to 0.6 gN $L^{-1}d^{-1}$ at day 35. Then, it remained stable at 0.8 gN $L^{-1}d^{-1}$. Later on, after the summer break, it increased up to 5.6 gN $L^{-1}d^{-1}$ while the fraction of real wastewater blended in the influent was 70% since 15 days. Then, at day 137, the SBR was stopped and left 5 days at room temperature without feeding; after this stop, at day 143 the measured NRR_{max} was 2.0 gN $L^{-1}d^{-1}$,

resulting in a 62% reduction. Then, it remained stable around 2.0 gN $L^{-1}d^{-1}$ until the percentage of real wastewater was increased to 100%. At this point the NRR_{max} decreased to 0.5 gN $L^{-1}d^{-1}$, corresponding to a loss of 75% of the anammox removal capacity. After this strong activity reduction, the SBR was washed with mineral medium and the NLR reduced to 0.3 gN $L^{-1}d^{-1}$; however the NRR_{max} did not recover significantly.
While performing tests to assess the NRR_{max}, the $\Delta NO_2/\Delta NH_4$ ratio was also computed and it resulted to be 1.27±0.22, suggesting that Anammox was the main N-removing process. However, this ratio increased to 1.63±0.09 during the last two tests. This fact may suggest an improved denitrification activity in the reactor, as already discussed in paragraph 5.3.2.

The decrease in the NRR_{max} observed between day 14 and 35 corresponded to a 70% reduction in the nitrogen removal capacity. After the lab shut down (days 53-69), NRR_{max} increased from day 70 to 77, as Anammox activity recovered slowly after 16-day storage at 4°C. While The NRR_{max} was 1.4 gN $L^{-1}d^{-1}$ at day 77, it increased to 5.6 gN $L^{-1}d^{-1}$ in two months (day 135, 400% increase).
A simplified model describing the Anammox growth process can be used to compute the expected variations in the Anammox concentration. The variation in the anammox concentration (C_{AN}) in time for a continuously stirred tank reactor is computed as:

$$\frac{dC_{AN}}{dt} = biomass_{AN}\ input - biomass_{AN}\ output + growth\ rate - decay\ rate$$

By considering the biomass input negligible, and discretizing, the mass balance becomes:

$$\frac{\Delta C_{AN}}{\Delta t} = \frac{C_{AN,t+1} - C_{AN,t}}{\Delta t} = \frac{-C_{AN,t}}{SRT} + NRR_t \times Y_{AN} - b_{AN} \times C_{AN,t}$$

where:

NRR_t= nitrogen (nitrite plus ammonium) removal rate measured in the reactor (gN $L^{-1}d^{-1}$) during the time interval;
Y_{AN}= 0.05 gVSS gN_{rem}^{-1} from stoichiometry (Strous et al. 1998), considering NH_4+NO_2 removal;
Δt =time interval (d);
b_{AN}= 0.0048 (d^{-1}) anammox decay coefficient (Scaglione et al., 2009);

Therefore, the anammox biomass concentration $C_{AN,t+1}$ (as gVSS L^{-1}) can be estimated for each time interval as:

$$C_{AN,t+1} = C_{AN,t} + NRR_t \times Y_{AN} \times \Delta t - \left(\frac{1 + b_{AN} \times SRT}{SRT}\right) \times C_{AN,t}$$

The average SRT assumed was 87 d for the first period (day 0-52, mainly mineral medium) and 406 d for the second period (day 70-170, increasing percentage of real wastewater). The first value

was computed by taking into account both the amount of solids extracted from the reactor for analyses (MLTSS, activity tests) and the solids that left the reactor with the effluent. During the second period, the SRT was calculated by considering only those solids that had been withdrawn from the reactor for activity and solids analyses, assuming that most of the solids in the effluent were not related to anammox biomass but came from the influent.

To compute the time trend of Anammox biomass concentration, an initial value needs to be known or estimated. Initial Anammox biomass estimation was computed as follows:

$$C_{AN(tO)=}MLVSS(t0) \times (\%\ anammox\ in\ VSS)$$

The initial MLVSS concentration was measured at the reactor start-up (2.7 gVSS L^{-1}). As for the fraction of anammox bacteria in VSS, a value of 60% was assumed, as suggested by van der Star et al. (2007).

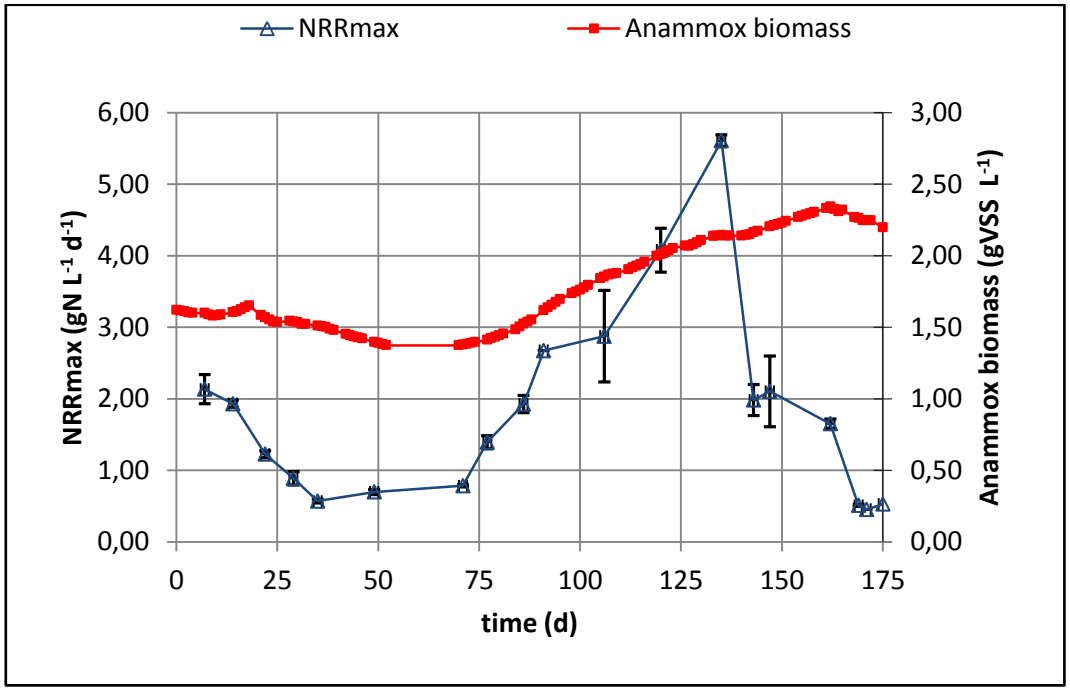


Figure 6.10 – Simulated anammox biomass concentrations and measured maximum nitrogen removal rate capacity (NRR_{max}) of the SBR.

The simulated anammox biomass concentration in the reactor is shown in Figure 6.10. Anammox biomass decreased only by 6% between day 14 and 35 because the low SRT and slightly low NLR applied, while it increased by 60% between day 77 and day 135 due to the higher SRT applied. Thus, variation on the measured NRR_{max} cannot be associated to a variation in biomass concentration only.

Once the anammox biomass concentration is estimated, it is possible to correlate it to the corresponding maximum nitrogen removal rates (NRR_{max}) as:

$$NRR_{max} = \frac{C_{AN} \times \mu_{react}}{Y_{AN}}$$

With: μ_{react} = maximum anammox specific growth rate (d^{-1}) in the SBR.
Therefore, the μ_{react} value can be calculated from the measured values of NRR_{max} and the estimated values of C_{AN}:

$$\mu_{react} = \frac{Y_{AN} \times NRR_{max}}{C_{AN}}$$

Results are shown in Figure 6.11. The corresponding doubling times (t_{2x}) are also indicated and calculated as:

$$t_{2x}(d) = \frac{\ln(2)}{\mu_{react}}$$

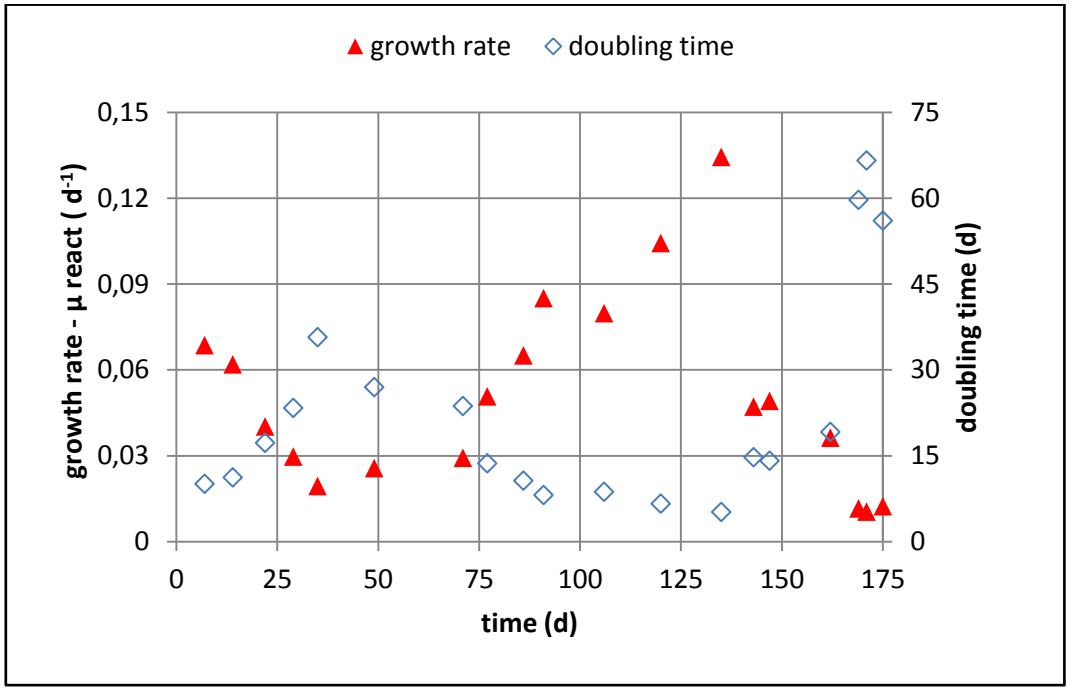


Figure 6.11 – Estimated specific anammox growth rate during the experimentation and corresponding doubling time.

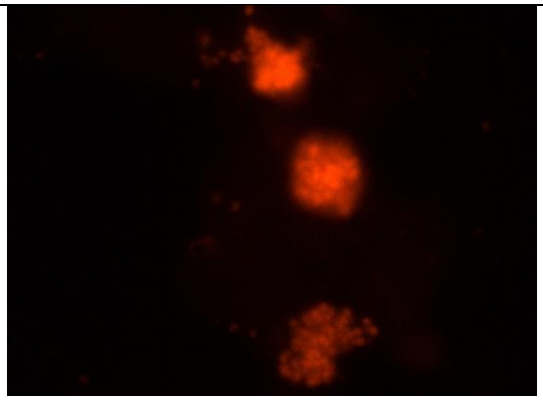
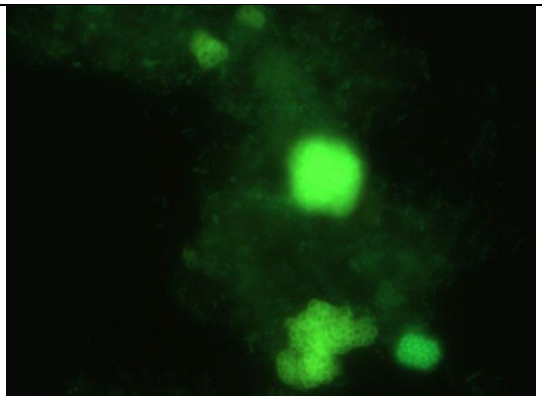
The estimated μ_{react} decreased from the value of 0.06 at day 14 to the value of 0.02 at day 35. Then, at day 71, it was 0.04 d^{-1} and from day 77 to day 135 it increased up to 0.13 d^{-1}.
The range of specific growth rate values obtained in the first 162 d corresponds to a doubling time between 5 and 36 days and are within the range of reported literature values (Strous et al. 1998, Fux et al. 2006, Tsushima et al. 2007, Van der Star et al. 2008). The low values of NRR_{max} during days 169-175 (100% real ww) corresponded to specific growth rates as low as 0.01 d^{-1} (doubling time 60 days).

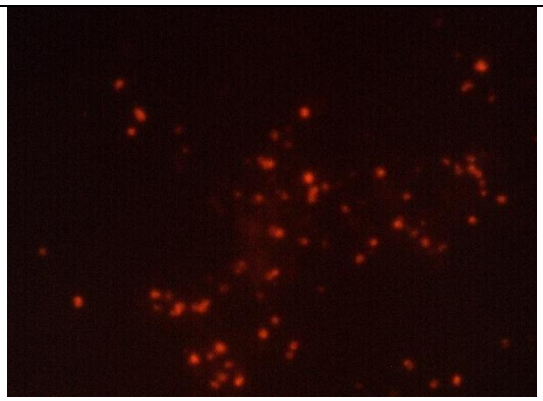
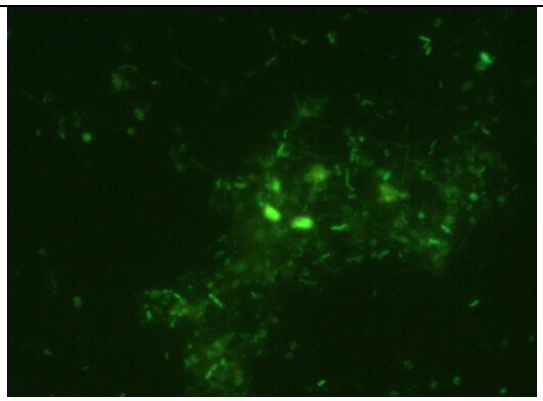
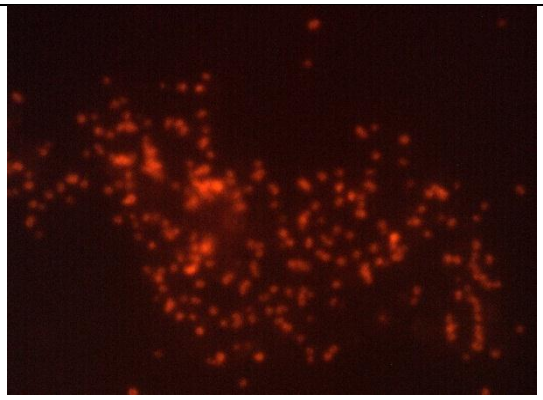
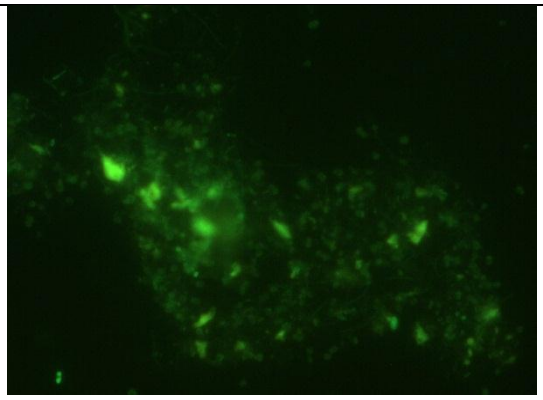
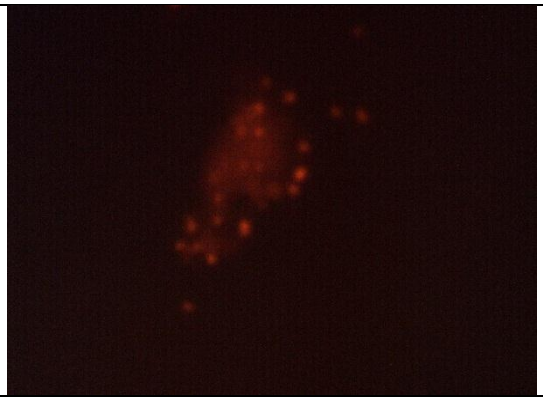
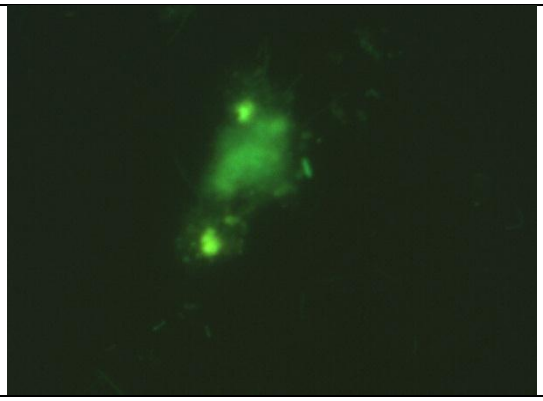
The decrease of NRR_{max} values in the first period might be related to sub-optimal environmental conditions in the reactor. At day 70 a problem in the T probe was detected: the real temperature in the reactor was 38°C while the probe was indicating 36°C, therefore the reactor operational temperature during the first 70 days of operation may have varied between 36°C and 38°C. As for any biological process, temperature affects anammox specific activity. According to experimental data on granular sludge reported by Dosta et al.(2008), the Anammox activity increases with temperature up to 37°C and decreases sharply for temperatures above 40°C, while the temperature effect is unclear between 37 and 40°C. A temperature of 38°C may have been sub-optimal for our granular sludge that had been grown at temperature between 32°C and 35°C (Van der Star et al. 2007).
The high NRR_{max} increase in between day 77 and 135 can be therefore correlated to a better control of the reactor temperature around 36°C. A higher availability of micro and macro-nutrients in the real wastewater fed in that period may also have favored the observed activity recovery.

6.4 Microbiological results

The results of FISH analyses showed the constant presence of the anammox organisms. It was also confirmed that all organisms are phylogenetically affiliated with the anammox detected species *Candidatus "Brocadia anammoxidans"*, reflecting the composition of the initial inoculum and no population shift was observed during the experimentation.
In Table 6.6 different picture of FISH images at different experimental time are reported and are compared with the results obtained on the same sample between the probe BAN162 for the Cand. “Brocadia anammoxidans” and the probes EUB338I+EUB338II+EUB338III detecting most bacteria. Except for the sample at day 0, all the following granular samples have been disrupted by using a “Potter homogenizer” before fixation.

Table 6.6 – FISH images at different experimental time

Time	Cand. “Brocadia anammoxidans”	most Eubacteria
0		

Time	Cand. “Brocadia anammoxidans”	most Eubacteria
50		
98		
113		

From FISH results on the sample collected after day 135 a sharp decrease in the presence of active anammox bacteria was evidenced (data not shown).

6.5 Discussion and conclusions

After 44 days of operation with the mineral medium as influent, the SBR was loaded with increasing fractions of real wastewater (effluent of the nitritation-denitritation pilot plant treating agricultural digestate supernatant) mixed with proportionally decreasing fraction of mineral medium. From day 70 to day 135 the fraction of real wastewater fed to the reactor was increased gradually up to 70%. The reactor efficiency remained stable and the maximum nitrogen removal rate capacity (NRR_{max}) in the reactor increased by 4 times up to 5.6 gN L^{-1} d^{-1}.
This fast increase in the NRR_{max} of the reactor can be related to:

- growth of the anammox biomass;
- increase in the specific nitrogen removal rate, probably due to the recovered optimal conditions (temperature and/or nutrients).

The observed increase in the NRR_{max} in the reactor between day 70 and 135 was contemporary to the increase in the wastewater fraction in the feed (from 10 to 70%). This suggests that no adverse effects of the real wastewater on anammox activity was taking place up to a fraction of 70% (v/v).

Later, two activity drops have been registered. The first NRR_{max} drop was registered after the reactor was kept in idle for 5 days at room temperature (15°C), after feeding a blend made of 70% of real wastewater. The second NRR_{max} drop took place after few days of feeding undiluted real wastewater. The organic compounds and solids fed to reactor increased a lot in these last days, favoring heterotrophic biomass growth.

It is noteworthy that the influent fed between day 167 and 170 (sample 3) was characterized by high suspended solids (1 gTSS L^{-1}) and COD concentrations (2600 mg solubleCOD L^{-1}). Moreover, in sample 3 the ratio of $sBOD_{20}$ to total soluble COD was 27%, while in sample 2 it was 12% (see Table 6.1).

Table 6.7 – Comparison between different literature studies on anammox process treating liquid fraction of piggery digestate

Reference	Reactor	Temp (°C)	HRT (d)	N influent concentrations mgN L^{-1}	Influent real wastewater (v/v)	NLR (kg N $m^{-3}d^{-1}$)	NRR (kg N $m^{-3}d^{-1}$)	pretreatment
Hwang et al. (2005)	Up-flow sludge bed reactor (1L)	35	2.5	NH_4^+-N: 213±32 NO_2^--N: 323±34	40%	1.36	0.72	Not indicated
Yamamoto et al. (2008)	Up-flow fixed bed reactor (2.85 L)	35	0.5	NH_4^+-N: 76±4 NO_2^--N: 111±4	25%	0.39	0.22	Clari-flocculation (cationic polimeric coagulant)
Yamamoto et al. (2011)	Up-flow glass column reactor (3L)	30	0.125	NH_4^+-N: 117±6 NO_2^--N: 130±15	10-15%	2.2	2.0	Filtration with polyester non-woven sheet
Quiao et al. (2010)	Granular reactor (PEG gel carrier) (0.73L)	30	0.2	NH_4^+-N: 213±94 NO_2^--N: 212±94	50%	4.1	3.12	No pretreatment
This study	**Granular SBR (6.5 L)**	**36-38**	**2.5**	**NH_4^+-N: 642±49 NO_2^--N: 737±43**	**10-70%**	**0.58±0.09**	**0.49±0.12**	**No pretreatment**

In Table 6.7, the main literature studies about the application of the anammox process to the treatment of the liquid fraction of livestock digestates after a partial nitritation step are reported. All the reported studies were related to piggery manure digestate, while in our study the digester was fed also with poultry manure and energy crops (mainly maize). All data reported in the literature concern reactors working with diluted wastewater (from 2 to 10 times), and with influent ammonium and nitrite concentrations 3 times lower than those maintained in our study. Some authors pretreated the influent for solids removal by means of clari-flocculation (Yamamoto et al. 2008) or filtration (Yamamoto et al. 2011), which was considered the best option. Others (e.g. Quiao et al., 2010) worked under extremely low HRT (0.2 d) to reduce the potential inhibitory effect of high concentration of slowly biodegradable organics on anammox activity.

The present study was conducted at stable influent nitrogen concentrations comparable to those expected in a full-scale plant. No pretreatment was applied and the fraction of real wastewater treated was gradually increased up to 100%. The nitrogen loading rate applied was maintained around 0.6 kg N $m^{-3}d^{-1}$ during the whole experimentation even though the maximum nitrogen removal capacity in the reactor reached 5.1 kg N $m^{-3}d^{-1}$ while the fraction of real wastewater in the SBR was 70%. The influent treated was the effluent of the nitritation-denitritation pilot plant modified by adding ammonium and nitrite salts. Although this is different from the actual effluent of a partial nitritation process, the experimental results can be considered as indicative of the anammox biomass response to these kind of wastewaters. As a matter of fact, the presence of potential inhibitors such as recalcitrant organics of heavy metals are not expected to vary significantly.

The following hypotheses can be made to explain the anammox activity losses experinced during this experimentation, which could be caused by the presence of a compound which may have been:

- already present in the real wastewater, and with an inhibiting threshold concentration that was reached when the dilution factor was reduced (e.g. antibiotics, humic/fulvic acids);
- produced in the reactor by hydrolisys of the slowly degradable influent organic matter. The rate of hydrolysis may have increased with time, as the hetherotrophic biomass growth was supported by the organic matter fed during the experimentation, with higher COD and volatile solids concentrations in the influent; this might have also be prompted during the 4-days idle phase at 15°C.

Moreover, the activity loss was experienced after 3 days feeding with undiluted wastewater and might have been favored by the high solids and COD content of the influent.

Available experimental data are insufficient to draw any definite conclusion on the causes of this inhibition. More tests are therefore needed to clarify this aspect. In the follow up, it would be also interesting to investigate whether the origin of the inhibition is caused by a soluble or particulate compound in order to identify effective detoxifying pretreatment options.

Nevertheless, the anammox process appears to be applicable to this real wastewater after a moderate dilution of 0.5: 1, without any further pretreatment. This result needs to be confirmed by a future long- term operation under stable conditions.

7 NITRITE INHIBITION AND RECOVERY RESPONSE OF ANAMMOX GRANULAR BIOMASS

All the results presented in this chapter were done in the LEQUIA (Laboratory of Chemical and Environmental Engineering) laboratory at University of Girona during a 6-month long research stage.

Most of the reported experiences on nitrite inhibition have been performed on anammox bacteria grown on synthetic medium, while little is known on the sensitivity of Anammox biomass grown on real wastewaters. In particular, mature urban landfill leachates are suitable to be treated by the anammox process. Leachates are high strength, complex wastewaters, moreover their composition is highly variable and strongly dependent on the landfill age. As leachate from old "mature" landfills is rich in ammonium nitrogen and has a low carbon to nitrogen ratio, it looks like a suitable feed for fully autotrophic nitrogen removal processes. However, very little experience on the application of the Anammox process to old-landfill leachate is yet available (Liang et al., 2008; Ruscalleda et al., 2008; Wang et al., 2010).

The aim of this work is the evaluation of the inhibition of Anammox activity at different nitrite concentration. The experimentation has been carried out on two different samples of granular anammox biomass, that have been started from the same inoculum, but that have been separately cultivated on a synthetic mineral medium (SBR-1), and on an urban landfill leachate (SBR-2). In particular, the research team was interested in evaluating whether the inhibitory response to high nitrite levels was affected by the nature of the wastewater that the anammox bacteria were used to treat. Moreover, the occurrence and relevance of the following activity recovery was also investigated on both Anammox biomass samples.

7.1 Analytical and microbiological methods

7.1.1 Analytical methods

The ammonium concentration was measured by distillation (BUCHI Distillation Unit B-324; Buchi Labortechnik AG, Flawil, Switzerland) followed by acid titration with sulphuric acid (Metrohm 719 S Titrino;Metrohm, Herisan, Switzerland).

Nitrite and nitrate concentrations were determined by ionic chromatography (Metrohm 761-Compact IC) and an autosampler IC (Metrohm 813 Compact Autosampler).

Total suspended solids (TSS) and volatile suspended solids (VSS), were measured according to the APHA Standard Methods for the Examination of Water and Wastewater (APHA, 2005).

Granular diameter distribution was assessed by using a laser diffraction particle size analyzer (Beckman Coulter, USA).

Images of the granular biomass were obtained using a digital camera (Nikon coolpix-4500) coupled to a stereomicroscope (ZEISS SteREO Discovery V12) using transmitted light (Schott KL2500).

7.1.2 Microbiological analysis

Biomass samples were collected and fixed in a 4% paraformaldehyde solution for subsequent fluorescence in situ hybridization (FISH). Hybridization was carried out according to Amann et al.

(1995) at 46 ºC during 90 minutes. The general probe targeting all bacteria was an EUBmix Cy3 labeled (EUB338-I, EUB338-II, and EUB338-III). The specific probe used was Amx-820 Cy5 labeled, targeting both Candidatus "Brocadia anammoxidans" and Candidatus "Kuenenia stutgartiensis". Moreover, some samples were also hybridized with a Fluo labeled Kst-157 probe that targets only Candidatus "Kuenenia stutgartiensis" in order to determine which anammox genera was present in the reactors.

For quantification, image acquisition was performed using a TCM SP5 confocal laser scanning microscope (CLSM) (Leica Microsystems, Germany). The percentage of anammox bacteria was estimated by determining the ratio of the specific bacterial biovolume of zones targeted as anammox to the total bacterial biovolume.

7.2 Inoculum and procedure

7.2.1 Anammox biomass

The granular biomass used in this experimentation came from two lab-scale anammox SBRs, both treating high N-strength wastewater. SBR-1 was a 20L reactor fed with synthetic mineral medium prepared according to Lopez et al. (2008), whereas SBR-2 was a 2L reactor treating real urban landfill leachate (Ruscalleda et al., 2010) pre-conditioned in a partial nitritation reactor (Ganigué et al., 2010). The SBR-2 was inoculated with anammox granules from SBR-1. The operational conditions of both SBR-1 and SBR-2 are summarized in Table 7.1. The temperature was maintained at 35 °C in both cases. 1M HCl-solution addition allowed pH control and adjustment between 7.1 and 8.0 in SBR-1, and 7.5 ± 0.1 in SBR-2. Both reactors were operated for more than two years before this study was carried out.

Table 7.1 - Characteristics of the two anammox SBR during the experimental period

Parameter	Units	SBR 1 Mineral medium (min – max)	SBR 2 Leachate (min – max)
NH_4^+-N influent concentration	mg L^{-1}	226.2 - 1232.5	309.4 - 530.6
NO_2^--N influent concentration	mg L^{-1}	302.1 - 1718.2	417.4 - 722.3
NH_4^+-N Removal Rate	gN L^{-1} d^{-1}	0.08 - 0.45	0.19 - 0.33
NO_2^--N Removal Rate	gN L^{-1} d^{-1}	0.03 - 0.60	0.25 - 0.44
Nitrogen Removal Rate	gN L^{-1} d^{-1}	0.14 - 1.02	0.41 - 0.75
Specific Nitrogen Removal Rate	gN $gVSS^{-1}$ d^{-1}	0.14 - 1.67	0.08 - 0.42
NH_4^+-N removed	%	78.3 - 98.9	83.4 - 99.2
NO_2^--N removed	%	87.1 - 99.9	85.1 - 99.6
N removed*	%	79.4 - 99.9	72.9 - 93.0
Influent total COD	mgCOD·L^{-1}	n.d.	572.3-993.1
Total COD removed	%	n.d.	6.1-37.4
HRT	d	2.28 - 2.92	1.58

*ammonium-N plus nitrite-N.

7.2.2 Specific anammox activity batch assay

To assess the specific anammox activity (SAA), a manometric method adapted from Dapena-Mora et al. (2007) was used. This technique is based on the principle described in par 3.2.2.
Batch assays were performed in 120 mL glass vials sealed with a rubber septum kept in place by an aluminum clamp. A thermostatic shaker (Bibby Scientific, UK) was used to keep the desired temperature (35°C) and mixing conditions (150rpm). A differential pressure transducer (Centerpoint Electronics) with a measuring range within 0–345 mbar and a resolution of 1.7 mbar was used for pressure measurements. This method was previously validated for its accuracy by performing injections of known amounts of water in closed vials, thus causing artificial pressure increases. Average errors in pressure measures were lower than 5%. SAA estimates on replicates showed an average variability of 8%.
Granular sludge samples were collected from the lab-scale reactors, washed and re-suspended in a the mineral medium (Lopez et al., 2008) to a final concentration of around 1 gVSS L^{-1} and supplemented with sodium bicarbonate (30 mM). Salinity in the SBR-2 samples was adjusted with NaCl to the same level to which the Anammox bacteria were normally exposed to. An anoxic gas mixture (95% Ar and 5% CO_2) was used to flush the reaction environment for de-oxygenation and to fix the working pH between 7.6 and 7.7. Then, vials were sealed and located in the thermostatic shaker. Concentrated solutions of NH_4Cl and $NaNO_2$ were injected through the rubber septum to set the initial ammonium and nitrite concentrations. To assess the N_2 production rate after the desired incubation time, pressure measurements were performed at 30 min intervals for 3 to 4 hours by piercing the rubber septum with the manometer needle. At the end of the test, ammonium nitrite and nitrate nitrogen concentrations were measured; the pH was checked to be within the optimal range for the Anammox activity. Finally the volatile suspended solid (VSS) concentration was assessed by filtration of the whole suspension volume to allow for the calculation of the specific anammox activity.
For each test, overpressure data were used to compute the cumulated volume of N_2 produced during time. From these data, the Maximum N_2 Production Rate (MNPR, $mgN_2\text{-}N\ L^{-1}\ d^{-1}$) was assessed as the maximum slope of the cumulative N_2 production curve. Finally, the specific anammox activity (SAA, $mgN\ L^{-1}\ d^{-1}$), was computed as the ratio between MNPR and the concentration of VSS ($gVSS\ L^{-1}$) in the suspension.

7.2.3 Procedure to assess nitrite inhibition

Different experimental batch tests were performed to assess the nitrite inhibitory response of granular anammox biomass sampled from both SBR-1 and SBR-2. The inhibitory response was assessed after 3-4 h (short-term response) and after 24 h (long-term response) of incubation at the desired nitrite concentration. Short term recovery of anammox activity after biomass washing was also assessed.

Each anammox sample was split into 6 equal aliquots that were used and ammonium chloride was added to achieve 50 mgNH_4^+-N L^{-1} in all vials, while different amounts of sodium nitrite were added in order to achieve the following nitrite concentrations: 50 mgNO_2^--N L^{-1} in the control vial to assess the maximum SAA, followed by 100, 200, 300, 400 and 500 mgNO_2^--N L^{-1} in the remaining vials. Then, the activity test was performed in all vials following the procedure described in the paragraph above. First, the SAA activity was assessed during the initial 3-4 hours of incubation (short exposure). At this point, vials were opened to adjust nitrite and ammonium concentrations to their initial value, deoxygenated and resealed. The SAA was assessed again after 24 h of further incubation (long exposure). Vials were kept in incubation for further 24 h to achieve a final nitrite exposure time of 48 h. At this point, all vials were opened and the granular anammox suspension was washed twice by making it settle and by replacing the supernatant with fresh mineral medium. A further assessment of the SAA was run during the following 3-4 h (recovery) by adding ammonium (50 mgNH_4^+-N L^{-1}) and nitrite (50 mgNO_2^--N L^{-1}) in all vials. The above described procedure is sketched in Figure 7.1.

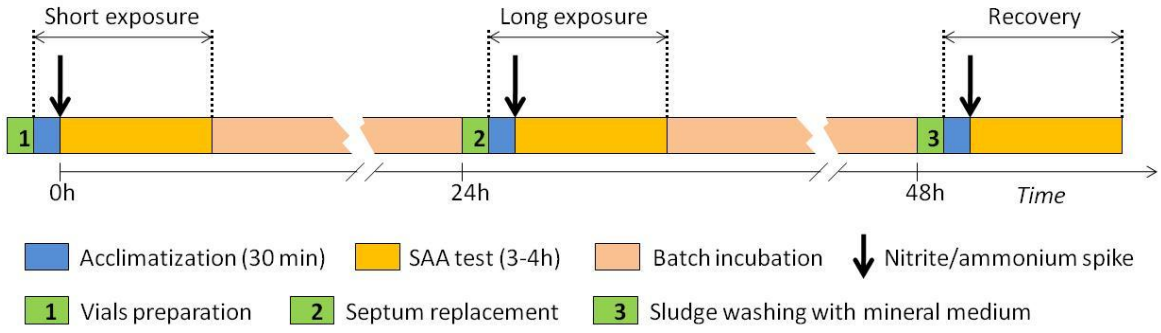


Figure 7.1 - Sketch of the procedure for the assessment of short and long term nitrite inhibition and recovery.

7.3 Biomass characteristics

In order to assess whether the chemical composition of the feeding influences the anammox community, several macro and micro properties of the granular biomass coming from each SBR were analyzed and presented in Table 7.2. Granules were first analyzed for their size distribution (Figure 7.2).

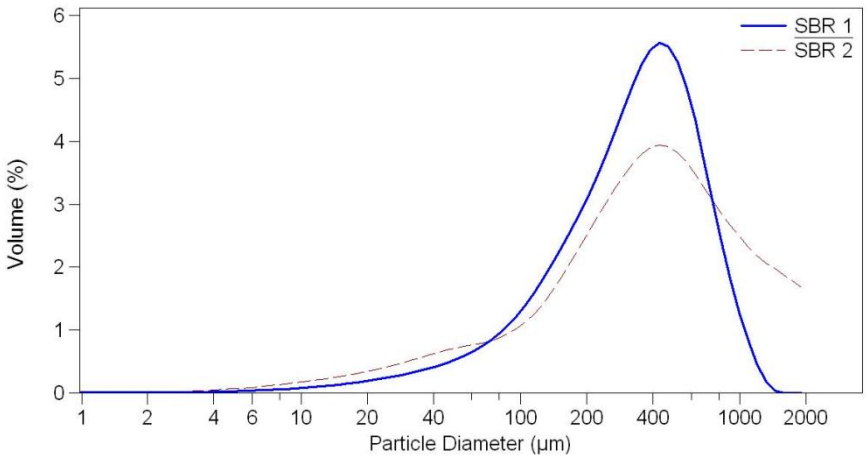


Figure 7.2 - Particle size distribution for SBR1 and SBR2 granules.

The results obtained showed that the size distribution of granules was quite similar in both reactors, with a similar median diameter but a lower mean value and variability for the SBR-1 granules. Despite similar mean and median the 90th percentiles were significantly different (720µm and 1220µm in SBR-1 and SBR2 respectively) confirming that in SBR-2 more granules with diameter bigger than 1,000µm were present. In summary, granules grown in SBR-1, fed with synthetic mineral medium, were averagely smaller and more homogeneous than those grown in SBR-2, fed with urban landfill leachate.

This result was confirmed by the stereomicroscope images (Figure 7.3). As it can be seen in Figure 7.3-A1, granules from SBR-1 are more homogeneous with a higher abundance of small granules if compared to the granules from SBR-2 (Figure 7.3-A2), which present some granules with a diameter over 2,000µm.

The pictures obtained with the stereomicroscope show granules with a red-brown color, typical in anammox sludge. However, the presence of anammox bacteria was confirmed by molecular techniques. FISH analysis revealed the presence of Candidatus “Brocadia anammoxidans” in both SBR (Figure 7.3, B1 and B2). FISH images were also used to evaluate the relative abundance of Anammox bacteria. The relative abundance of anammox bacteria in the microbial community of SBR-1 and SBR-2 was measured to be 86% ± 11% and 61% ± 17%, respectively. Granules from SBR-1 were richer in Anammox bacteria, and this is reasonably due to the fact that the SBR-2 influent may have supported a more abundant heterotrophic community, growing on the slowly biodegradable COD of the leachate.

Specific anammox activity was assessed in control samples during inhibition tests and was found to be averagely higher for granules collected from the SBR-1, fed on the synthetic medium, as presented in Table 7.2. This may be due to the higher percentage of Anammox bacteria on total bacteria in this reactor (86% ±11% in SBR-1 compared to 61% ±17% in SBR-2), which can be due to the different type of wastewater fed to the two reactors.

However, anammox activity resulted to be practically the same when taking into account the percentage of Anammox bacteria, i.e. 0.50 $\pm$ 0.14 and 0.52 $\pm$ 0.26 gN_2-N $gVSSanammox^{-1}d^{-1}$, for

SBR-1 and SBR-2, This was obtained by assuming that all bacteria have similar density and therefore that the volumetric percentage equates the percentage in weight.

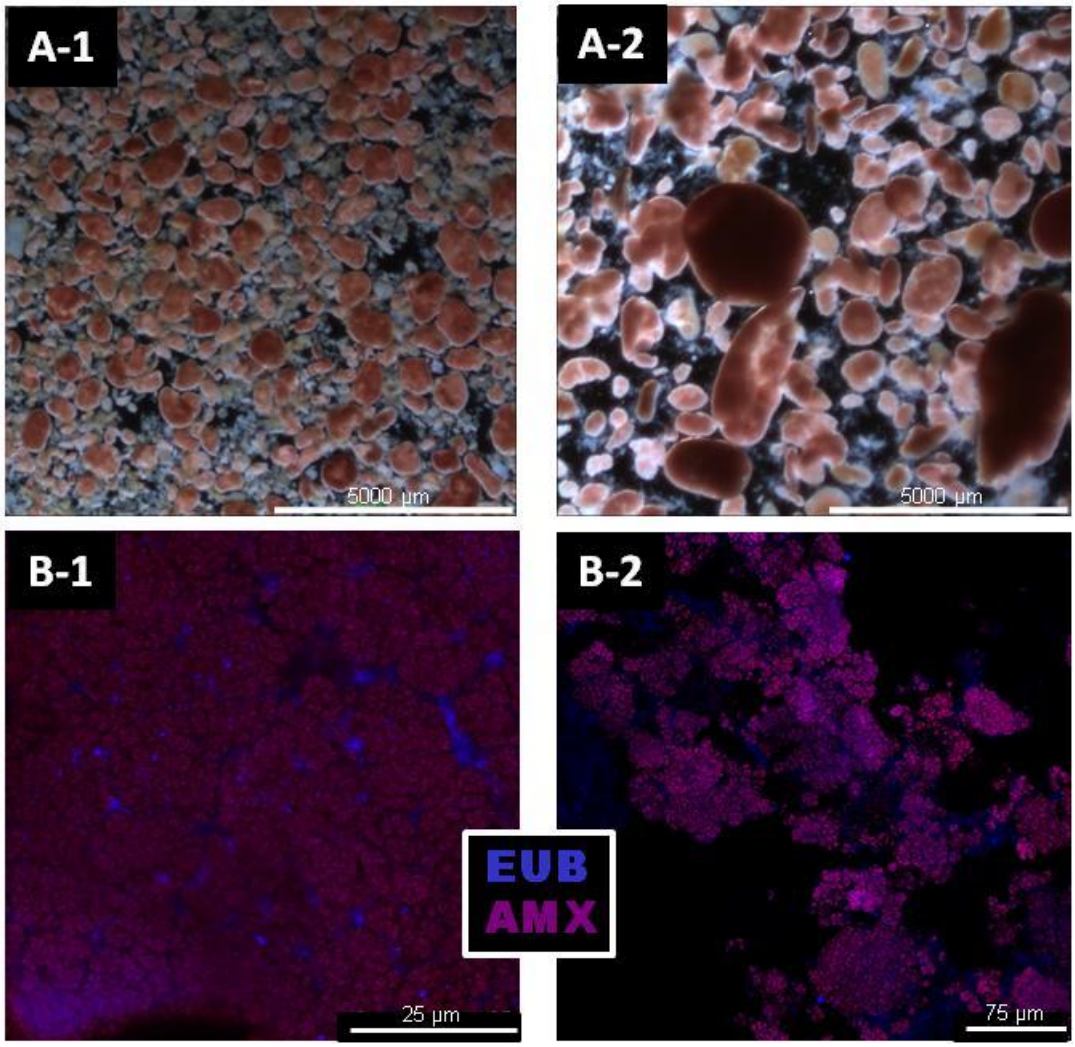


Figure 7.3 - Stereomicroscope images (A), FISH images, EUB=all the bacteria, AMX=Anammox bacteria (B), Left side: SBR-1. Right side: SBR-2

Table 7.2 - Main characteristics of anammox granules from SBR-1 and SBR-2.

Parameter		SBR-1	SBR-2
Specific anammox activity (gN_2-N $gVSS^{-1}d^{-1}$)	Average	0.43 ± 0.11	0.32 ± 0.13
	Number of repetitions	10	12
Granule size distribution(μm)	Mean	379	518
	Standard deviation	247	455
	Median	340	382
	90th percentile	720	1223
Anammox/total bacteria volumetric ratio	Average	86% ±11%	61% ±17%
	Number of repetitions	22	16

Granules were first analyzed for their size distribution. The two size distributions are quite similar, with SBR-1 granules showing lower average and lower variability than SBR-2 granules, but similar median diameter. This result is confirmed by the stereomicroscope images (Figure 7.3 A1 and A2).

7.4 Inhibition results and discussion

7.4.1 Response to short and long term nitrite exposure

As described previously, the anammox inhibition response to nitrite concentrations ranging from 100 to 500 mgNO_2^--N L^{-1} was tested within the first 3-4 h (short exposure) and after 24 h of exposure (long exposure). A total of 61 and 63 inhibition tests were performed on SBR-1 and SBR-2 anammox samples, respectively.

Figure 7.4A shows the results of the short term inhibition tests performed at various nitrite concentrations. The percentage of residual SAA with respect to the blank sample (i.e. exposed to 50 mgNO_2^--N L^{-1}) is reported as a function of the nitrite concentration. Within the tested range, residual activity decreased almost linearly with increasing nitrite concentration. By comparing the response of anammox samples collected from the two SBRs, one can see that they showed a very similar and partial inhibition to almost all the tested nitrite concentrations. Up to 100 mgNO_2^--N L^{-1} almost no inhibition effect can be observed On the overall, all tested conditions caused a loss of activity lower than 40%.

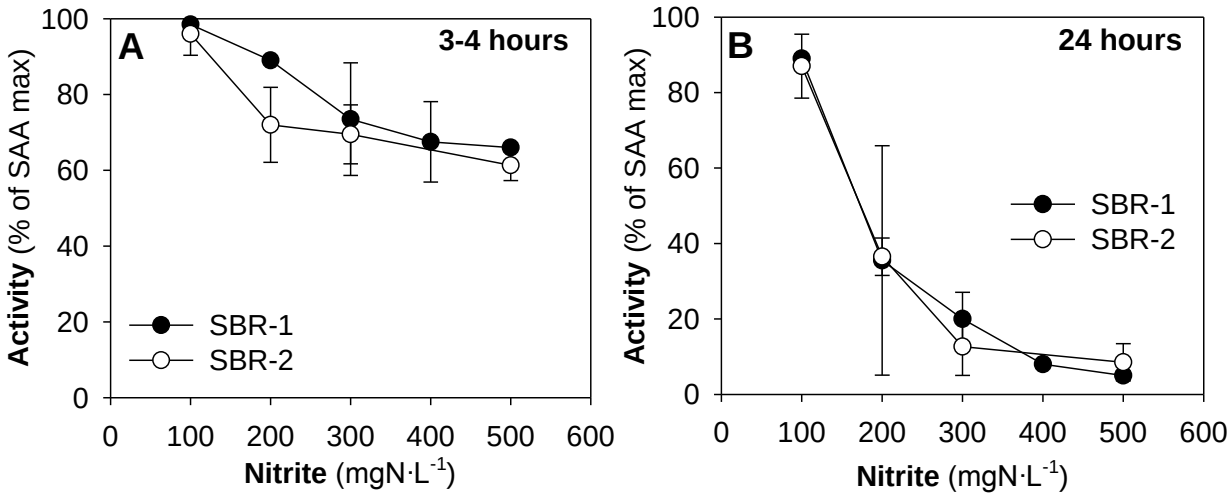


Figure 7.4 - Short term (A) and long term (B) response of anammox sludge from SBR-1 (black dots) and from SBR-2 (white dots).

After 24-h exposure, the inhibition response was found to be more severe, as suggested by Figure 7.4B. Residual activity after 24-h exposure was substantially lower than after 3-4 h exposure for all tested concentrations, with inhibition exceeding 50% at concentrations higher than 200 mgNO_2^--N L^{-1}. The activity measured in SBR-2 in the 100 mgNO_2^--N L^{-1}long-term activity test was still high (87 $\pm$ 3.5% of the maximum SAA) but dropped to only 36 $\pm$ 4.9% of the maximum SAA after 24h-exposure at 200 mgNO_2^--N L^{-1}. Similar results were obtained for SBR-1 granules, so that one can conclude that anammox bacteria from SBR-1 and SBR-2 had comparable activity loss after long-term exposure to nitrite.

According to these results, the effect of nitrite inhibition on anammox bacteria strongly depends on the duration of the exposition. A short-term exposition up to 500 mgNO_2^--N L^{-1} had a relatively low impact on the nitrogen removal capacity of the system. However, after 24h the treatment capacity was dramatically affected at nitrite concentration around 200 mgNO_2^--N L^{-1}. These results suggest that anammox reactors can deal with nitrite shocks, but corrective measures must be rapidly applied.

Long-term dose response data were fitted by means of the minimum squared errors method with a S-shaped curve (extended non-competitive inhibition model, Kroiss et al., 1992) to estimate the IC50 (50% inhibition concentration) value. To this purpose, the following equation was used:

$$I(\%) = 100 \times \left(1 - \frac{1}{1 + \left([NO_2^- - N] / a \right)^b} \right)$$

Where I% represents the inhibition response (I% = 100 - residual activity), while *a* and *b* are fitting parameters. Note that parameter *a* is the model estimation of IC50. Dose-response data and their model interpretation are reported in Figure 7.5. The estimated IC50 values were 173 $\pm$ 23 mgNO_2^--N L^{-1} and 171 $\pm$ 8 mgNO_2^--N L^{-1} for SBR-1 and SBR-2, respectively, confirming similar inhibition response for both Anammox communities, as already commented before. These long-term IC50

values are lower than 350 mgNO_2^--N L^{-1}, as reported by Dapena Mora et al. (2007) in batch test lasting 5-7 hours and tested on a different anammox specie (Candidatus Kuenenia Stuttgartiensis).

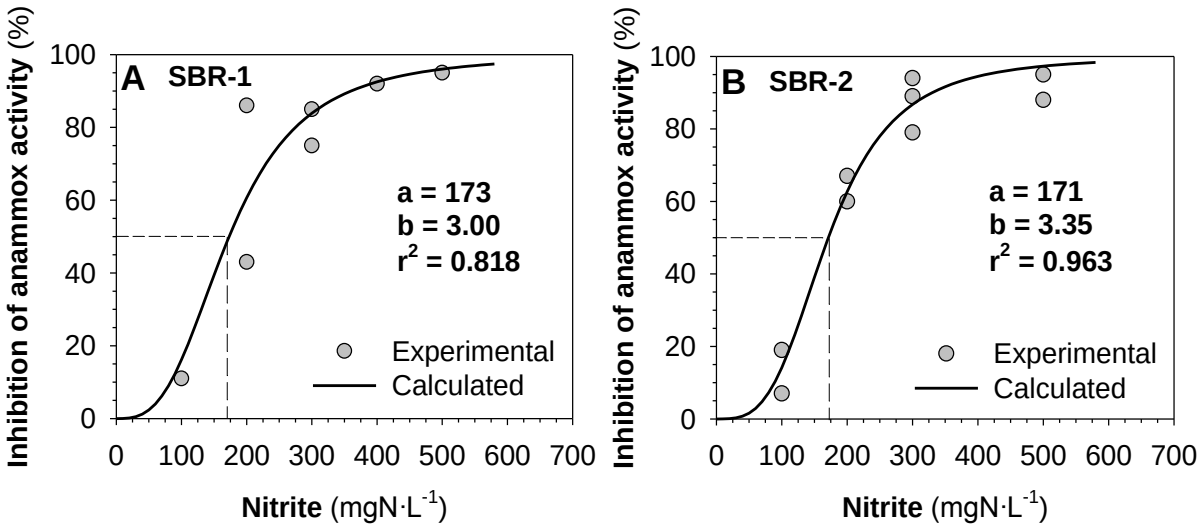


Figure 7.5 - Dose-Response curve for long term exposure to nitrite for the SBR-1 (left) and SBR-2 (right) anammox granules.

Interestingly, these experimental data suggest that the short and long term inhibition response is independent from the chemical nature of the influent that is fed to the anammox biomass. Moreover, the anammox granules from both reactors were proven to be quite tolerant to moderate and high nitrite concentrations, as long as the exposure time was limited to few hours. On the contrary, after prolonged exposure, the activity loss was substantial and 24 hour exposure to 500 mgN-NO_2^- L^{-1} resulted in a almost complete inhibition of the anammox activity. These results confirm that nitrite inhibition is highly dependent on the exposure time and are in accordance with the results of IC50 reported by Dapena-Mora et al. (2007), since their results at "medium term" exposure (7h) fit and confirm the trend found in this study.

The dependence of the activity loss on the exposure time to high nitrite levels was also reported by other authors in short-term batch tests and in long-term continuous-feed tests (Kimura et al., 2010; Fernandez et al., 2010). This suggests that major activity loss in anammox reactors can be avoided by a timely identification of process operational conditions causing nitrite build-up in the reactor. It is now interesting to evaluate whether this activity loss is reversible or not, since irreversible toxicity would result in long recovery periods due to the slow growing rate of these microorganisms.

7.4.2 Activity recovery after nitrite inhibition

To evaluate the reversible or irreversible nature of the SAA reduction caused by nitrite exposure, samples were let incubate for 48h at various nitrite concentrations. Then, vials were opened and the granular anammox suspension washed and re-suspended in a fresh mineral medium with an initial nitrite concentration of 50 mgNO_2^--N L^{-1} (no inhibition concentration). After washing, the

SAA was once again assessed. Results are summarized in Figure 7.6 for both reactors. After washing, the anammox activity recovered substantially (white dots, Figure 7.6) in all range of nitrite concentration exposed previously. Samples previously exposed to 300 mgNO_2^--N L^{-1} reached 68 ± 2.1% (SBR-1) and 73 ± 11.3% (SBR-2) of the control value activity. Similarly, the washing procedure caused a partial recovery in the activity of samples previously exposed to 500 mgNO_2^--N L^{-1} and whose residual SAA was found to increased from 10% or less, to around 60% after washing. A very similar recovery of activity was registered for samples taken from both SBRs, consistently with the similar inhibitory response. On the overall, residual activities after 48h exposure and washing were practically the same as those assessed after the short-term exposure.

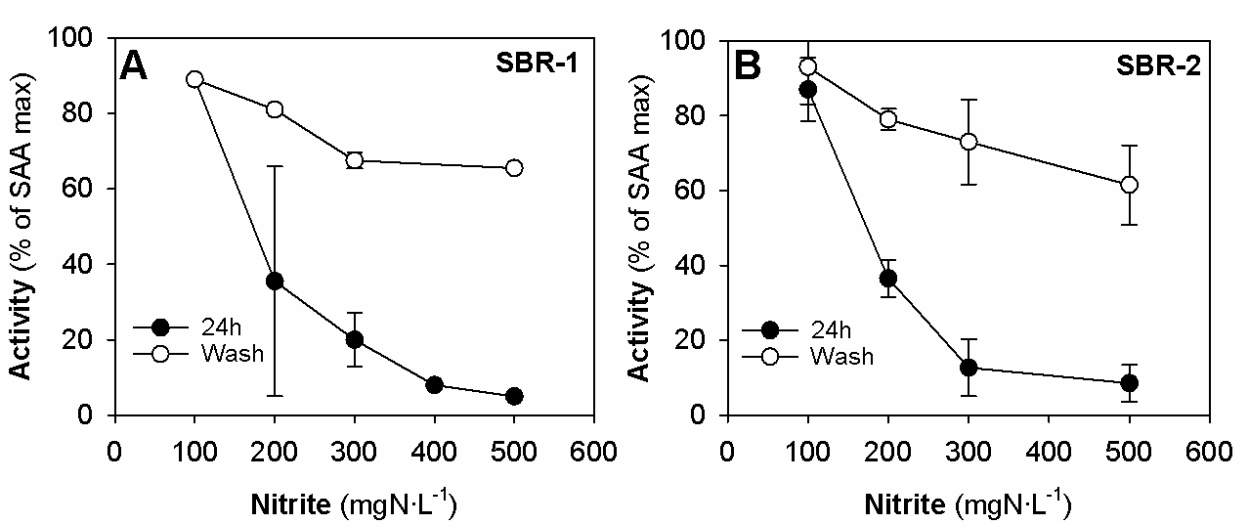


Figure 7.6: Comparison of anammox activity from SBR-1 and SBR-2 granules exposed to various nitrite concentrations for 48h and then washed with a nitrite free mineral medium (white dots). Black dots depict the residual activity after 24h as shown in fig. 6.5 B.

7.4.3 Effect of leachate on anammox bacteria

A sample from SBR 2 was taken to test the stress on anammox activity during leachate treatment. The activity test (see par 7.2.2) was performed in duplicate leaving the real medium in 2 bottles (Leachate1 and Leachate2) and replacing it with synthetic medium in other 2 bottles (Synthetic1 and Synthetic2).

The same conditions of temperature, pH, salinity (8.5 gNaCl L^{-1}), bicarbonate (2.5 g$NaHCO_3$ L^{-1}) and nitrogen compounds (50 mgNH_4^+-N L^{-1} and 50 mgNO_2^--N L^{-1}) were maintained in the 4 bottles.

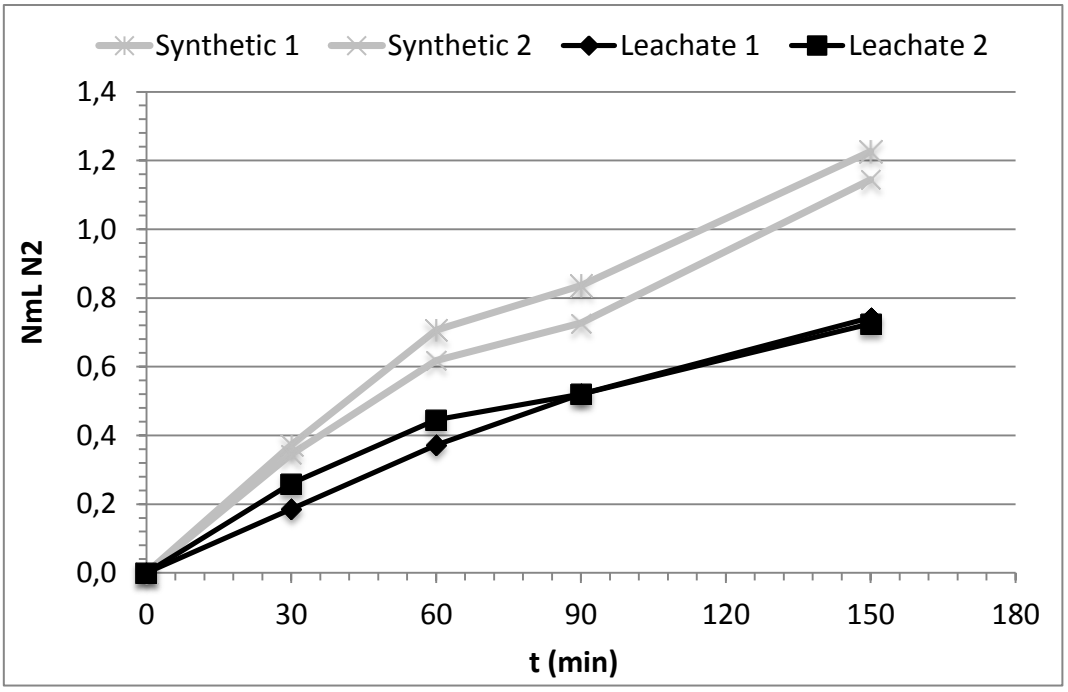


Figure 7.7 – Activity test to verify leachate effect on anammox activity

The results (Figure 7.7) clearly showed that activity was higher (+130%) in the batch tests with synthetic medium than in the tests with real wastewater (leachate). This would mean that one or more compounds, yet unknown, present in the leachate, partly inhibits anammox bacteria.

7.5 Conclusions

Anammox granules from two SBRs fed on very different wastewaters (synthetic medium and landfill leachate) shared many similarities in terms of microbial population and kinetics, resulting in similar response to increasing concentrations of nitrite (100, 200, 300, 500 $mgNO_2^-$-N L^{-1}). Despite the feeding of SBR-2 with leachate, both reactors presented granules enriched with Candidatus Brocadia anammoxidans. SBR-2 presented a more heterogeneous granule size distribution compared to SBR-1, but the behavior of the biomass to nitrite exposure was similar in both reactors. SBR-2 presented more abundance of heterotrophic bacteria due to the COD content in the leachate, but similar maximum SAA were measured in SBR-1 and SBR-2 (0.43 ± 0.11 and 0.32± 0.11 gN_2-N $gVSS^{-1}$ d^{-1}, respectively).

Anammox granules from both reactors were proven to be quite tolerant to moderate to high nitrite concentrations, as long as the exposure time was limited to 3-4 hours with less than 40% activity loss at 500 $mgNO_2^-$-N L^{-1}. However, after prolonged exposure (24 h), the activity loss was substantial, with IC50 values of 173 $mgNO_2^-$-N L^{-1} and 171 mg $mgNO_2^-$-N L^{-1} for Anammox granules grown on synthetic medium and on leachate, respectively). After washing with nitrite-free medium, anammox activity recovered substantially in the granules from both the SBRs, reaching the 60-80% of the initial maximum SAA. This confirms that major activity losses in

anammox reactors can be avoided by a timely identification of process operational conditions causing nitrite build-up in the reactor.

8 GENERAL CONCLUSIONS

General aim of this thesis was to explore the feasibility of advanced biological processes to reduce nitrogen from agricultural digestate to comply with the European directive on nitrates (91/676/CEE). This thesis is part of a bigger project (BRAIN) and the two biological processes considered to treat the liquid fraction of agricultural digestate are the nitritation-denitritation process (DENO2) and the fully autotrophic nitrogen removal process in two separate reactors (partial nitritation and anammox). A pilot scale study on the application of the DENO2 process and three different lab-scale studies on the anammox process have been performed.

Digesters operated at farm level are more and more managed with the main aim of maximizing biogas production. Co-digestion of animal wastes, agro-wastes and energy crops is commonly applied and this matrixes are mixed in variable proportions according to their seasonal availability. Moreover, operational parameters of digesters of agro-wastes may vary significantly and unpredictably. As a consequence, the chemical composition of digestate is expected to be highly variable.

Part of the experimentation presented in this thesis was performed in a piggery farm where co-digestion of piggery wastewater, poultry manure and energy crops was operated. During a 7-month experimentation, the composition of the digested liquid fraction was monitored. Experimental data confirmed that the influent variability was very high: ammonium varied between 600 and 1600 $mgNH_4^+$-N L^{-1}, COD between 1300 and 7500 $mgCOD\ L^{-1}$, BOD_5/COD between 34 and 56%, COD/TKN between 0.9 and 6.3.
To deal with highly variable wastewaters, sequencing batch reactors are more adapted than continuous processes for their inherent operational flexibility. An SBR was therefore designed to test the DENO2 process at pilot-scale.

Results of the DENO2 experimentation allowed to draw the following conclusions regarding the nitritation-denitritation process:

- The NOB suppression resulted to be easily and quickly achievable during the experimentation. A satisfactory nitritation efficiency has been maintained: in fact, the NO_2/NO_x ratio at the end of the aeration phase remained around 80-90% at temperature 25°-30°C, SRT 18-30 d and oxygen concentration in the range 0.5-1 $mgO_2\ L^{-1}$;

- As for nitritation, a stable AOB population could be maintained in the SBR when the reactor SRT was adequate to prevent biomass wash-out. In the definition of the minimum operational SRT, substrate limitation factors as well as inhibition effects are to be taken into account. In this regards, useful information were obtained. The free ammonia and free nitrous acid inhibition functions and IC_{50} were assessed for AOB activity. According to these data and under stable operation, the overall FA and FNA inhibition was calculated to be averagely 8±4% and always lower than 20%. Dissolved oxygen limitation was proven to strongly affect AOB activity especially during the first part of the aeration phase, i.e. when heterotrophic oxygen uptake takes place simultaneously to nitrification. Therefore, while typical Monod term for DO limitation correctly describes the AOB sole respiration, it does

not fit experimental data obtained in the presence of active heterotrophic respiration and overestimates the AOB actual respiration rate. This phenomenon may explain the observed differences between nitritation rates measured in the SBR and those measured in the activity tests which were always higher than actual rates. A deeper understanding of this phenomenon would allow a more accurate prediction of the actual AOB activity under practical operational conditions. Finally, results of activity tests suggest that the digestate liquid fraction did not have significant inhibitory effects on AOB activity.

- N_2O emissions have been detected in preliminary tests accounting for 14-20% of the N treated. These high N_2O production may be caused by the low C/N during the pre-denitrification phase. Many other factors that are reported to favour N_2O emissions are present in $DENO_2$ process configuration (e.g. transient periods, high nitrite concentrations and low dissolved oxygen concentrations), therefore this aspect needs to be considered and evaluated in deeper detail.

The SBR configuration confirms to be a flexible solution to treat such variable wastewaters. During more than 200 days of experimentation the pilot plant worked with nitrogen loading rates between 0.3 and 0.6 gN L^{-1} d^{-1}. Soluble nitrogen removal efficiency ranged from 60-70% up to 90-95% despite the wide variability of influent concentrations and operational parameters (HRT 2-4 d, SRT 4-30 d, DO 0.5-1.0 mgO_2 L^{-1}, temperature 25-30 °C). Conductivity showed a good correlation with ammonium concentration (270 mg NH_4^+-N $(mS/cm)^{-1}$, R^2=0.99) during the nitritation phase confirming to be a useful process monitoring tool in future applications.
Fixed cycle phases is not an optimal operational strategy. Nevertheless, even under non-optimal external acetate addition, the DENO2 efficiency was generally satisfactory. Process optimization may be addressed in future tests, as it is challenging when dealing with highly variable wastewaters. However, optimization requires a more complex plant instrumentation and operational procedures. Moreover, it is difficult to provide well trained operators with a deep knowledge of the biological processes in rural areas. The actual basic configuration may be seen as a good compromise between simplicity and effectiveness in this context.

The lab-scale studies performed on the anammox process were: (i) anammox enrichment from conventional sludge samples; (ii) tests to assess the applicability of anammox to treat the liquid fraction of agro-waste digestate; (iii) nitrite inhibition and recovery of anammox biomass.
These experimentations allowed to draw the following conclusions about anammox process and its applications:

- a simple fed-batch method to enrich anammox was applied to conventional sludge samples from Italian wastewater treatment plants treating municipal, yeast-production or swine effluents. Anammox bacteria successfully grew from all tests samples and their activity became evident after 75 to 220 days. The following enrichment phase in a SBR allowed to increase the nitrogen removal rate 10 times in 80 days, reaching 0.22 gN $L^{-1}d^{-1}$;

- monitoring anammox activity with conventional or manometric tests resulted to be a powerful tool in order to evaluate the effective nitrogen removal capacity, detect inhibitions or activity reduction and thus assisting in load definition;
- anammox granules from 2 different SBR were proven to be quite tolerant to moderate to high nitrite concentrations, as long as the exposure time was limited to 3-4 hours, with less than 40% activity loss at 500 mgNO_2^--N L^{-1}. A prolonged exposure (24 h) causes a substantial activity loss (IC_{50} around 170 mgNO_2^--N L^{-1}). However, after washing with nitrite-free medium, anammox activity recovered, demonstrating that nitrite inhibition is reversible. Also during the continuous operation of anammox SBR, after temporary nitrite accumulation up to 400 mgNO_2^--N L^{-1} (due to technical failures) the anammox process quickly recovered without the need of reducing the nitrogen load applied;
- lag-phases and periods of variable growth rates were experienced in all the lab-scale experimentations, probably related to temporary sub-optimal conditions in the reactor (e.g. high temperature, lack of nutrients) but not always attributable to specific causes;
- A lab-scale SBR was inoculated with anammox granules and fed with the effluent of the DENO2 reactor diluted with a mineral medium; the dilution ratio was gradually decreased from 0.1:1 to 1:1. A satisfactory nitrogen removal rate of 0.54±0.07 gN $L^{-1}d^{-1}$ was achieved as far as the dilution factor was 0.4:1 and during the first 15 days of feeding with a dilution of 0.7:1. During this period, the DENO2 effluent seemed to exert a stimulation effect on the anammox growth since the nitrogen removal capacity increased 4 times in 60 days reaching 5.1 gN L^{-1} d^{-1}. Later, while still feeding with a dilution of 0.7:1 and also when treating the undiluted wastewater, the anammox activity dropped. Inhibition could be caused by one or more compounds already present in the real waste water (e.g. antibiotics, humic/fulvic acids) or that have been produced in the reactor (e.g.: by hydrolysis of the slowly degradable organic matter). Therefore, at this stage of the research, the anammox process seems applicable to such wastewater, provided that at least a 50% dilution is applied.

Concerning the future perspectives and research needs:

- more tests on N_2O emissions for the DENO2 process are needed to check whether different modes of operation may reduce the N_2O emissions (e.g.: avoiding excessively low C/N ratios in denitrification, by adding more external carbon). To better understand the mechanism of N_2O production and confirm our hypothesis, dissolved N_2O concentrations analyses during the whole cycle should be performed allowing to calculate a complete mass balance and detect the most critical phase. Should those high emissions of N_2O be confirmed even under optimal operative conditions, the environmental feasibility of DENO2 process in this configuration may be questionable.
- long term experimentation is needed to fully confirm the feasibility of complete autotrophic process to treat agricultural digestate. A pilot-scale experimentation is already planned. The DENO2 reactor will be switched into a partial-nitritation SBR reactor, followed by a granular SBR anammox reactor. A challenging issue will be to

obtain a suitable effluent for the anammox reactor in terms of a stable nitrite/ammonium molar ratio and low residual biodegradable organic matter. As for the anammox reactor, further research is needed to indentify the cause of the observed anammox inhibition. It would be also interesting to investigate whether the origin of the inhibition is caused by a soluble or particulate compound, and to identify effective detoxifying pretreatment options to overcome the need for dilution as well as possible long term acclimation.

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