Università degli Studi della Basilicata

Scuola di Dottorato di Ricerca

Dottorato di Ricerca in

METODI E TECNOLOGIE PER IL MONITORAGGIO AMBIENTALE

TITOLO DELLA TESI

MONITORAGGIO DEL PROCESSO ANAMMOX: ASPETTI FISIOLOGICI PER UN UTILIZZO IN PIENA SCALA

Settore Scientifico-Disciplinare

ICAR03

Coordinatore del Dottorato

Prof. Ignazio Mancini

Università degli Studi della Basilicata

Dottorando

Dott. Tommaso Lotti

Tutor

Prof. Claudio Lubello

Università degli Studi di Firenze

Co-Tutor

Prof. Mark C. M. van Loosdrecht

Delft University of Technology

A.A. 20010/2011 Ciclo XXIV

TABLE OF CONTENTS

CHAPTER 1

General introduction

Outline

This chapter aims to provide a general introduction to the main topics addressed in this thesis. In the first part the anammox process and the problems related to its implementation, as well as the physiology of anammox bacteria are introduced. In the second part an overview of the environmental aspects related to animal breeding is illustrated, followed by the possible solutions and mitigations that biotechnology proposes.

1.1 ANAMMOX PROCESS

Nitrogen is one of the most abundant elements in nature. Formally divided into organic and inorganic nitrogen, it's present in the biosphere, lithosphere, hydrosphere and atmosphere. Many industrially important compounds, such as fertilizers, ammonia, nitric acid, organic nitrates (propellants and explosives) and cyanides also contain nitrogen. Nitrogen occurs in all living organisms (organic nitrogen), since it's a constituent element of amino acids and thus of proteins and nucleic acids (DNA and RNA). It resides also in the chemical structure of almost all neurotransmitters, and is a defining component of alkaloids, biological molecules produced by many organisms. For instance the human body contains about 3% mass-percentage of nitrogen, a larger fraction than all elements apart from oxygen, carbon, and hydrogen. Inorganic nitrogen atoms exist in different oxidation states ranging from -3 (ammonium/ammonia) to +5 (nitrate/nitric acid).Through redox reactions, catalyzed by microbial activity, most of the nitrogen compounds representing these oxidation states can be converted from one to another. These conversions were linked by microbiologists with the so-called Nitrogen Cycle (Fig. 1.1). As nitrogen fixation, nitrification and denitrification had already been described for a long time (Winogradsky, 1949), at the beginning of the 20th century the N-Cycle was assumed to be complete. 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, -358 kJ mol^{-1} (Broda, 1977), with nitrite as electron acceptor. Moreover, based on mass-balance studies, oceanographers had already noticed that the accumulation of ammonium in anoxic fjords was far less than was to be expected according the assumption that ammonium is inert under anoxic conditions (Richards 1965). A few years later this was likely stacked by a thermodynamic justification (Cline and Richards, 1972). These predictions were put forward however in an era in which the general belief was that biological ammonium oxidation under anoxic conditions was simply impossible. 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 (Heijnen 1998; Mulder 1989; Mulder et al., 1995; van de Graaf et al., 1990). Succeeding this evidence, numerous attempts to isolate the organism responsible for this process failed (Strous et al., 1999) in the years following. About ten years after the initial observations in Delft, in Germany (Hippen et al., 1996) and in Switzerland (Binswanger et al., 1997; 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 consisting of a modernization of the Winogradsky/Beyerinck strategy of selective enrichment and based on the introduction of the molecular toolbox and modern bioreactor engineering 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).

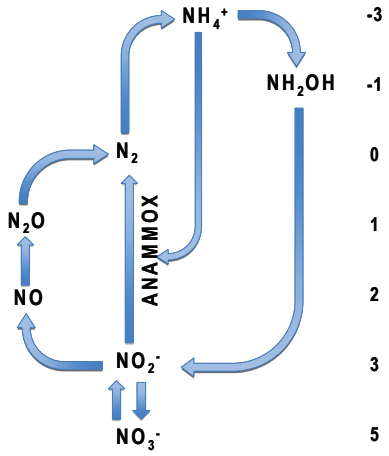


FIG. 1.1
The Nitrogen Cycle. The arrows represent microbial catalyzed reactions; on the right is reported the oxidation states of the N-compounds.

1.2 PHYSIOLOGY

For the application of the anammox process in wastewater treatment, fifteen-years of research had provided a sufficient overall view of physiological parameters needed. Surprisingly however, many of the parameters that were identified as critical are not known quantitatively or their mode of action is largely unknown. This is probably a result of the experimental complications of slow growth and the absence of pure cultures. Therefore, this paragraph includes a discussion on the present knowledge of the values of the important parameters of anammox bacteria for use in process and reactor design. Such physiological parameters for ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) are of importance for the proper evaluation of the one-reactor nitrification-anammox process. As a comprehensive description of these values is reported elsewhere (Anthonisen et al., 1976; Wiesmann, 1994), they will not be explicitly discussed here.

Stoichiometry and growth rate

Although anammox bacteria are autotrophic and gain similar energy from their catabolic reaction as AOB, their maximum specific growth rate was reported to be considerably lower. Instead of 1 to 1.2 d^{-1} (typical for AOB, Anthonisen et al., 1976; Sin et al., 2008), the growth rate of anammox bacteria is at 0.05 to 0.2 d^{-1} (Strous et al., 1999; Tsushima et al., 2007; Van der Star et al., 2008). In Chapter 3 a maximum specific growth rate of 0.21 d^{-1} (at 30°C) is reported. Due to the increased maintenance requirement associated with growth at these rates, the maximum biomass yield varies considerably as well: 0.12 C-mol biomass/mol NH_4^+ for AOB, but 0.066 C-mol biomass/mol NH_4^+ for anammox bacteria, resulting in the following overall reaction (Strous et al., 1998):

(1.1)

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

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 on ammonium (R_NiAm) and nitrate on ammonium (R_NaAm) of (circa) 1.1 to 1.3 and 0.1 to 0.25, respectively, are another characteristic for the anammox process. A higher ratio between nitrite and ammonium and/or the reduced nitrate production generally is an indication of the (co)occurrence of 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. Although the overall catabolic reaction in this case is the same as in denitrification, anammox organisms have never been shown to use the fatty acids directly as C-source for growth, but still use the energy-expensive CO_2 fixation. Therefore, the biomass yield (i.e. sludge production) is expected to be extremely low compared to “classical” denitrification. This low yield, in turn leads to lower sludge production. The decay rate (b_{AN}) 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 35ºC (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 have a very high affinity for their substrates nitrite and ammonium with half-saturation constants estimated at <100 µg N L^{-1} (Strous et al., 1999) and 3 to 50 µg N L^{-1} (Van der Star et al., 2008) (evaluated for nitrite only), respectively. These values are lower than those commonly found for AOB, NOB, or denitrification on nitrite and are thus a competitive advantage. Chapter 3 shows the nitrite half saturation constant has been estimated to be 35 µg N L^{-1}.

1.1.3 INHIBITION AND TOXICITY

In addition to feasibility studies and reactor design, knowledge of the adverse effects of compounds possibly present in anammox reactors is also of great importance to the development of the start-up strategy.

While nitrite and oxygen (among others like phosphate, sulfide, etc.) are compounds known to have toxic effects in anammox reactors, their mode of action, be it reversibility, the combination with other substances, the effect of exposure time or the relation of compound toxicity to the activity of cells, is still unknown, as well as the concentrations at which they occur. A brief introduction of those toxic compounds (i.e. nitrite and oxygen) studied in this work (Chapter 3 and 4) is reported below.

Nitrite

The most striking inhibitor to the anammox process is its own substrate: nitrite. Unlike the inhibition of AOB and NOB by the nitrous acid HNO_2 (the undissociated form that is most easily transported over the cell wall by passive transport), there are indications that not HNO_2, but the ion itself, NO_2^-, is toxic to the annamox bacteria (Strous, 2000; Chapter 4). The level at which toxicity occurs and its reversibility remains unclear and seems strongly dependent on exposure time. When only short-term effects on nitrite removal rate are evaluated, relatively high values are found (50% reduction only at 400 mg N L^{-1} for Strous et al. (1999), 630 mg N L^{-1} for Dapena-Mora et al. (2007), or 37% reduction at 430 mg N L^{-1} for Kimura et al. (2010), respectively). A 50% inhibition at a nitrite concentration of 350 mg N L^{-1} (IC_{50}) for anammox granules is reported in Chapter 4. However, at these nitrite levels an immediate deviation occurs in the ratio between nitrite and ammonium consumption (R_NiAm), possibly indicating ammonification. During longer periods of evaluation, toxicity is observed already at much lower concentrations. Prolonged experiments (40 h) showed this stoichiometric change started already above 70 mg N L^{-1} (Strous et al., 1999), probably indicating a negative response of the culture. In contrast to inhibition at these levels, operational conditions in a full-scale anammox reactor and in a full-scale nitritation-anammox reactor took place at typical concentrations of 40 to 80 mg N L^{-1} (Van der Star et al., 2007) and of 20 to 30 mg N L^{-1} of nitrite (Abma et al., 2010) indicating growth can take place at these values. Much lower inhibition concentrations were reported in an intermittently aerated one-reactor nitritation-anammox process, where irreversible toxicity occurred at 50 mg N L^{-1} and where values as low as 5 mg N L^{-1} (Wett et al., 2007) were reported to already have a detrimental effect on the process. The finding of these lower toxicity values in predominantly aerated systems suggests an effect of operation on loss of conversion capacity. Also, the extent to which nitrite inhibition is reversible is subject to much discussion. Anammox on gel-carriers recovered fully in 3 days from a 7-day exposure to 700 mg N L^{-1} (resulting in a temporary reduction of conversion of 90%). In view of the low growth rate of anammox bacteria, such an increase in conversion can be only due to recovery rather than growth. In Chapter 4 nitrite inhibition was shown to be completely reversible and an extensive discussion on the topic is given.

Oxygen

The (reversible) inhibition by oxygen is only noted in enrichments where insufficient nitrification or endogenous respiration occurs to successfully remove it, which is generally the case at very high enrichment levels. If this is the case, toxicity occurs already at the lowest measureable oxygen concentrations (0.5% of oxygen saturation Strous et al., 1997; Van der Star et al., 2008). In systems where anammox and aerobic bacteria (e.g. AOB) coexist in a biofilm, inhibition levels of oxygen are much higher. In Chapter 3 influence on anammox aggregation and activity is discussed.

1.1.4 MOLECULAR TECHNIQUES

Molecular techniques such as polymerase chain reaction (PCR) and fluorescence in situ hybridization (FISH) have been used in order to trace anammox bacteria in home-made and natural environments. Fluorescence in situ hybridization is a tool to both detect and quantify anammox bacteria. Anammox bacteria can be successfully detected with domain, phylum, genus and species specific probes. Anammox bacteria belong to the phylum Planctomycetes and they can therefore be identified with a Planctomycetes-specific probe like Pla46 (Neef et al., 1998). Several oligonucleotide probes have been developed in the past years for the specific detection of anammox microorganisms. Below we list just

those oligonucleotide probes used in this work: probe Amx368 was designed to detect all anammox microorganisms (Schmid et al., 2003); probe Amx820 was designed to detect *Kuenenia spp.* and *Brocadia spp.* (Schmid et al., 2001); probe Kst157 was designed to detect *Kuenenia spp.* (Schmid et al., 2001); Bfu613 was designed to detect *Brocadia Fulgida* (Kartal et al., 2007). Nowadays specific probes for all known anammox bacteria species are available and the increasing amount of available validated anammox sequences will lead to the design of improved probes for detection of the anammox bacteria. The detection of anammox bacteria by PCR depends strongly on the proper choice of PCR primers. The relative amount of retrieved anammox sequences from a given sample, can be increased by using Planctomycetes or anammox specific primers (such as Pla46F used in Chapter 3) (Schmid et al., 2005; Penton et al., 2006). Tsushima et al. (2007) developed and tested in quantitative PCR assays anammox specific primer sets. With further optimizations this technique could be applied in the future to detect and quantify anammox bacteria.

1.1.5 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 existing plants with the application of patented processes such as SHARON-ANAMMOX (SHARON: Single reactor system High Ammonium Removal Over Nitrite, ANAMMOX: ANaerobic AMMonium OXidation), CANON (Completely Autotrophic Nitrogen removal Over Nitrite) and OLAND (Oxygen-Limited Autotrophic Nitrification-Denitrification) (Li et al., 2008). An alternative is represented by gradual anammox enrichment of a mixed culture taken from sludge digesters or activated sludge reactors. There are several evidences of the wide diffusion of the Anammox microorganisms both in natural and in man-made environments, such as wastewater treatment plants (Kuenen, 2008). Enrichment techniques aim to enrich anammox bacteria from environmental sludge samples within a reasonable time frame (3 to 6 months, e.g. Pynaert et al., 2004, Noophan et al., 2009, Chamchoi et al., 2010), to be used subsequently as inoculum for a reactors start-up. 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 one hand it allows the cultivation of a specifically adapted biomass on the specific substrate to be treated, and on the other hand it doesn't rely on the purchase of biomass specimen abroad. In Chapter 2 the enrichment of anammox bacteria in samples of different origin is reported and discussed.

1.1.6 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 so 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 placed in series. An extensive review on the engineering aspects and practical applications of the anammox technology has been published recently (van Hulle et al., 2011) and therefore will not be discussed here in depth. To help the comprehension however, a brief introduction on the two different configurations is presented below. The "two-reactor" configuration will be discussed more thoroughly since it is the one applied in Chapter 3 and 6.

Nitritation-anammox in One Reactor

When the nitritation and the anammox process take place in the same reactor, oxygen acts both as a substrate (for AOB) and a toxin (for anammox bacteria). Since even very low oxygen levels cause (reversible) inhibition of anammox bacteria, it requires for the presence of 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 growth of anammox bacteria. 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). 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.

2. Time-dependent aeration, in which oxygen levels vary in time (Third et al., 2005; Wett, 2006). In such system, 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 full inundation and emerged presence above the water level (Kuai and Verstraete, 1998) or (II) transporting the biomass between aerated and non-aerated zones of a reactor (Beier et al., 2008).

Besides conditions that favor growth of AOBs and anammox bacteria, successful operation of single-stage nitritation-anammox reactors requires also 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 performing the one-reactor nitritation-anammox process in several of its operating strategies. These include granular sludge systems (sequencing batch reactors, air lifts, bubble columns) as well as systems with biomass on carrier (moving bed reactors or with carrier materials fixed in the reactor). The main common feature is the ability to achieve high SRTs and substantial mixing.

Nitritation-anammox in Two Reactors

In two-reactor configurations, the nitritation and the anammox process are taking place 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 treated 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 thinkable based on oxygen limitation or addition of specific inhibitors (Wyffels et al., 2003). An alternative and nitrite-level-independent way of producing nitrite uses the difference in maximum specific growth rate between AOB and NOB (Hellinga et al., 1998). At temperatures above 25°C, specific growth rate is higher for AOB than for NOB and therefore it's possible to operate the reactor at a biomass retention time, which enables growth of AOB while preventing growth of NOB (typically 1 day). In the calculation of this retention time in intermittently aerated reactors, only the time in which the reactor is actually aerated should be taken into account, as this is the only period that the AOB are growing. 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 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 stripping of CO_2.

(1.2)

$$NH_4HCO_3 + 1.5O_2 \rightarrow CO_2 + H_2O + NH_4NO_2$$

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

Anammox Reactor

Critical factors for the stable operation of anammox reactors are consistent and sufficiently long biomass retention and good mixing. The latter is mainly important at the location where the influent enters the reactor, as the concentrations of nitrite in the influent are

generally high enough to be toxic. Biomass retention is important in regard to the slow growth of anammox bacteria. It should be noted that the required sludge age in typical cases is not extreme, 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 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 and which consist of separate mixing and settler zones. In the latter zone, a stable upflow velocity (>1 m h^{-1}) strongly selects for stable granules. The advantage of this type of reactor is the very high volumetric loading rates possible due to existence of specific biomass areas of up to 3,000 m^2 m^{-3} and, 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. Reactors characterized by lower specific biofilm surface area in which the anammox process was implemented (such as low-weight biofilm carrier materials with a 1-cm diameter, Cema et al., 2006, or biofilm sheets, Fujii et al., 2002) showed lower volumetric conversion rates.

1.2 ANAMMOX APPLIED TO ANIMAL WASTEWATERS

Intensification of livestock production during the past decades led to the development of large indoor animal houses, especially pigs and poultry. An important consequence of this intensification is the concentration of animals in limited areas in order to reduce the production costs (Tregaro and Lossouarn, 2004). Livestock manure may be comprised of excreta, hair or feathers, spilled water and feed, process-generated wastewater (water used for flushing gutters, etc.) and bedding (sand, sawdust, wood shavings, peanut hulls, composted manure, and other substances).

Animals do not flush toilets, take baths, or wash dishes or clothes. Therefore, animal manure may not have the same characteristics and behavior as municipal wastewater. In general, animal manure is much more concentrated than municipal wastewater. It's obvious that in areas where farming is highly developed, serious risks for the environment exist. Carbon and nutrients natural cycles can be strongly alterated and as a consequence, natural ecosystems can be negatively affected. Focussing on the N-Cycle (topic of this Thesis), it has to be realized that farming activities play a primiry role and can therefore alter its equilibrium. Atmospheric nitrogen is fixed by natural (microbial nitrogen fixation) or industrial processes (fertilizer production) and fed to the livestock in the form of cereal or other N-containing industrial products. Eccessive fertilizer 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 effect, and wastewaters highly concentrated in nitrogen. A schematic overview of the role that livestock farming plays in the N-Cycle is given in Figure 1.2.

To reduce the environmental impact of livestock farming, the manure (and wastewater originating from manure processing) has to be handled and/or treated in proper ways.
Treatment of animal manure and wastewater is carried out by biological, chemical, and physical processes, to reduce potential degradation of natural resources prior to a designated end use of the treated product and byproducts. Animal manure treatment systems have historically been selected to recover or use valuable fertilizer constituents or feed ingredients and to protect soil, air, and water quality. Over time, however, the protection of soil, air, and water quality has evolved to include such considerations as the management of potentially toxic materials, such as copper, zinc, and antibiotics, concerns about proper nutrients management, and increased emphasis on air quality. Odor, ammonia volatilization (the release of nitrogen, in the form of ammonia, during storage or spreading), the release of hydrogen sulfide, methane, and other gases, and the potential of dust to transport odors and produce bio-solids (nutrient-rich organic matter) have become major public concerns.
In the following material a brief introduction to the biological treatment of livestock effluents will be given with emphasis on swine effluents. Among the variability of treatment schemes proposed in literature, the attention will be posed on the treatment train comprising anaerobic digestion and the consequent biological treatment of the produced digestate.

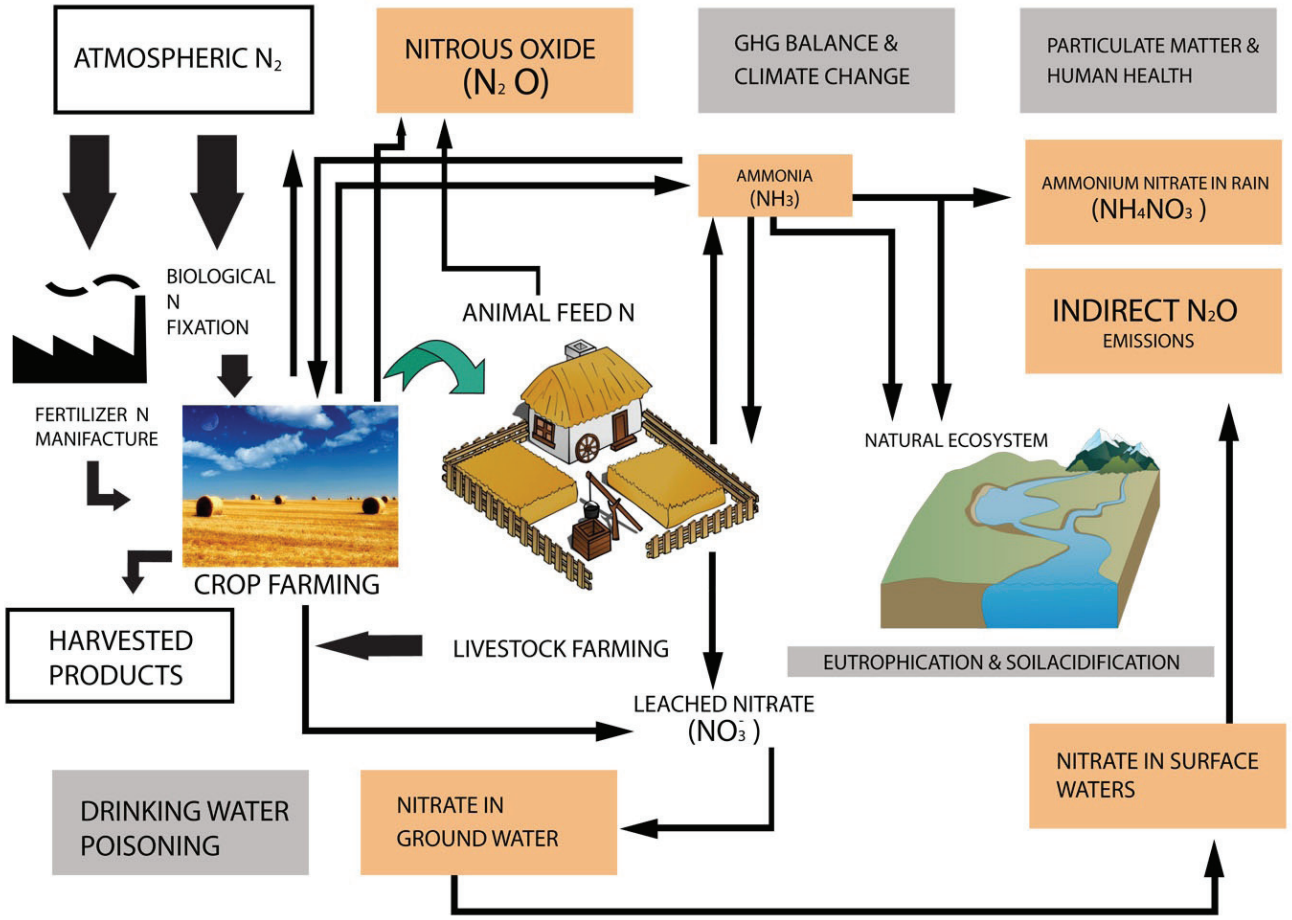


FIGURE 1.2
Nitrogen cycle in agriculture

1.2.1 Biological treatment of livestock effluents

Among the alternatives to improve manure management, the biological processes are often seen as (a part of) the solution due to their implications in the N, C and P cycles. Studies on biological treatment of livestock effluents initially dealt with organic matter degradation and odor reduction only (Owens et al., 1973; Evans et al., 1979, 1983; Smith and Evans, 1982; Blouin et al., 1988), but lately the nitrogen removal (Evans et al., 1986; Oleszkiewicz, 1986) and the energy recovery by anaerobic digestion (Chynoweth et al., 1999) were 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;
Bernet et al., 2000; Mace and Mata-Alvarez, 2002);
(**f**) Anaerobic digestion (Chynoweth et al., 1999; Burton and Turner, 2003).

Aerated lagoons and fixed-bed processes have been shown to be difficult to manage and failed to give satisfying results compared to other systems, because of the varying specific properties of different manures, like high nitrogen content, chemical oxygen demand (COD), suspended solid (SS) content and the variability through time of the concentrations (Oleszkiewicz, 1986; Westerman et al., 2000). Moreover, anaerobic lagoons are responsible for high ammonia and greenhouse gases (GHG) emissions (Aneja et al., 2001; Vanotti et al., 2008). An activated sludge with anoxic tank was reported to give good results in terms of removal efficiency 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 commonly adaptable (Germirli et al.,

1993). Consequently, most full scales processes are single tank technologies (Burton and Farrent, 1995, 1998; 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).

Anaerobic digestion is the anaerobic conversion of organic matter to a biogas containing methane (CH_4) and carbon dioxide (CO_2). Four successive biological processes are involved in the anaerobic degradation of organic matter including hydrolysis, acidogenesis, acetogenesis and methanogenesis. Complex polymers are converted into monomers by extra-cellular enzymes during hydrolysis while these monomers are transformed into volatile fatty acids (VFAs) and hydrogen (H_2) during acidogenesis. Acetate, CO_2 and H_2 are produced from VFA (acetogenesis) and finally converted into methane during methanogenesis. The biogas produced during anaerobic digestion, mainly composed of CH_4 (55–80%) and CO_2 (20–45%) can be used as an energy source, generally as heat or/ and after conversion to electricity by cogeneration. This process has been widely applied for years to the treatment of organic wastes, including manure (Chynoweth et al., 1999; Burton and Turner, 2003). Beside nitrogen, phosphorus is also responsible for eutrophication and therefore has to be considered in the manure management as well. Since the phosphorus present in wastewater mainly occurs in particulate organic form and/or adsorbed on the solid fraction, most of the phosphorus can be separated from the effluent liquid phase by solid/liquid separation (Greaves et al., 1999). Actually, up to 80% of the phosphorus can be extracted from the wastewater in the solid phase by means of mechanical separation using a centrifuge decanter (Burton, 2007). In this case, phosphorus is mixed with most of the organic matter of the manure and a further composting stage is required before being sold and exported as an organic fertilizer. Agriculture and manure management are involved in gas emissions, producing ammonia (NH_3) and two GHG, nitrous oxide (N_2O) and methane (CH_4). Livestock are responsible for 64% of anthropogenic NH_3 emissions, 37% of anthropogenic CH_4 and 65% of anthropogenic N_2O (Steinfeld et al., 2006). About 30% of the GHG emitted by livestock production are attributed to manure management. These GHG emissions can be efficiently reduced by substituting open storage of manure for anaerobic digestion in a closed system.

Taking into account these considerations, a treatment scheme comprising anaerobic digestion, efficient solid/liquid separation and nitrogen removal from the digestate appear attractive from both an environmental and an economic point of view. In this work (Chapter 5 and 6) the attention is posed on the nitrogen removal step of the aforementioned treatment scheme. A brief summary of the technologies applied in the treatment of the digestate originating from livestock waste anaerobic digestion is reported.

Biological treatment of digestate

Vanotti et al. (2007) proposed a full scale system combining liquid/solid separation, nitrification/denitrification and soluble phosphorus removal processes to replace an anaerobic swine lagoon, reporting satisfactory results. Preez et al. (2005) reported a pilot-scale study of a treatment scheme including anaerobic digestion, ammonia stripping, advanced membrane technology based on ultrafiltration and reverse osmosis. Beside the efficient removal obtained, the implementation of membrane technologies turns out to be expensive and difficult to operate due to characteristics of the wastewater matrix. A similar scheme is now proposed by some companies in Germany and Italy. Although there are a few full-scale installations, no data about economical costs and maintenance needs are yet available. A modification of the SHARON-ANAMMOX process scheme (van Dongen et al., 2001) was applied in lab-scale for resources recovery (phosphorus, organic carbon and biogas) and autotrophic nitrogen removal from slurry-type piggery waste (Hwang et al., 2006). The combined ADEPT-SHARON-ANAMMOX process scheme (ADEPT: Anaerobic Digestion Elutriated Phased Treatment) was applied in lab-scale for resources recovery (phosphorus, organic carbon and biogas) and autotrophic nitrogen removal from slurry-type piggery waste (Hwang et al., 2006). Although the process performance looks promising as a solution for pig manure treatment, development and optimization of the full-scale technological process is still needed to prove its sustainability. Karakashev et al. (2008) proposed an innovative scheme for treatment of high strength organic waste such as pig manure. The scheme included full-scale anaerobic digestion, decantation, UASB (up-flow anaerobic sludge blanket) post-digestion, partial oxidation and OLAND process. It resulted in removal of total organic matter, nitrogen and phosphorus contents of pig manure with 96%, 88% and 81% respectively. However, more investigations are needed to clarify the economical and environmental sustainability of such process scheme at pilot-scale and full-scale.

Using the anammox process for nitrogen removal from sludge liquors has shown to be a promising novel technology (van Dongen et al, 2001). Already applied in full-scale in municipal wastewater treatment plants (WWTP), this process is likely to be attractive also for digestates of different origin, both from an economic and environmental point of view. In the field of livestock wastewaters, the autotrophic nitrogen removal implementation appears to have potential to benefit as well, as here the development of anaerobic digestion is hindered by difficulties connected with the nitrogen removal from the produced digestate. Considering the massive and spatially concentrated presence of pig breeding in Europe (especially in Italy) and the potentially toxic compounds contained in such manure (i.e. antibiotics and heavy metals) this study will focus on swine wastewater, which is one of the most problematic livestock wastewaters to treat. In this thesis a feasibility study on the implementation of the anammox process was performed at lab-scale for the treatment of the liquid fraction of swine wastewater anaerobic digestion effluent (Chapter 5 and 6).

Outline of the thesis

The study described in this thesis is part of the research program "*Biotecnologie per la Riduzione dell'Azoto dai digestati con processi INnovativi per promuovere la sostenibilità economica ed ambientale della produzione di biogas"* (BRAIN, innovative biotechnological processes for nitrogen reduction from digestate to promote the economic and environmental sustainability of biogas production). The project was financed with Decree of the Italian Ministry of Agriculture DM no.16917/7303/10 issued on 23/07/2010 and is in collaboration with the Department of Civil and Environmental Engineering of the University of Florence (DICeA, Università degli Studi di Firenze) and the Dept. of Environmental, Hydraulic, Infrastructures and Surveying Engineering of the Milan University of Technology (DIIAR, Politecnico di Milano). This thesis describes the research executed partly in Florence and partly at the Department of Biotechnology of Delft University of Technology under the guide of Prof. Mark C.M. van Loosdrecht (co-tutor of this research). The aim of the research was on one hand to investigate the scientific fundamentals of the anammox process and derive physiological parameters useful for engineering applications, on the other hand to perform a more applicative study on the feasibility of the implementation of the anammox process for the treatment of the liquid fraction of swine wastewater anaerobic digestion effluent. The thesis has been divided in several chapters, each one reporting one of the topics addressed in this work. Apart from Chapter 1 (general introduction), all chapters present a small introduction where some background on the topic of the chapter is given to help the reader's understanding. The thesis is concluded with a summary of the main results obtained during the research work, followed by the list of references used.

Chapter 2 describes the enrichment of anammox bacteria in fed-batch systems starting from sludges of different origins. The research was conducted in parallel between the DICEA and the DIIAR.

Chapter 3 describes the obtainment of a highly enriched anammox suspended culture in a membrane bioreactor (MBR) and the operational strategies that enable anammox growth as free cells. The enriched biomass is used to assess physiological parameters relevant for the engineering of the process as well as for the ecophysiology of anammox bacteria.

In *Chapter 4* an in depth study of the effect of nitrite on anammox activity is performed. The mode of action, reversibility, the combination with other substances, the effect of exposure time and the relation of compound toxicity to the activity of cells are assessed.

Chapter 5 describes the effect on anammox activity and metabolism of heavy metals, such copper and zinc, as well as of antibiotics, such as sulphathiazole and oxytetracycline, characteristic for swine wastewaters.

In *Chapter 6* the feasibility of the anammox process applied to the treatment of digestates of swine origin is assessed at lab-scale. For this evaluation a biofilm gas-lift suspension reactor (BGS) was designed and fed with a synthetic medium containing rising proportions of digestate.

CHAPTER 2

Anammox enrichment in conventional sludge samples via a simple fed-batch procedure with activity measurements

Part of this Chapter has been presented in IWA World Water Congress 2010, Montreal (CAN) 19-24 settembre 2010 as Scaglione D., **Lotti T.**, Ficara E., Caffaz S., Canziani R., Lubello C., Malpei F.

Anammox enrichment in conventional sludge samples via a simple semi-batch procedure with activity measurements

Outline

In this Chapter, results from an anammox enrichment campaign are presented. The enrichment set-up was kept as simple as possible and consisted in 1-L glass bottles, featured with two lateral openings sealed with rubber septa for substrate injection, gas discharge or sampling, magnetically mixed and kept in a thermostated chamber (35-37°C). A literature mineral medium was manually fed in a fed-batch mode averagely every 2 to 7 days. Nitrite, nitrate, ammonium and pH were measured at the end of each reaction cycle. As inocula, 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 anaerobic ammonium oxidation was considered to take place as soon as ammonium and nitrite decreased simultaneously during at least two subsequent cycles. 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. The slowest sample to show-up an anammox activity was the one collected from the WWTP fed on digested swine manure. As soon as the anammox activity was detected, its rate was periodically monitored by measuring the rate of N_2 production by means of a manometric device. Its initial value was included within 0.2 and 0.83 mg N L^{-1} h^{-1} for all samples. In conclusion, the proposed simple set-up proved to be adequate for anammox enrichment and all tested inocula developed the ability to oxidize ammonium anaerobically.

2.1 INTRODUCTION

In Italy, the enforcement of the EU Nitrate directive (91/676/CEE) is stimulating the search for cost-effective N removal technologies, such as the fully autotrophic nitrogen removal. This very attractive biological treatment consists in the partial aerobic ammonium oxidation to nitrite followed by anaerobic ammonium oxidation and nitrite reduction via the anammox process (Van Dongen et al., 2001).

The core and limiting step of this solution is the anaerobic ammonium oxidation that is carried out by different microorganisms related to Planctomycetes, according to the following stoichiometric equation (Strous et al.,1998):

(2.1)

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

As these microorganisms grow very slowly (Strous et al., 1999), the availability of a suitable biomass inoculum is very important. 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 reactors start-up.

Different types oh 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). The aim of this work was to verify the efficacy of a simple, low-tech fed-batch procedure of enrichment coupled with activity measures. Results of an experimental campaign on 6 sludge samples collected from Italian wastewater treatment plants are here presented.

2.2 MATERIALS AND METHODS

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 Casteggio (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 Casaletto di Sopra (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).

From each sludge sample, approximately 4 gTSS were drawn, settled and re-suspended in a mineral medium containing micro and macro-nutrients (from van de Graaf et al. 1996) to a final volume of 1L. Each 1L-sample was poured into 1140 mL glass bottle featured with two lateral openings sealed with rubber septa for substrate injection, gas discharge or sampling. 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.

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, 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 (Dr.Lange test kits) 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 mg N 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 mg N L^{-1}. Nitrite concentration was adjusted at the beginning of each batch cycle to remain between 5 and 20 mg N L^{-1}. Higher loads were tested once a stable anammox activity was observed.

Anammox activity was periodically monitored by measuring the rate of N2 production. Gas production was assessed directly in the glass bottles used for biomass enrichment by featuring the main bottle opening with a manometric data-logger, as sketched in Figure 2.1. A detailed description of this procedure can be found in Scaglione et al., (2009) and Bettazzi et al. (2009).

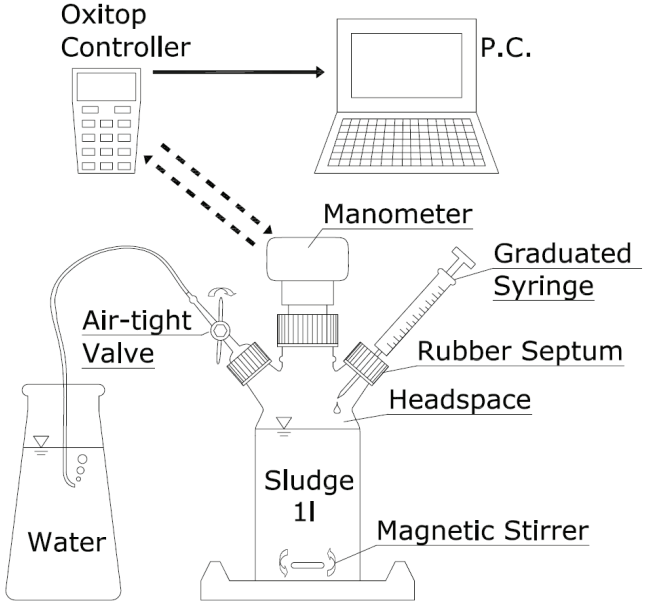


FIG. 2.1
Experimental equipment for manometric determination of the N2 production rate.

2.3 RESULTS AND DISCUSSION

In Figure 2.2, ammonium and nitrite trends for the six sludge samples are presented. 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 released by sludge hydrolysis. At the same time, denitrification of the solubilized organic matter took place, leading to nitrate consumption and to pH increase (data not shown). 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 variation 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 ratio between nitrite and ammonium consumption stabilized during time around the expected stoichiometric ratio of 1.32 ($N\text{-}NO_2^-/N\text{-}NH_4^+$ normally ranged between 0.8÷2.3) suggesting that a prevailing anaerobic ammonium oxidation was finally reached. FISH analyses confirmed anammox microorganisms presence (data not shown).

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 1 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 around 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 wastes. 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. This hypothesis needs to be confirmed by further experimentation on similar inocula.

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.

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 2.2, a stable ammonium reduction in sample S2b (with hydrazine) was observed later then in sample S2 (without hydrazine), although this may also be attributed to 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.

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 (Fig. 2.3). An increase in the gas production rate during cultivation time was observed, indicating an increase in anammox activity. Moreover, peculiar behaviors, such as remarkably long

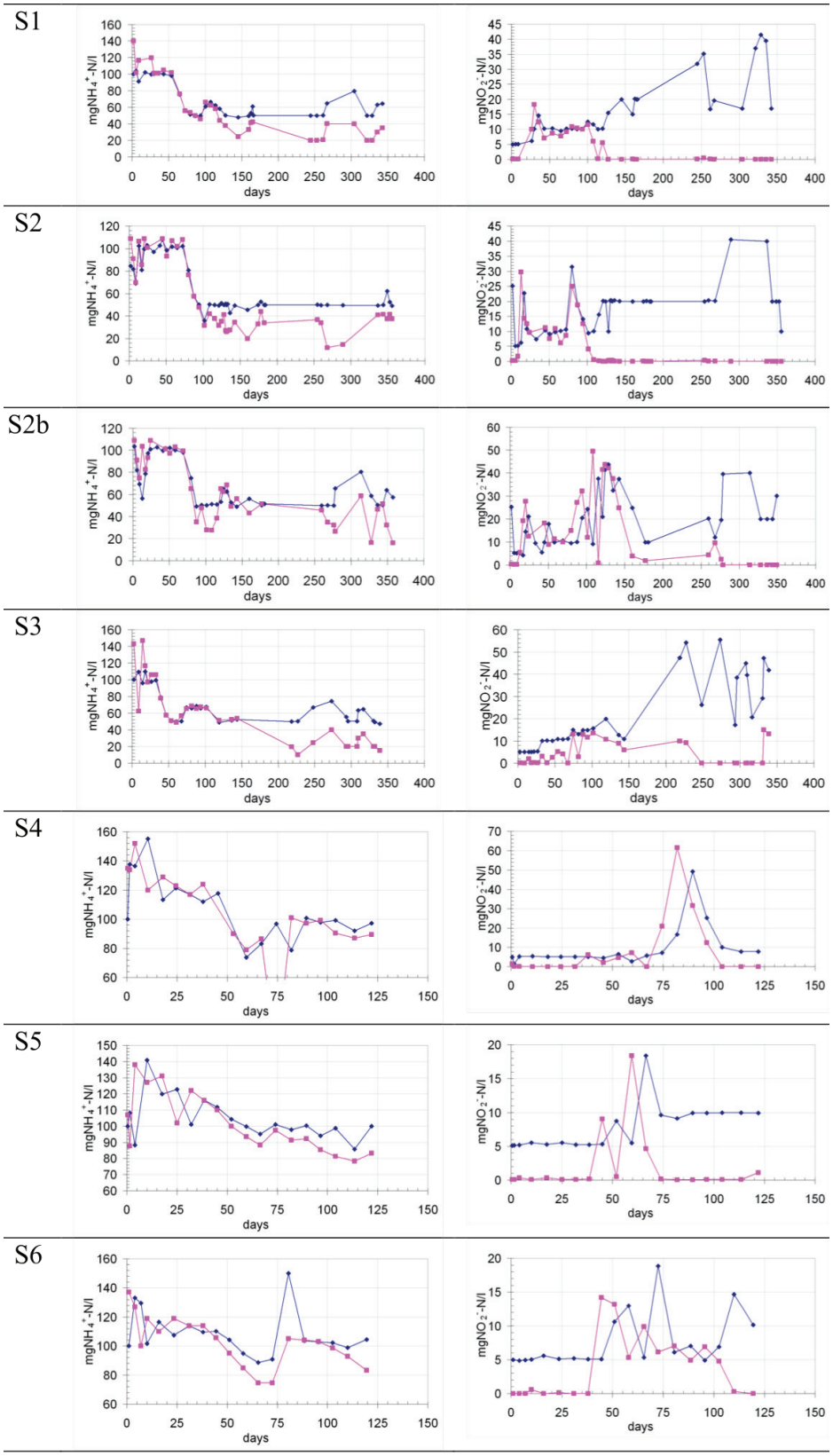


FIG. 2.2
Ammonium and nitrite trends during the anammox enrichment for the tested samples. Data series refers to the initial (diamonds) and final (squares) concentrations across each fed-batch cycle.

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.

In Table 2, the values of the anammox activity, calculated form the N2 production rates in batch tests, 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 gas production in time. It is worth noting that all activities were of the same order of magnitude

Table 2.1: 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 sludge	120	SBR	Chamchoi & Nitisoravut, 2006
Methanogenic anaerobic sludge	54	SBR	Dexian et al., 2007
Mix of different sludge	60	SBR	Lopez et al., 2008
Anaerobic granular sludge	83	UASB	Tang et al., 2010
S1: Suspended anaerobic sludge from the sludge digester	*108*		
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*	*Semi batch*	*This experimentation*
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*		

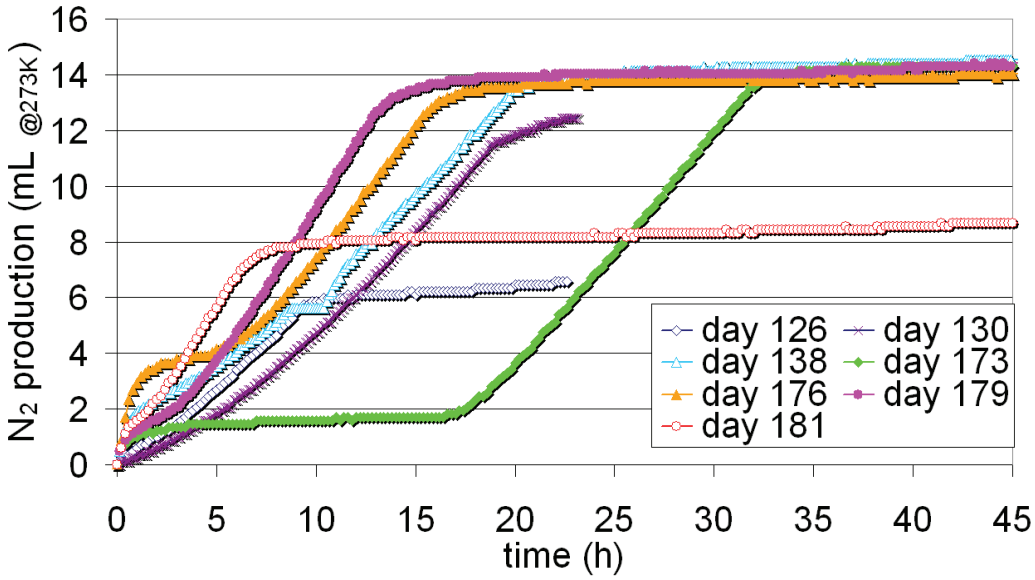


FIG. 2.3
Cumulated gas production curves for S2 sludge at various enrichment time (day 126→181) in response to the addition of 10 to 20 mg N-NO_2^-. The lag phase observed at day 173 is likely due to the 18 days endogenous time for summer break.

in all samples and around 10-70 mg N-NH_4^+ 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.

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 mm of diameter (Van Niftrik et al. 2008),

(**iii**) the protein cell density in the Anammox cell is 0.6 gprot $gbiomass^{-1}$ (derived from Strous et al., 1998),

(**iv**) the settled sludge volume was around 300mL for all the 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 the sludge samples. By further considering that the spatial distribution of anammox microorganisms within the sludge aggregates was not homogeneous (FISH images evidence, data not shown), the calculated cell density is in accordance with that one suggested by Strous et al. (1999).

Table 2.2: Anammox activity as calculated from N2 production rates in batch manometric tests.

Sample	Enrichment time (d)	Anammox activity (mg $N\text{-}NH_4^+$ L^{-1} h^{-1})
S1	132	0.13
S2	126	0.42
S4	108	0.10
S5	108	0.25
S6	125	0.10

2.4 CONCLUSIONS

The following main conclusions can be drawn from this experimental campaign on the enrichment of anammox biomass from Italian sludge samples:

— a very simple, low-tech fed-batch procedure was successful in growing anammox biomass;

— hydrazine addition did not favor anammox development;

— anammox biomass is likely to be present in most Italian wastewater treatment plants;

— the anammox activity achieved after about 110-130 days was within 0.13 and 0.44 mg $N\text{-}NH_4^+$ L^{-1} h^{-1} and appears to be sufficient to inoculate a continuously operated anammox reactor;

— simple 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 over-loadings.

CHAPTER 3

High purity anammox suspension in MBR: physiological and kinetic characterization

Part of this Chapter is yet to be submitted for publication as

Lotti T., Kleerebezem R., Lubello C., van Loosdrecht M.C.M. **High purity anammox suspension in MBR: physiological and kinetic characterization**

3.1 INTRODUCTION

The anammox process is the biological conversion of ammonium and nitrite to dinitrogen gas (Van de Graaf et al., 1996) and is performed by slow-growing deep-branching Planctomycetes (Strous et al., 1999a). Anammox bacteria are autotrophic and have a notoriously low growth rate with minimum doubling times of several days (Strous et al., 1998; van der Star et al., 2008). Despite considerable interest in their cultivation, only enrichments (which typically contain 60-80% anammox bacteria) are available consisting of agglomerates or biofilms. This might even lead to the perception that anammox bacteria are preferentially growing in biofilms or granules. However, the high abundance of free anammox bacteria at the oxic-anoxic interface in several marine systems (Schmid et al., 2007) indicates that growth as free cells is (also) a natural mode of growth. The cultivation of slow-growing microorganisms requires efficient retention of biomass and relies mostly on the ability of microorganisms to form biofilms or aggregates such as flocs or granules. Startup of these reactors can be impeded by insufficient biomass build-up: a continuous loss of ‘‘small’’ amounts of biomass via the effluent might lead to significantly longer observed biomass doubling times in the case of slow-growing microorganisms (Strous et al., 1998). Granular sludge-based reactor design (Nicolella et al., 2000) leads to compact reactors, which combine a short hydraulic retention time (HRT) with a long and stable solid retention time (SRT). Processes employing slow-growing organisms like nitrification (Tanaka and Dunn, 1982), anaerobic digestion (Lettinga et al., 1980), ferrous ion oxidation (Ebrahimi et al., 2005), and phosphate removal (De Kreuk and Van Loosdrecht, 2004) all can be implemented successfully, on lab-scale as well as on full-scale, in retention-based reactors with a high volumetric loading rate. Typical examples of such reactors are airlifts (Heijnen et al., 1990), sequencing batch reactors (SBR, Irvine et al., 1977; Wilderer and McSwain, 2004), internal circulation reactors (Pereboom and Vereijken, 1994), and upflow anaerobic sludge bed reactors (UASB, Lettinga et al., 1980; McHugh et al., 2004). Although particle-based bioreactors are advantageous for the cultivation of slow-growing microorganisms, and thus valuable from a technological point of view, the produced granules are not the most suitable forms for the study of these microorganisms (van der Star et al., 2008). Bio-kinetic parameters such as substrate affinities, maximum growth rate or maintenance need cannot be well assessed due to diffusion limitations within the floc or granule itself (Chu et al., 2003; Harremoës, 1977). Finally, agglomeration potentially also leads to underestimation of the observed maximum specific growth rate (μ) (Characklis, 1990) because of the energy required for bacteria to agglomerate (e.g., additional production of extracellular polymers, EPS). In the membrane bioreactor (MBR), biomass retention is not based on the settling properties of biomass. The effluent is withdrawn via a membrane which is impermeable for microbial cells. Unlike the reactors with granular biomass, the MBR enables cultivation of slow-growing microorganisms with full biomass retention but without a selection on settling ability allowing the uncoupling of HRT and SRT. This reactor type is currently employed for the growth of sensitive cells like plant/animal cells as well as for cell tissue production (Drioli and De Bartolo, 2006). The MBR is also employed in full-scale wastewater treatment (Sutton, 2006; Yang et al., 2006), where the membrane separation reduces the surface area that is normally required for settling of flocculated sludge.

All over the world, research groups are working on diverse aspects of the molecular biology, biochemistry, ultrastructure, physiology and metabolism, and ecology of anammox bacteria, as well as assessing the impact of their activities on the environment and their applications in waste-water and waste-gas treatment. The availability of a highly enriched suspended culture of anammox bacteria represents a pre-condition necessary to accurately investigate all these fascinating research topics (Kuenen et al., 2008). Recently the MBR has been pointed as a novel tool for the enrichment of suspended culture of anammox bacteria (van der Star et al., 2008). In that study the reasons why a suspension culture was achieved were reported to be (i) the low levels of bivalent ions (i.e., calcium and/or magnesium) and (ii) the addition of yeast extract in a system characterized by (iii) the absence of selective pressure for settling, (iv) a high growth rate and (v) low shear stress. That study could not differentiate between the effect of addition of yeast extract on one hand and lowering of calcium and magnesium levels on the other since they coincided in time. The first aim of the present work was to obtain a highly enriched suspension culture verifying the suitability of the tool proposed by van der Star (2008) and identifying the key-factor(s) responsible for the growth as free-cell. The obtained suspension culture would then have been used for an (still lacking) accurate kinetic characterization of the anammox process and for the in detail study of parameters of interest (i.e., affinity for substrates, interactions with oxygen).

Physiological and Kinetic Carachterization

Proper bioprocess design requires detailed kinetic information on the microorganism utilized and the stoichiometry of the process they catalyze. For slow growing autotrophic organisms like the ammonium oxidizing bacteria described here, kinetic characterization is a laborious and time consuming task. Full kinetic characterization in a chemostat bioreactor setup of a microorganism that has a maximum specific growth rate similar to anammox (≈0.003 h^{-1}) will take likely several months. Kinetic characterization in batch reactors is much faster, but the interpretation of the generated data is often complicated by the rapidly changing process conditions (e.g., substrate and product concentrations) the organisms are exposed to and by undefined operational parameters (e.g., SRT). We have developed a method for kinetic characterization that is based on a continuous process operated at a variable nitrogen-loading rate that combines the advantage of a short experiment as in a batch, with relatively slow changes in environmental conditions as in a stirred tank type reactor. Herewith full kinetic characterization of a slow growing organism can be established in a few days, provided that the experiment is started in a stirred tank reactor in steady state. In the study described in this chapter we have used the kinetic characterization method based on a variable nitrogen-loading rate for investigation of ammonium anaerobic oxidation by an anammox bacteria closely related (≈97% sequence similarity) to Candidatus *Brocadia Fulgida*. The anammox culture was previously enriched in a MBR and at the moment the experiment was conducted a free-cell suspension at steady state conditions was obtained. Based on on-line and off-line measurements, the catabolic and anabolic fluxes could be identified independently, allowing for a complete stoichiometric and kinetic characterization. The stoichiometry currently used for bioprocess design and modeling purpose was reported by Strous (1998). In that study stable conditions (comparable to a steady state in a chemostat) were achieved in a SBR, enabling mass-balancing under defined conditions. However the degree of enrichment was only the 74% and the carbon balance relied on a set of data (collected over a period of about 200 days) of dry weight measurements affected by high standard deviation (up to ±50% of the averaged data). The system biomass retention (entering in the carbon balance) was also experimentally evaluated by the same data set to be equal to approximately 90%. Concerning physiological and kinetic parameters of interest such as the affinity for nitrite and the inhibitory concentration of oxygen, only indications and/or intervals of values are reported in literature (van der Star et al., 2008 and Strous et al., 1997, respectively). Even if these informations are satisfactory for design purposes, they may lead to systematic inaccuracy in modeling studies. For the correct evaluation of such physiological and kinetic parameters the availability of a highly enriched (negligible amount of non-anammox bacteia present) suspended culture (none mass transfer limitation) of anammox bacteria represents a pre-condition. Same considerations account for the correct evaluation of the anammox macro-chemical equation (stoichiometry of the bio-catalyzed reaction).

The membrane bioreactor used in the present study enabled for the achievement of a highly enriched anammox suspended culture characterized by high growth rate at steady state and for an accurate control of the operational parameters during the experimentation (i.e., SRT), resulting in a more accurate and reliable physiological and kinetic characterization of the anaerobic ammonium oxidation process than previously reported. Furthermore in this Chapter the key-factor (i.e., oxygen load) for successful enrichment of the anammox bacteria in a lab-scale MBR to high purity single-cell suspension is identified. The anammox bacteria enriched in this study presented the highest specific growth rate (and the highest specific ammonium removal rate) ever reported.

3.2 MATERIALS AND METHODS

3.2.1 Inoculum

The reactor 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 biomass was withdrawn to inoculate the MBR, the anammox reactor was operated at the design volumetric load of 7.1 kg N m^{-3} d^{-1} (van der Star et al., 2007). During 2010 the average reactor conditions were: temperature 34±2.5 ºC, pH 7.2±0.4 and concentrations of nitrogen in effluent were 50±20 mg NH_4^+-N/L, 15±15 mg NO_2^--N/L, 95±20 mg NO_3^--N/L. The biomass, the day of the MBR inoculation, was confirmed to

consist of a "*Brocadia*" enrichment by *fluorescence in situ hybridization* (FISH), the sludge hybridized with AMX820 and not with KST157 oligonucleotide probes (Schmid et al., 2001). The reactor was inoculated with 1.8 L of granular biomass (Fig. 3.1).

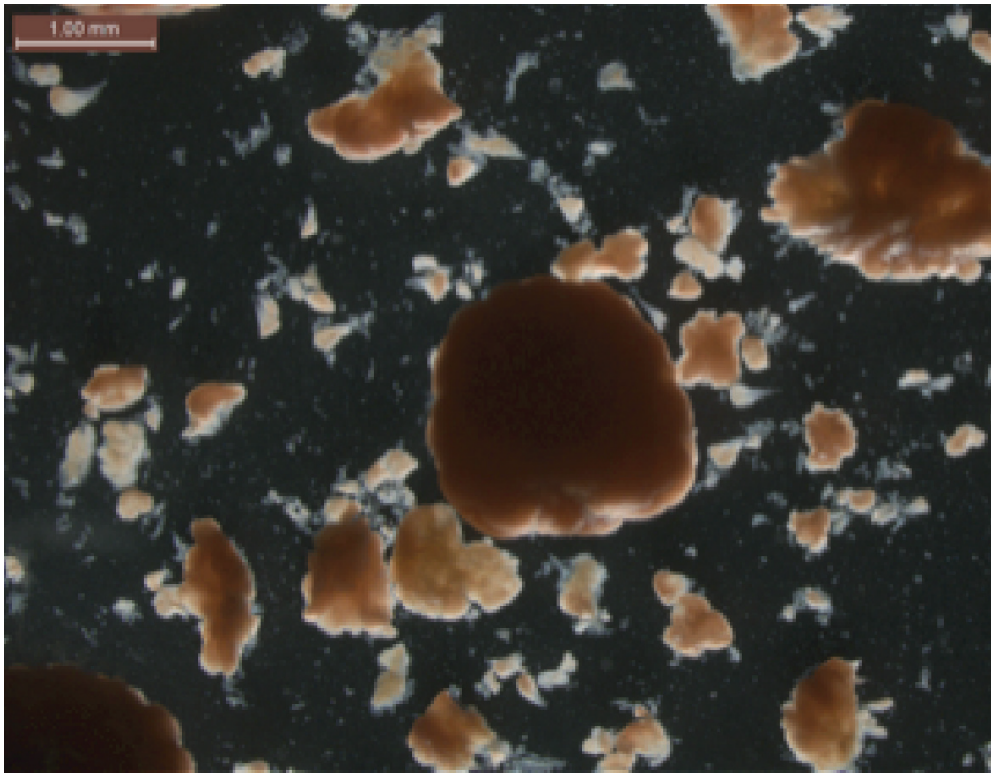


FIG. 3.1
Anammox granules from the inoculum (microscope Leica DFC420); scale bar is 1 mm.

3.2.2
Reactor operation

A 15 L reactor was used for the cultivation (van der Star et al., 2008; see Fig. 3.2). The liquid volume was 10 L (V_L) and the reactor was fed continuously with 6 L day^{-1} medium with different compositions, resultin g in a HRT of 1.67 days. The liquid level was maintained via a liquid level-controlled (peristaltic) effluent pump connected to a membrane microfiltration module type Zeeweed (GE Power & Water, USA) completely submerged in the liquid in the reactor vessel. The membrane fiber (absolute pore size: 0.1 µm) was designed for operation in MBRs for wastewater treatment and is impermeable for microbial cells. The lab-scale module that was used (ZW1) consists of about 100 tubes (diameter ca. 1 mm, length ca. 300 mm). The module was replaced every 2-3 weeks to prevent biofilm growth on the membrane surface, and was subsequently cleaned (outside the reactor) with diluted (2-3 times) commercial bleach (the cheapest found in supermarket). After one day immersed in stirred diluted bleach, the membrane was cleaned by means of prolonged and intensive rinsing with water. Replacement of the membrane took only 1–2 min and mixing was stopped during the replacement to avoid the entrance of large amounts of air into the reactor. The headspace volume was 5 L (V_H). To maintain anoxic conditions and to provide buffering capacity and carbon source, the reactor was sparged continuously at 50 mL/min with an industrially prepared mixture of Argon and CO_2 (95 and 5%, respectively). The gas entering the reactor was supplied from pressurized bottles with Brooks mass flow controller (MFC). The pressure inside the reactor was maintained constant at 20 hPa by connecting the outflow gas-tube to the bottom of a water-filled vessel to act as a water-lock. Since day 333 on the headspace-pressure in the reactor was set at 40 hPa by rising the level of water into the water-filled vessel (40.7 cm of water-column) to ensure no oxygen leakage into the reactor during sampling or SRT control. Since day 333 on a dithionite solution (200 mM) in which the gas (Argon/CO_2, 95/5%) was bubbling (through a "fine-bubbles" porous sparger) before entering the reactor was also implemented in the set-up.

The reactor was fed with a concentrated medium according to van der Star et al. (2007) containing 60 mM ammonium and 60 mM nitrite (Table 3.1). The bicarbonate concentration was 15 mM till day 450. Then, since day 451, bicarbonate was not added to the medium and therefore the carbon dioxide present in the gas (5% at 50 mL min^{-1}) was the only carbon-

FIG. 3.2
Photograph of the set-up of the membrane bioreactor (MBR) for enrichment of anammox bacteria as free cells.

source fed to the system. Between day 125 and 231 a vitamin solution was added to the medium (10 mL/L). The vitamin solution contained (mg/L): Folic acid (2.0), Riboflavin (5.0), Biotin (2.0), Thiamine (5.0), Nicotinic acid (5.0), Calcium Pantothenate (5.0), Vitamin B12 (0.1), p-Arninobenzoic acid (5.0), Thioctic acid (5.0), Monopotassium phosphate (900.0). Every time a new vessel of feeding-medium (20-50 L) was prepared, it was intensively sparged with nitrogen gas for 2-4 hours before connecting it to the reactor. During normal operation pH was not controlled, but was always between 7.1 and 7.5. Since day 451 the addition of bicarbonate was discontinued and a phosphate buffer (tot-P 17 mM) was added to the medium in order to set the pH at 7.0. The temperature was controlled at 30°C, and the stirring speed was 200 rpm. To avoid growth of phototrophic organisms (and the related oxygen production, which would enable growth of other non-anammox microorganisms like nitrifying bacteria), the reactor was covered completely by a PVC cover (1 mm thickness) to prevent penetration of light.

Table 3.1: Different medium compositions for the enrichment of anammox bacteria.

Nutrient	Dimension	Van der Star et al., 2008	Days 0-88	Days 89-124	Days 125-231	Days 232-612
Ammonium	mM	120	60	60	60	60
Nitrite	mM	120	60	60	60	60
Calcium	mM	1.0	1.0	0.5	0.5	0.5
Magnesium	mM	0.4	0.4	0.4	0.4	0.4
Vitamin solution	mL L^{-1}	0	0	0	10	0
Yeast extract	mg L^{-1}	1	0	0	0	0

The start-up period, from the day of the inoculation (the designated experimental day 1) till the design volumetric nitrogen loading rate (1 g NO_2^--N L^{-1} d^{-1}) was achieved, lasted one month. During the start-up period the nitrite-loading rate was increased step-wise increasing the medium flow entering the reactor (medium composition remained constant)

each time the nitrite concentration was verified to be stably below 10 mg N/L for few days. The solid retention time (SRT) was controlled during the all experimental period by means of a remote-controlled (peristaltic) pump removing every day 0.83 ($Q_{w,12}$, SRT 12 days) or 0.67 L ($Q_{w,15}$, SRT 15 days) from the reactor using an excess-sludge pump which was not connected to the membrane, but which pumped out the reactor-suspension directly into a sludge withdrawal vessel (pump was operated 5 minutes every two hours). During start-up period the SRT was controlled at 15 days. Then, after 63 days of operation the SRT was controlled at 12 days and kept constant throughout all the experimentation. The reactor was operated for more than 610 days.

3.2.3
Size distribution of MBR aggregates

The size distribution of the aggregates from the MBR was determined with the aid of image analysis (Tijhuis et al., 1994).

3.2.4
Measurements

3.2.4.1 Biological Oxygen Monitor (BOM)
Biological oxygen monitor (BOM) measurements were conducted to determine the maximum volumetric oxygen uptake rate (OUR^{max}) and the maximum biomass specific oxygen uptake rate (q_{O2}^{max}). BOM-measurements are small-scale (≈43 mL) batch experiments conducted in closed reactors equipped with a DO electrode. The reactor was incubated with an oxygen- saturated sample from the reactor and after substrate dosage the oxygen uptake rate was measured. The OUR^{max} value was subsequently determined by estimating the slope of the oxygen depletion curve. By relating the OUR^{max} to the actual biomass concentration, q_{O2}^{max} can be calculated.

3.2.4.2
Manometric test

Manometric measurements (methodology according to Chapter 4, par 4.2.3) were conducted to determine the maximum biomass specific N_2 production rate (q^{max}_{N2}). Considering the overall accepted assumption that during anammox catabolism one mole of ammonium is converted to one mole of di-nitrogen gas, q^{max}_{N2} is equivalent to the maximum biomass specific NH_4^+ consumption rate (q^{max}_{NH4}). Manometric measurements were also used to identify the oxygen concentration at which anammox activity is completely inhibited. Increasing volumes of oxygen saturated (at 20°C) demineralized water were injected in the OxiTop vessel while anammox reaction was taking place (actual production of dinitrogen gas). The oxygen concentration inhibiting the reaction was identified as the crucial factor for stopping the production of dinitrogen gas.

3.2.4.3
Maximum nitrite removal rate (MNRR)

For the evaluation of the maximum volumetric nitrite removal rate ($Rate_{NO2}^{max}$, g NO_2^--N L^{-1} d^{-1}) batch tests were conducted into the reactor. A solution containing ammonium 13 M (as $(NH_4)_2SO_4$) and nitrite 10 M (as $NaNO_2$) was injected into the reactor and the concentrations were monitored in time (starting ammonium and nitrite concentrations 2 mM and 2.6 mM, respectively). Maximum biomass specific nitrite removal rate (q^{max}_{NO2}) was calculated by relating $Rate_{NO2}^{max}$ to the biomass concentration in the reactor.

3.2.4.4
Gas measurements

CO_2, N_2O, NO, N_2, O_2 in the gas-phase CO_2, N_2O, NO and O_2 concentrations in the dewatered gas leaving the reactor were measured on-line using a Rosemont Analytical multicomponent gas analyzer. Off-gas dewatering was achieved in a reflux condenser operated at 4°C with a Permapure filter. Measured partial pressures were converted to molar fluxes by correcting for the air pressure and the gas flow rate applied. N_2 was measured off-line on an Agilent 6890 gas chromatograph. Mass transfer limitations could be neglected due to the high stirrer speed (200 rpm) and the maximum aggregates diameter (≈30 µm).

3.2.4.5 Mass flow

The gas flow rate of the dewatered outflowing gas was measured with a Bronkhorst MFC Calibrator, Bronkhorst Hi-Tec, Veenendaal, Netherlands.

3.2.4.6 Biomass concentration

TSS and VSS were determined according to the Standard Methods (APHA 2005). Total organic carbon (TOC) was measured in a SHIMADZU 5050A as the difference between total carbon (TC) and inorganic carbon (IC). TC present in the sample was combusted or decomposed to CO_2 in a combustion tube filled with oxidation catalyst, heated to 680°C and supplied by high purity oxygen. Resulting CO_2 was then detected in a non-dispersive infrared gas analyser (NDIR). For IC measurement the sample was acidified and resulting CO_2 was detected in a NDIR gas analyser. During the experimentation on kinetic characterization biomass concentrations were estimated from CO_2 uptake in the bioreactor and checked by TOC measurement.

3.2.3.7 Elemental composition of biomass

The carbon, hydrogen, nitrogen and sulfur content of the enriched sludge was measured using a PerkinElmer CHNS ANALYZER 2400 (Waltham, Massachusetts, USA) after washing the sludge with a physiological salt solution and drying under vacuum at -50°C. Phosphorus content of the biomass was measured after digesting (according to standard methods, APHA 2005) one gram of freeze-dried sample using commercial test kits according to the protocol of the manufacturer (brand: Dr.Lange test kits, Hach-Lange GmbH, Düsseldorf, DE, kits LCK348) and determined on a designated spectrophotometer (DR 2800). Oxygen content was calculated as the remainder after subtracting the ash and CHNSP content of the samples.

3.2.4.8 Soluble nitrogen compounds

Ammonium, nitrite and nitrate were measured via spectrophotometric flow injection analysis (QuickChem 8500 series 2 FIA System, Lachat Instruments, Loveland, Colorado, USA). The methods applied were QuikChem®Methods 10-107-06-5-E for ammonium (range 0.1 to 10.0 mg N/L, measurement of NH_3 after increasing pH and volatilization) and 10-107-04-1-C for nitrate/nitrite (range 0.01 to 2.0 mg N/L, direct measurement of nitrite, or measurement proceeded by reduction of NO_3^- to NO_2^- to yield the concentration of "NO_3^-+NO_2^-") according to the protocol of the manufacturer. The length of the sample loop of the nitrate/nitrite detection was increased in order to obtain a measurement range from 0.005±0.001 to 10±0.01 mg N/L.

3.2.5 Sampling Procedure

Samples were taken directly from the reactor by means of a 30 mL syringe filled with deep-frozen (-26°C) steel spherical beads (0.5 mm diameter, specific heat 0.107 cal g^{-1} K^{-1}). The amount (grams) of beads contained in the syringe was adapted to the sample volume in order to achieve the necessary temperature decrease (from 30°C to 2°C) and therefore to stop the microbial conversion. All samples were filtered at 0.45 µm before analysis.

3.2.6 Molecular Methods

Fluorescence in situ hybridization

Samples were fixed for fluorescence in situ hybridization (FISH) as described by Pernthaler et al. (2001). Briefly, cells were washed in phosphate buffer, fixed in paraformaldehyde and spotted onto Teflon-coated multi-well slides. After dehydration by immersion into ethanol solutions (50, 80, 98%), the cells were hybridized with the following fluorescently-labeled oligonucleotide probes: EUB-mix (mix of oligonucleotides EUB-338, EUB-338 II and EUB-338 III), Nso-190, Neu-653, AMX-820, KST-157 or Bfu-613. Details on the target organisms and the sequences can be found in Table 3.2. Probe and hybridization details are available at Probebase (Loy et a., 2003). Microscopic observations were performed with a Zeiss Axioplan epifluorescence microscope (Zeiss, Stuttgart, Germany). Fixation took place evry two-three weeks. On day 472, the enrichment level was estimated by counting those cells which were visible under the microscope, but which did not hybridize with the AMX-820 probe (the number of non-anammox cells). This number was compared to the total number of visible (anammox and non-anammox) cells (circa 10,000).

Table 3.2: Oligonucleotides used in this Chapter

Oligonucleotide	Target organisms	Sequence (5'-3')	Reference
Eub-338	Bacteria	GCT GCC TCC CGT AGG AGT	Amann et al. (1990)
Eub-338 II	Bacteria	GCA GCC ACC CGT AGG TGT	Daims et al. (1999)
Eub-338 III	Bacteria	GCT GCC ACC CGT AGG TGT	Daims et al. (1999)
Nso-190	AOB of the beta-Proteobacteria	CGA TCC CCT GCT TTT CTC C	Mobarry et la. (1996)
Neu-653	Halophilic and halotolerant *Nitrosomonas* sp.	CCC CTC TGC TGC ACT CTA	Wagner et al. (1995)
Amx-368	Anammox bacteria	CCT TTC GGG CAT TGC GAA	Schmid et al. (2003)
Amx-820	"Kuenenia"/"Brocadia"	AAA ACC CCT CTA CTT AGT GCC C	Schmid et al. (2000)
Kst-157	"Kuenenia"	GTT CCG ATT GCT CGA AAC	Schmid et al. (2001)
Bfu-613	"Brocadia Fugida"	GGA TGC CGT TCT TCC GTT AAG CGG	Kartal et al. (2007)
Pla-46f	Planctomycetes	GAC TTG CAT GCC TAA TCC	Neef et al. (1998)
Univ-1392r	Bacteria	ACG GGC GGT GTG T	Ferris et al. (1996)

DNA Extraction, PCR Amplification, and Phylogenetic Analysis

On day 451 a sample (5 mL cell suspension) was taken from the reactor and directly centrifuged for 5 min at 13,000 rpm at 4°C. The cell pellet was stored at -20°C. Genomic DNA was extracted from the cells using the UltraClean Soil DNA Extraction Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's protocol. The quality of the extracted DNA was analyzed by agarose gel electrophoresis. Subsequently, the extracted DNA was used to amplify the nearly complete 16S rRNA gene using primers Pla-46f and Univ-1392r (see Table 3.2 for details). The PCR products were analyzed with agarose gel electrophoresis, purified using the Qiaquick PCR purification kit (Qiagen, Düsseldorf, Germany) and sequenced by a commercial company (BaseClear, Leiden, The Netherlands). The sequences were first compared to sequences stored in GenBank using blastn. Thereafter, they were imported into the SILVA database (Pruesse et al., 2007) with the ARB software program (Ludwig et al., 2004). The sequences were automatically aligned and alignments were corrected by hand after which a tree was created using the neighbor-joining algorithm with Felsenstein correction.

3.2.7 Kinetic Characterization Experiment

Experimental Set-Up

Two additional vessels (*vessel-a* and *vessel-b*) were connected to the feeding-medium vessel (*vessel-c*) by two distinct tubing lines equipped with two (peristaltic) pumps as indicated in figure 3.3 (before entering in *vessel-c* the two tubing lines were connected for practical reasons). The medium contained in *vessel*-c entered the reactor by means of a (peristaltic) pump at a flow rate of 6 L d^{-1} throughout the experimentation. *Vessel-a* contained a concentrated medium (20 L) prepared according to par. 3.2.2 (Table 3.1, days 232-612) with 300 mM ammonium and 300 mM nitrite (*medium-a*). *Vessel-b* contained a concentrated medium (20 L) prepared according to par. 3.2.2 (Table 3.1, days 232-612) without substrates (*medium-b*). *Medium-a* entered *vessel-c* (starting volume of 20 L) by means of a (peristaltic) pump at a flow rate of 6 L d^{-1} during the first 24 hours of the experimentation. At hour 24 (corresponding to the maximum imposed load) the composition of the medium contained in *vessel*-c (20 L) was according to par. 3.2.2 (Table 3.1, days 232-612) with 120 mM ammonium and 120 mM nitrite. The pump operating *medium-a* (final volume 14 L) was then stopped and the pump operating *medium-b* was started. *Medium-b* entered *vessel-c* by means of a (peristaltic) pump at a flow rate of 18 L d^{-1} during the second 24 hours of the experimentation (hours 24-48). At hour 48 the composition of the medium contained in *vessel*-c was back to steady state condition (according to par.

3.2.2, Table 3.1, days 232-612) and the dynamic part of the experiment was therefore concluded. The pump operating *medium-b* (final volume 2 L) was then stopped and steady state operations were applied. pH was constant at 7.0 throughout the experimentation due to phosphate buffer (tot-P 17 mM).

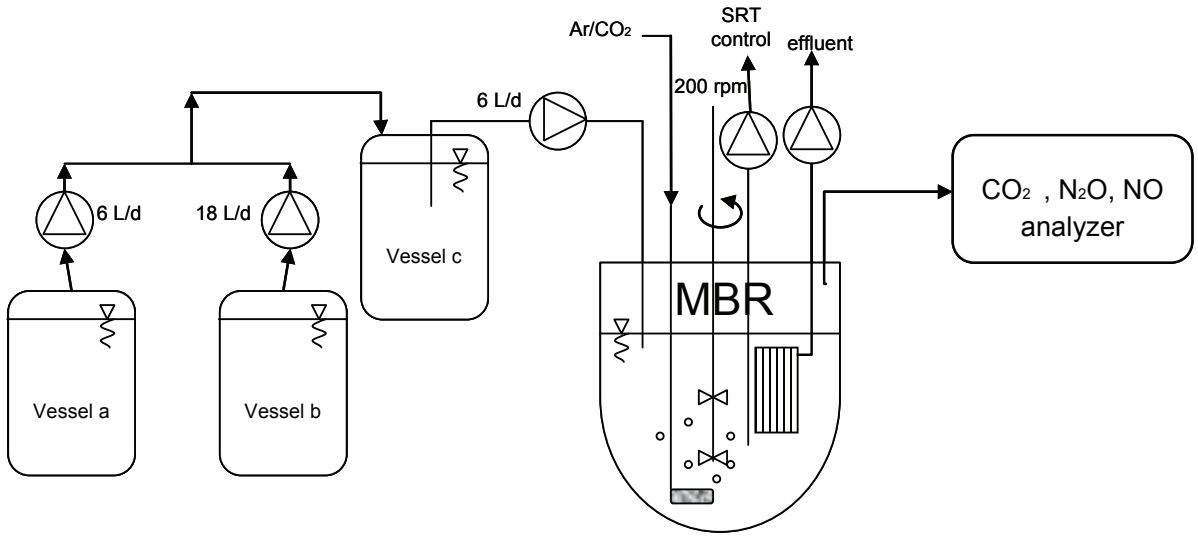


FIG. 3.3
Experimental set-up for the kinetic characterization experiment.

Experimental Procedures

Prior to conducting an experiment, the bioreactor was operated at constant loading rate, HRT, SRT until steady state (at least five volume changes). The experiment subsequently consisted of a change in the operation of the bioreactor (i.e., ammonium and nitrite loading rates) and on-line registration/off-line measurement of the response of the key variables in the system (on-line: N_2O, NO, CO_2, pH; off-line: NH_4^+, NO_2^-, NO_3^-, N_2, TOC). The off-line measurements were conducted on samples taken directly from the reactor at regular time intervals (5 to 10 mL sample volume). Samples were taken by means of a 30 mL syringe filled with deep-frozen (-26°C) steel spherical beads (0.5 mm diameter, specific heat 0.107 cal g^{-1} K^{-1}). The amount (grams) of beads was adapted to the sample volume in order to achieve the necessary temperature decrease (from 30°C to 2°C) and therefore to stop the microbial conversion. All samples were filtered at 0.45 µm before analysis. The measured response of the system variables allowed for calculation of important state variables such as the biomass yield, the biomass specific substrates uptake rate and the biomass growth rate as a function of the actual process conditions as described below in the *Calculations* section.

3.2.8 Modeling

A model representing the MBR operations was implemented in AQUASIM (Reichert 1994). Anammox was the only microbial activity modeled. The stoichiometric and kinetic parameters of anammox bacteria were obtained from the *kinetic characterization experiment* (par. 3.2.7). Other parameters like Henry constants or acid dissociation constant were taken from literature. The soluble compounds considered were ammonium, nitrite, nitrate, dinitrogen gas, carbon dioxide, bicarbonate, carbonate, protons. The only particulate compound considered was anammox biomass. The compounds considered in the gas phase were Argon, dinitrogen gas, carbon dioxide.

3.2.9 Calculations

Mass Balances

Since the carbon dioxide flux entering the bioreactor was known, on-line measurement of the CO_2 concentration in the dewatered gas leaving the bioreactor allows for direct estimation of the biomass production rate ($R_X = -R_{CO2}$, *C*-mol h^{-1}). Measured CO_2 uptake rates (R_{CO2}) were corrected for the dissolved carbon dioxide and bicarbonate in the

reactor effluent: on-line measurement of the operational pH allows for the estimation of the CO_2 and bicarbonate concentration in the bulk. Measurements of the soluble nitrogen compounds (NH_4^+, NO_2^-, NO_3^-) together with the measurement of the volatile nitrogen compounds allows for estimation of the actual substrates uptake rate (R_{NH4}, R_{NO2}) and the actual products production rate (R_{NO3}, R_{N2}). R_{N2O} and R_{NO} were also estimated even if their contribution to the total nitrogen balance was smaller than 0.1%. Using all these measurements a full mass balance can be established over the reactor. A metabolic system that catalyzes one dominant catabolic and anabolic reaction has two degrees of freedom and requires measurement of two rates for system identification. Since more measurements are available with the system described here, the individual measurements can be checked.

State Variables

Based on the observed carbon dioxide uptake rate (R_{CO2}, *C*-mol h^{-1}) and ammonium uptake rate (R_{NH4}, N-mol h^{-1}), the biomass yield can be calculated according to:

(3.1)

$$Y_{X/NH_4} = \frac{R_X}{R_{NH_4}} = \frac{-R_{CO_2}}{R_{NH_4}}$$

It is assumed that all CO_2 taken up is converted into biomass. Since both CO_2 and NH_4^+ are taken up in the system Y_{X/NH_4} has a negative value according to Equation (3.1). The experiments described in this Chapter were conducted starting from steady state. The steady state biomass concentration (X*) can be calculated from R_{CO2} and the reactor-suspension outflow rate (Q_W, L h^{-1}; $Q_W = V_L\ SRT^{-1}$):

(3.2)

$$X^* = \frac{-R_{CO_2}}{Q_W}$$

During the dynamic (non-steady state) experiment the biomass concentration (X) is calculated by integration with respect to time of the biomass mass balance:

(3.3)

$$X_{t+\Delta t} = X_t - \frac{\left(Q_W \cdot X_t + R_{CO_2}\right) \cdot \Delta t}{V_L}$$

During the experiments biomass concentrations calculated based on R_{CO2} were verified by TOC and gravimetric (TSS and VSS) measurements at regular intervals. Knowing the actual biomass concentration allows for calculation of the actual specific ammonium uptake rate (q_{NH4}, N-mol C-mol^{-1} h^{-1}) and the actual specific growth rate (μ, h^{-1}):

(3.4)

$$q_{NH_4} = \frac{R_{NH_4}}{X \cdot V_L}$$

(3.5)

$$\mu = \frac{R_X}{XV_L} = \frac{-R_{CO_2}}{XV_L}$$

Actual specific nitrate, dinitrogen gas, nitrous oxide and nitric oxide production rate (q_{NO3}, q_{N2}, q_{N2O}, q_{NO}, N-mol C-mol^{-1} h^{-1}) can be calculated with Equation (3.4) considering the respective non-specific production rate (R_{NO3}, R_{N2}, R_{N2O}, R_{NO}, N-mol h^{-1}). Actual specific nitrite uptake rate (q_{NO2}, N-mol C-mol^{-1} h^{-1}) can be equally calculated with Equation (3.4) considering the respective non-specific uptake rate (R_{NO2}, N-mol h^{-1}).

Nitrite Half-Saturation Constant

The nitrite half-saturation constant (or nitrite half saturation constant, $K_{S,NO2}$, mg N L^{-1}) was estimated from the reactor data during steady state operation (i), from an experiment in which the nitrite loading rate (mg N L^{-1} d^{-1}) was suddenly increased of 11% (ii) and from the data of the *kinetic characterization experiment* (iii) (par. 3.2.7):

(**i**) Before the evaluation of the maximum nitrite uptake rate ($Rate_{NO2}^{max}$) by means of batch

tests (see par. 3.2.4.3), the nitrite level at steady state operation was measured in three replicates by sampling as described in par. 3.2.5 in a 2 hours period. The traditional Monod-based Equation (3.6) represents the dependency of the biomass specific substrate uptake rate (q_S) on the substrate concentration (C_S) and on the substrate half-saturation constant (K_S).

(3.6)

$$q_S = q_S^{max} \frac{C_S}{C_S + K_S}$$

$K_{S,NO2}$ can then be calculated with Equation (3.7) by assuming the biomass concentration (X) as constant throughout the batch test (reasonable assumption given the low growth rate and low yield of anammox bacteria) and considering that the reactor was previously operated under nitrite limitation at steady state:

(3.7)

$$K_{NO_2^-} = C_{NO_2^-} \frac{Rate_{NO_2^-}^{max} - Rate_{NO_2^-}}{Rate_{NO_2^-}}$$

Where the actual nitrite uptake rate was equal to the nitrite loading rate (nitrite removal efficiency >>99%).

(**ii**) On day 147 the nitrite half-saturation constant was evaluated according to equation 3.7. After the MNRR measurement one liter of mixed liquor was withdrawn from the reactor (V_L=9 L) causing an immediate decrease of the HRT and therefore an immediate increase of the imposed nitrite loading rate (11%, from 502 to 558 mg N L^{-1} d^{-1}). The others operational parameters (i.e., feeding medium composition, liquid discharge, SRT) were maintained constant. Six hours later the evaluation of the nitrite half-saturation constant was repeated according to Equation (3.7).

(**iii**) The results from the *kinetic characterization experiment* (par. 3.2.7) were fitted by the model output (par. 3.2.8) calibrating the nitrite half saturation constant. The best data fitting was evaluated by minimizing the sum of squared residuals according to the method of least squares. The nitrite half saturation constant was therefore identified.

3.3 RESULTS

3.3.1 Reactor Operation

Since the day of the inoculation of the reactor (the designated experimental day 1) the nitrite-loading rate was increased step-wise increasing the medium flow entering the reactor (medium composition remained constant) each time the nitrite concentration was verified to be stably below 10 mg N L^{-1} for few days. Within one month the reactor could be operated at the design conversion rate of 0.5 g NO_2-N L^{-1} d^{-1} (total nitrogen-loading rate of 1 g N L^{-1} d^{-1}) at 30°C and at sludge retention time (SRT) of 15 days. On day 63 the SRT was decreased to 12 days and maintained constant during all the experimentation. Few days after the design nitrite-loading rate was applied the reactor was operated under nitrite limitation and nitrite consumption was generally complete throughout the study (Fig. 3.1). Since day 100 throughout all the study the reactor was in steady state with a nitrogen removal efficiency of 80-85%. Ammonium and nitrate concentration were on average both equal to 140 mg N L^{-1}. During the whole period at steady state (days 100-552), the molar ratio between nitrite removed and ammonium removed (R_NiAm) and the molar ratio between nitrate produced and ammonium removed (R_NaAm) were 1.219±0.08 and 0.211±0.07, respectively (Fig. 3.2). Averaged R_NiAm and R_NaAm during different experimental periods corresponding to different average bacterial aggregation status (granules, flocs, micro-flocs/free cells) are reported in Table 3.3. Oxygen concentration in the outflowing gas was occasionally measured on-line for 2-15 consecutive days and it was always below the analyzer detection limit (0.001% of oxygen saturation at 30°C). Maximum volumetric oxygen uptake rate (OUR^{max}) was also measured occasionally by means of BOM-measurement since day 121 and no oxygen removal capacity was detected.

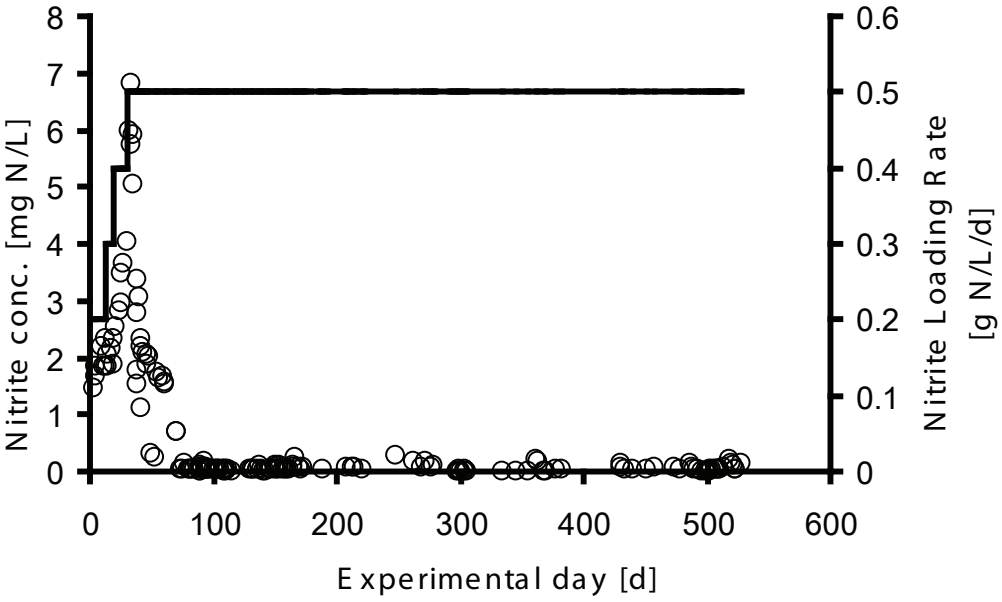


FIG. 3.1
Nitrite concentration in the reactor (circles, left vertical axis) and nitrite-loading rate applied (continuous line, right vertical axis).

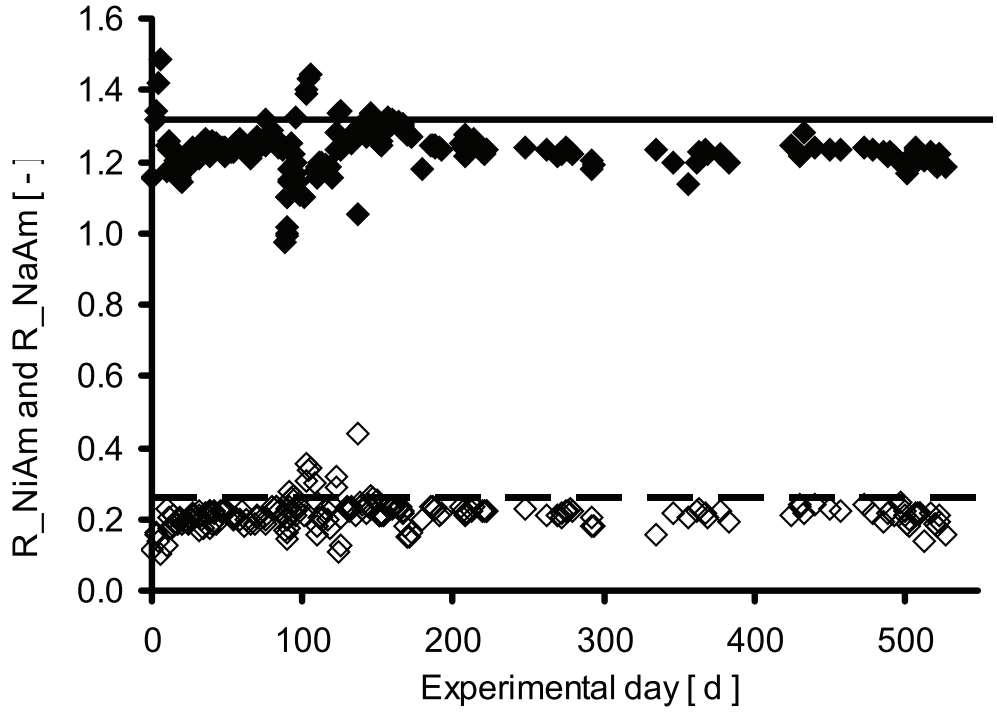


FIG. 3.2
Molar ratio between nitrite removed and ammonium removed (R_NiAm, close diamonds) and molar ratio between nitrate produced and ammonium removed (R_NaAm, open diamonds). The continuous and dashed lines represent R_NiAm and R_NaAm for anammox bacteria (Strous et al., 1998).

Table 3.3: Molar ratio between nitrite removed and ammonium removed (R_NiAm) and molar ratio between nitrate produced and ammonium removed (R_NaAm) during different experimental periods corresponding to different average bacterial aggregation status (granules, flocs, micro-flocs/free cells).

Bacterial aggregation status	Period [days]	R_NiAm	R_NaAm
Granules	0-100	1.246	0.211
Flocs	100-300	1.243	0.223
Micro-flocs/free cells	300-612	1.214	0.209

The granules of the inoculum (diameter distribution mode equal to 1 mm) progressively changed to small flocs during the first 100 days and after the first 140-150 days the floc-dimension distribution stabilized at floc-diameter size smaller than 90-100 µm (95th percentile equal to 50 µm, Fig. 3.3). From the aggregate size distribution evaluated on day 0, 98 and 164, the weighted mean diameter was calculated using the bin frequency as weight. These three data points were interpolated with an exponential equation in order to estimate the mean diameter of the aggregates in time (Fig. 3.4).

The biomass concentration inside the reactor continuously decreased during the first three months of operation. After approximately day 100 biomass concentration was stable throughout the experimentation at about 0.5 g TSS/L and 0.46 g VSS/L (Fig. 3.5).

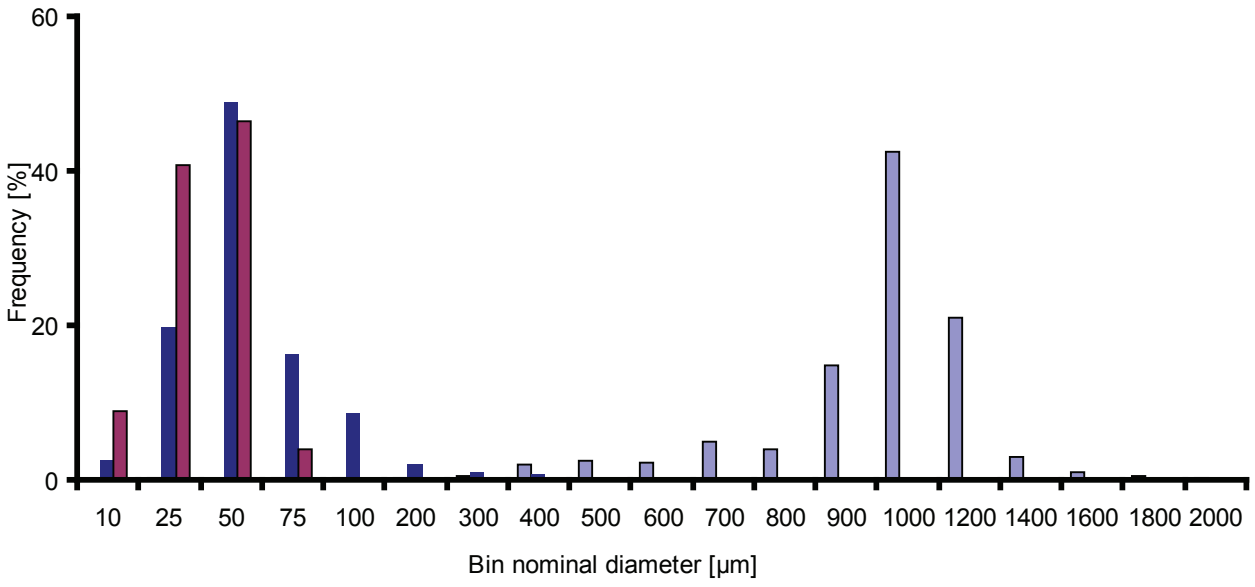


FIG. 3.3
Aggregate size distribution as frequencies ([%], number of aggregates on total number of aggregates investigated) in discrete intervals ([µm], in figure the middle point of each bin is reported). Vertical bars describe the aggregate size distribution on day 0 (inoculum, grey), on day 98 (blue) and on day 164 (red).

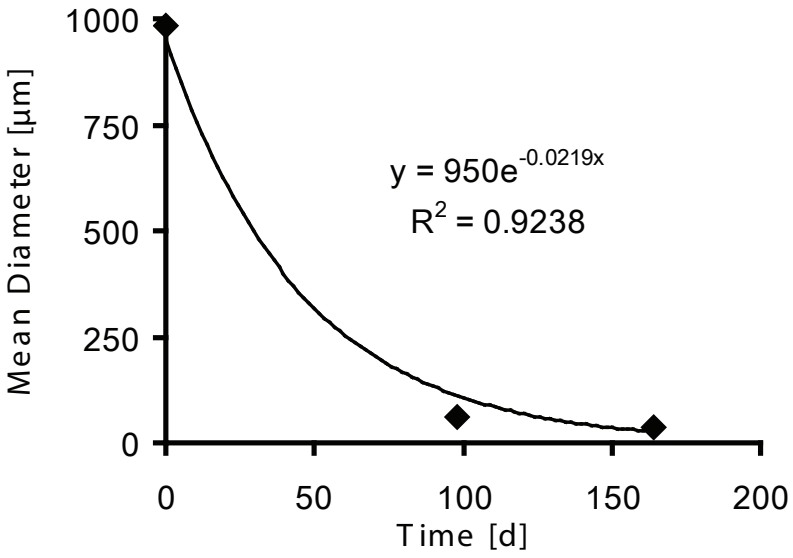


FIG. 3.4
Exponential relation between the weighted mean diameter of the aggregate size distribution (bin frequency as weight) measured by image analysis and day of measurement.

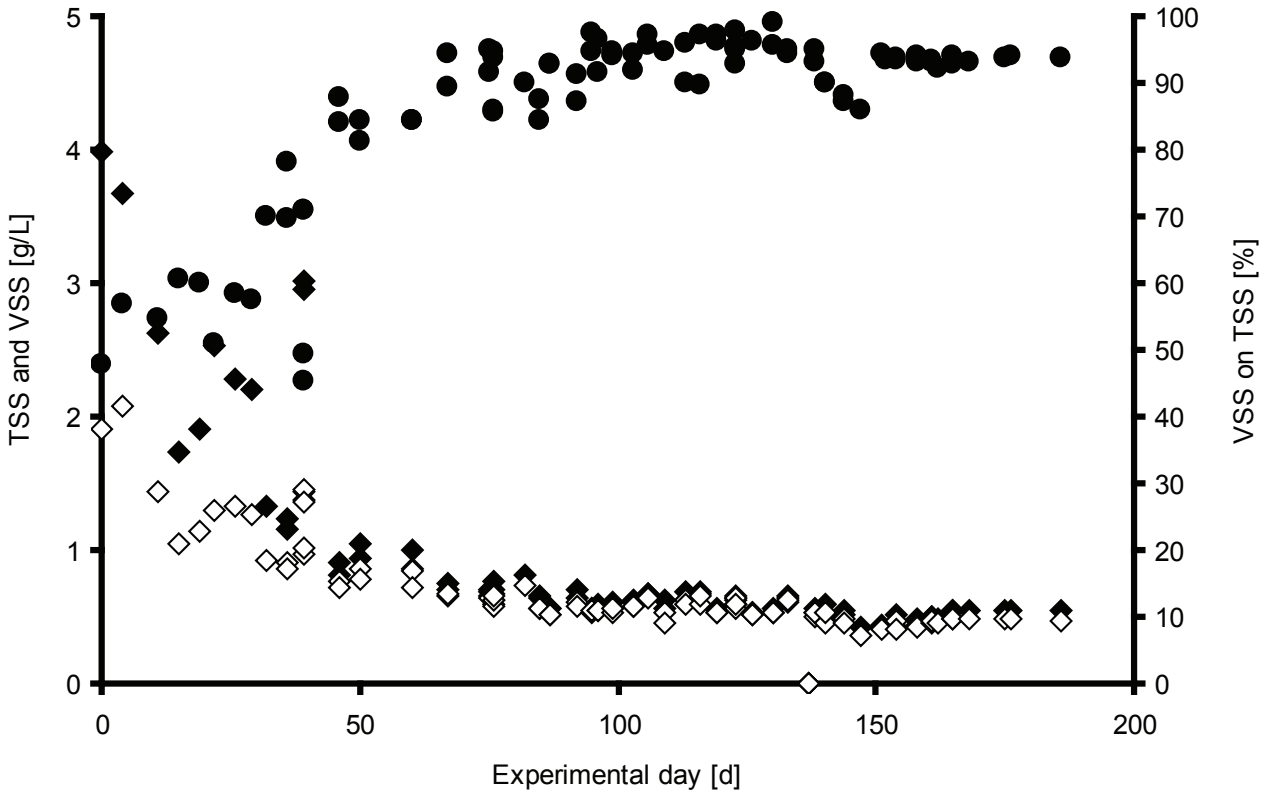


FIG. 3.5
Biomass concentration inside the reactor as TSS ([g/L], closed diamonds, left vertical axis), VSS ([g/L], opened diamonds, left vertical axis) and VSS/ TSS ratio ([%], circles). Only dynamic part of the data set (first 200 days) is shown.

The medium fed to the reactor during the first 88 days of operation was characterized by low calcium and magnesium ion level (1.0 and 0.4 mM, respectively, see Table 3.1). Further lowering of calcium ion level in the medium till 0.5 mM (on day 89) didn't result in any appreciable change in flocs dimension or settling properties. 35 days (approximately 3 SRT) after the lowering of calcium and magnesium a solution of vitamins (mimicking the vitamin content of yeast extract) was added to the medium, but again the average aggregate dimension didn't change remarkably. Yeast extract was finally dosed as spike addition directly into the reactor (final concentration of 2 mg/L) in addition to the vitamin solution in the medium in several occasions, but any change in the flocs structure or in the settling velocity could be noticed in the following days. On day 297 an experiment was conducted in which the applied nitrogen-loading rate was double for one week. Few days after the loading rate was set back to normal value (day 304) the biomass lost any settling capacity and microscope images confirmed that a suspension (almost all of the bacteria were as free-cell) was achieved. After two days free-cells were noticed for the first time, small flocs appeared again and the biomass came back to the previous aggregation status allowing settling. An attempt in which the medium was intensively sparged with helium (certified pure gas for analytical purposes) for 24 hours was conducted on day 330 and bacterial suspension was achieved again in 10-15 hours time span. 20 mL of air were then injected in the feeding medium vessel (50 L) to verify the influence of oxygen and free-cells aggregated into small flocs in few hours. On day 333 measures were taken in order to ensure complete anaerobic conditions inside the reactor. These changes in medium preparation and gas-circuit were effective to obtain an almost stable suspension for more than 250 days: in several occasions the suspension turned temporarily back to the previous small flocs-status without apparent reason probably due to uncontrolled oxygen leakage into the reactor or oxygen content in the feeding medium; furthermore, each time the membrane was replaced (every 2-3 weeks) and therefore the reactor vessel was opened letting air enter the headspace, small flocs appeared within few hours. Growth in suspension could be restored within few hours by increasing temporarily the flow rate of the inflowing gas. Since day 395 on the vitamin solution was no longer added to the medium without any effect on the settling properties of the biomass showing that vitamins lack wasn't the reason for aggregation. As oxygen concentration in the reactor headspace was constantly below the analyzer detection limit, the oxygen-loading rate (μmol O_2/L/d) causing free-cells to aggregate into small flocs was investigated. On day 450, after biomass was in suspension for ten consecutive days, a gas-bag (10 L) filled with air and N_2 at room temperature (23±2°C) was connected to the vessel containing degassed feeding medium. Oxygen loading rate supplied to the reactor was increased stepwise rising the air content of the gas-bag. Biomass aggregation status was monitored by the settling velocity in a 50 mL syringe connected by norprene tube (tube total internal volume of 4 mL) to the reactor mixed liquor (Fig. 3.6). As settling properties due the presence of micro-flocs were noticed, the biomass aggregation status was identified through microscope analysis on mixed liquor samples taken directly from the reactor with air-tight syringes previously flushed with nitrogen gas. Great care was taken when the sample was poured on the microscope slide in order to minimize exposure to oxygen; the whole microscope analysis procedure took no more than 3 minutes. An oxygen loading rate of 0.68±0.06 μmol O_2 L^{-1} d^{-1} (oxygen in the medium fed to the reactor equal to 1.1 μmol O_2 L^{-1}) was found to be sufficient to make the free-cells aggregate into small flocs (<100 μm) within one hour. In two distinct occasions, on day 481 and on day 502, the experiment was repeated and the result was confirmed. In figure 3.7 the phase-contrast pictures relative to the aforementioned oxygen loading rate experiments are reported. At room temperature, a degassing method with 99.6% oxygen removal efficiency was therefore required to allow bacteria as free-cells.

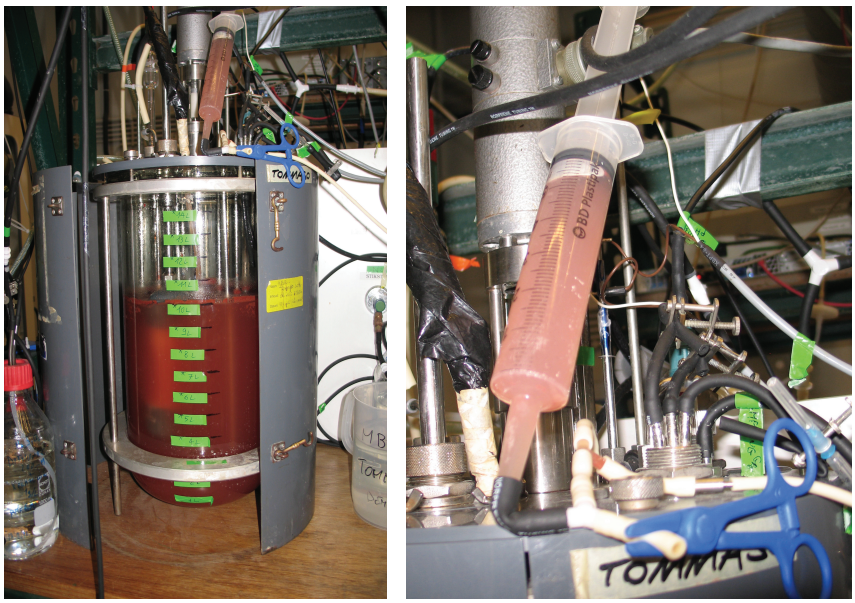


FIG. 3.6
syringe connected to the reactor mixed liquor by means of which the biomass settling properties were monitored.

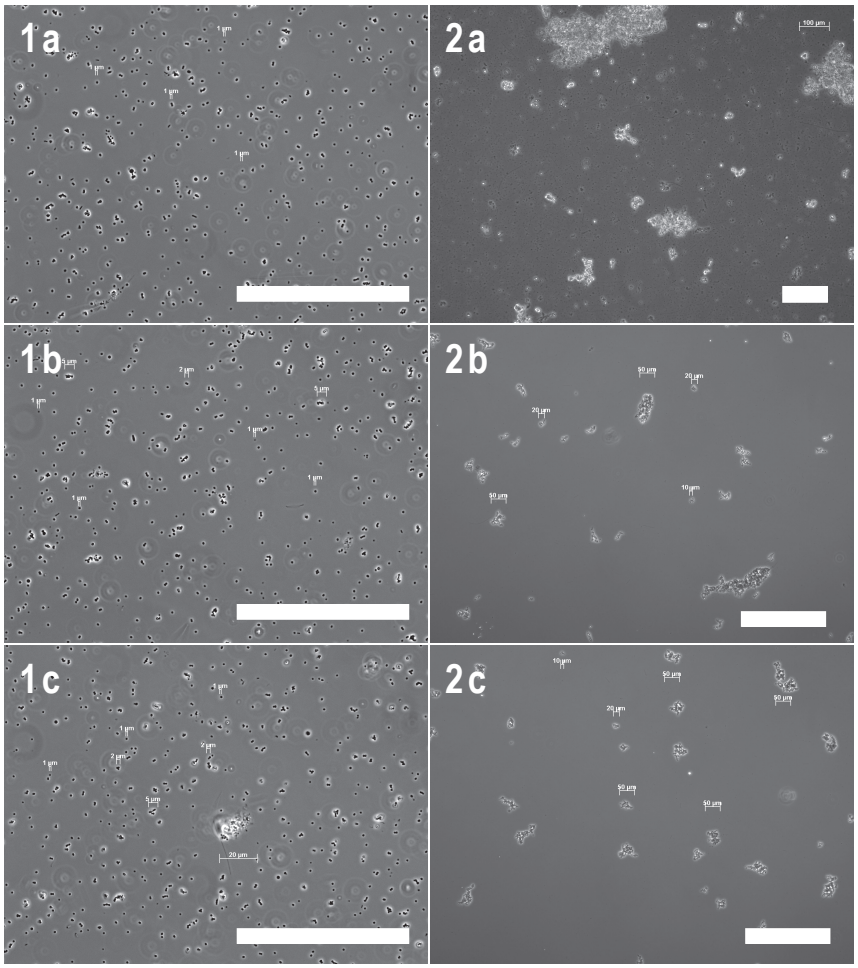


FIG. 3.7
Phase-contrast pictures taken under the microscope representing the biomass aggregation status before and after the critical oxygen loading rate was supplied to the reactor. The experiment was repeated on day 450 (a), 481 (b) and 502 (c). Bar=100 µm.

3.3.2
MICROBIAL COMMUNITY AND ENRICHMENT LEVEL

In 2007 the anammox bacteria of the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant (WWTP, see par. 3.2.1), was shown to consist of Candidatus ‘‘Brocadia’’ (Van der Star et al., 2007). When the MBR was inoculated (May 2010) this was still the main population since hybridization took place with the ‘‘Kuenenia’’/’’Brocadia’’-specific probe (AMX-820), but not with the “Kuenenia”-specific probe (KST-157). Throughout the whole experimental period no change in the main population could be noticed and ‘‘Kuenenia’’ could never be detected (day 444, Fig. 3.8, center). The level of enrichment increased from 70±5% to 85±5% in the first 90 days to more than 90% after day 165 (Fig. 3.8, top). No cells could be found at any time that did not hybridize with the ‘‘Kuenenia’’/’’Brocadia’’-specific probe (AMX-820), but which did hybridize with the all-anammox-specific probe (AMX-368) in any of the samples. This indicates that other described anammox bacteria were not present (or only present in very low amounts). On day 312 all cells hybridizing with the ‘‘Kuenenia’’/’’Brocadia’’-specific probe (AMX-820) were also found to hybridize (>99.5%) with the *Candidatus* Brocadia Fulgida-specifc probe (Bfu-613) indicating that the population was mainly composed by *Candidatus* Brocadia Fulgida anammox bacteria (Fig. 3.8, bottom). Since free cells were obtained, quantification using FISH was possible by viewing each individual cell and determining whether this cell had hybridized with the Brocadia Fulgida-specific probe (Bfu-613). The enrichment level at day 483 was 98±1% (ca. 10,000 cells counted).

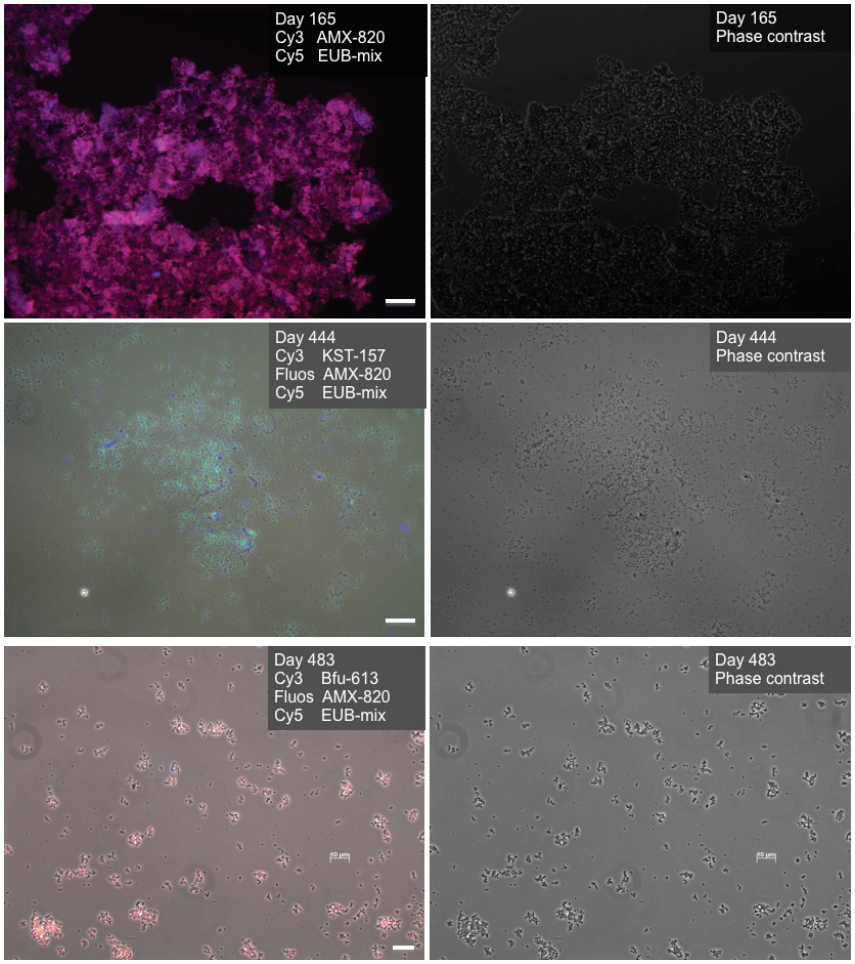


FIG. 3.8
The bacterial population in the membrane bioreactor as shown by FISH (left) and phase contrast microscopy (right). Fixation took place on day 165 (top), 444 (center), 483 (bottom). Scale bar is 100 µm (top and center) and 10 µm (bottom). The oligonucleotide probes used are specified on the figure.

The bacterial population composition was confirmed by 16S rRNA sequence analysis. The sequences of the sample on day 451 showed the strongest similarity (97.5%) with *Candidatus* ''Brocadia'' sp. 40 (Kieling et al., 2007) and 97% similarity with *Candidatus* Brocadia Fulgida (Kartal et al., 2007). Figure 3.9 shows a phylogenetic tree based on the 16S rRNA sequences of the strains.

3.3.3
Elemental biomass composition

The elemental biomass composition evaluation was conducted on a dried sample taken on day 485, when the anammox enrichment purity was shown to be 98±1% (par. 3.3.2). Both the C, H, N, S content and the P content were measured in two replicates and the elemental composition of the biomass was determined to be $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.09}$. The biomass molecular weight results therefore equal to 22.14 g C-mol^{-1}.

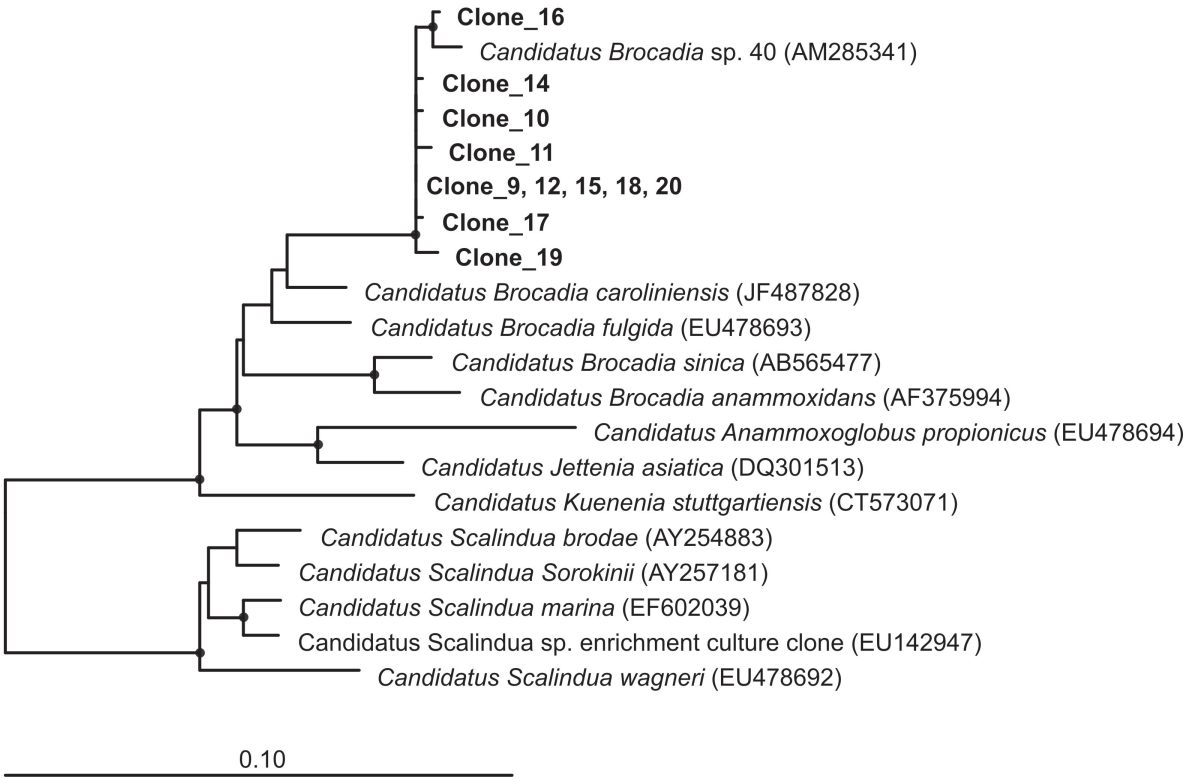


FIG. 3.9
Phylogenetic tree of 16s rDNA gene sequences showing the affiliation of anammox bacteria. The tree has been calculated using a maximal likelihood method, RAxML which is implemented in the ARB v5.2 software package. Bootstrap was performed for 250 round and values of >90% are indicated by a solid black dot. For calculations the bac_var_ssuref:bacteria filter (SSU_ref_108_silva database) has been applied to the data set to filter out noise. In total 1271 positions (ecoli pos. 594-1528) were used for calculation. Clones were calculated in the tree using 'quick add sequences using parsimony' tool. In total 711 positions (ecoli pos. 811-1528) were used. All Clones are highlighted in bold. The scale-bar represents 10% sequence divergence.

3.3.4
Affinity for Nitrite

From reactor operation and MNRRs

Throughout the whole experimental period the maximum nitrite removal rate (MNRR, g NO_2^--N L^{-1} d^{-1}) was measured as described in par. 3.2.4.3. The nitrite affinity constant was calculated as described in par. 3.2.9. The results from the nitrite half-saturation constant evaluations are reported in Figure 3.10 relative to the experimental day on which the MNRR measurement took place. The nitrite half-saturation constant was evaluated to be between 0.5 and 2.5 mg N L^{-1} the first 70 days, 0.047±0.04 mg N L^{-1} on days 70-504 and 0.035±0.011 on days 200-504. Taking into account the biomass aggregate size distribution relative to the periods in which the nitrite half saturation constant was evaluated (Fig. 3.3), a direct link between higher mass transfer limitation (associated to bigger aggregate size) and higher half saturation constant is demonstrated.

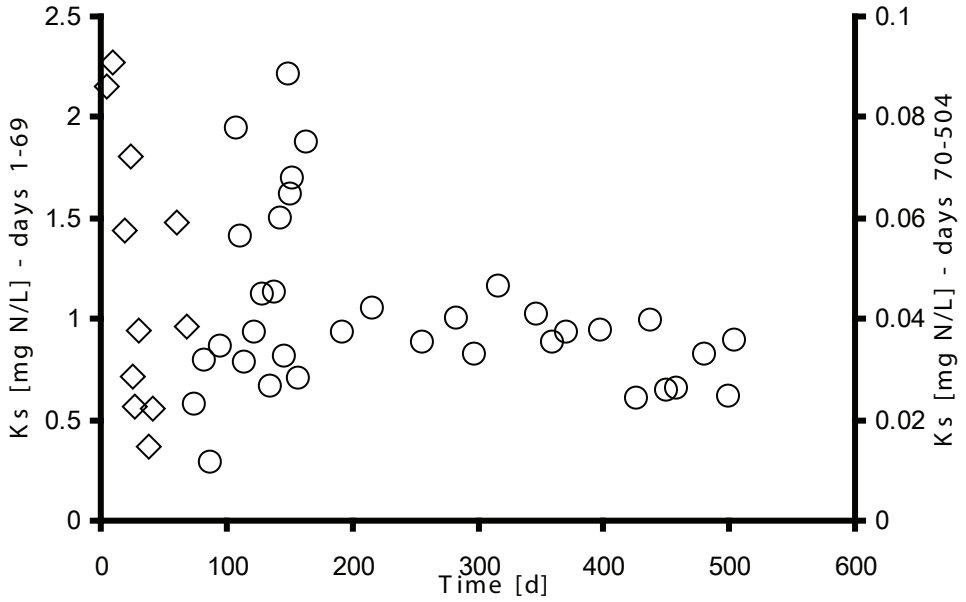


FIG. 3.10
Nitrite half-saturation constant from in reactor maximum nitrite removal rate (MNRR) measurements in different experimental days. Results on days 1-69 (diamonds) are reported on the left vertical axis, results on days 70-504 (circles) are reported on the right vertical axis.

On day 147 an experiment to evaluate in another way the nitrite affinity constant was conducted according to par. 3.2.9. The results are reported in table 3.4.

Table 3.4: Results from the evaluation of the nitrite affinity constant at different nitrite loading rate corresponding to different liquid volume in the reactor: 9 and 10 L.

Reactor liquid volume		**10 L**	**9 L**
$Rate_{NO2}$	**[mg N L^{-1} d^{-1}]**	436.1	484.5
$Rate_{NO2}^{max}$	**[mg N L-1 d-1]**	788	783
C_{NO2}	**[mg N L^{-1}]**	0.040	0.051
Ks	**[µg N L^{-1}]**	32.3	31.4

Kinetic Characterization by Changing the Loading Rate in Time

In order to determine the nitrite affinity constant for anammox bacteria, the nitrogen loading rate (NLR, sum of ammonium and nitrite loading rate) supplied to the reactor was continuously increased for 24 hours starting from the steady state-nitrogen loading rate (NLR*, 1 g N L^{-1} d^{-1}). The experiment started on day 587, after more then two weeks in which bacteria were stably as free cells. Mass transfer limitation could therefore be considered negligible. After 24 hours from the beginning of the experiment the nitrogen loading rate reached 210% of the NLR*. NLR was then continuously decreased for 24 hours at the end of which the steady state-nitrogen loading rate was constantly supplied again. Results are shown in Figure 3.11. The dynamics in the imposed NLR on the system (Fig. 3.11, C) resulted in corresponding dynamics in the actual CO_2 (Fig. 3.10, B), NH_4^+ and NO_2^- uptake rate (Fig. 3.11, D) and in the actual NO_3^- production rate (Fig. 3.11, D), as well as in the ammonium, nitrite and nitrate concentration inside the reactor (Fig. 3.11, E). Throughout the experiments no limitations in ammonium or carbon dioxide were evident while nitrite was the limiting substrate. From the actual CO_2-uptake rate the biomass concentration as a function of time was estimated using Equation (3.3) as shown in Figure 3.11, A. The biomass concentrations calculated from CO_2-uptake correspond adequately to the biomass concentration measured as TOC indicating that the CO_2-data are representative for the biomass production. The ratio between nitrite and ammonium uptake (R_NiAm) and the ratio between nitrate production and ammonium uptake (R_NaAm), were calculated from off-line concentration measurements and from the known ammonium and nitrite loading rate. Throughout the experiment (34 data) R_NiAm and R_NaAm were equal to 1.205±0.009 and 0.200±0.008, respectively (Fig. 3.11, F). Throughout the experiment nitric oxide (NO) and nitrous oxide (N2O) were produced at rate of 0.14±0.03 and 2.6±1.5 µmol N L^{-1} h^{-1}, respectively, accounting for the 0.003±0.002 and 0.056±0.02% of the imposed nitrogen load respectively. In Figure 3.12 the NO and N_2O production dynamics are shown. Using all the off-line and on-line measurements and considering the elemental composition of the biomass, a full mass balance could be established over the reactor as described in par. 3.2.9. The carbon and nitrogen balance were closed at 88% and 92%, respectively.

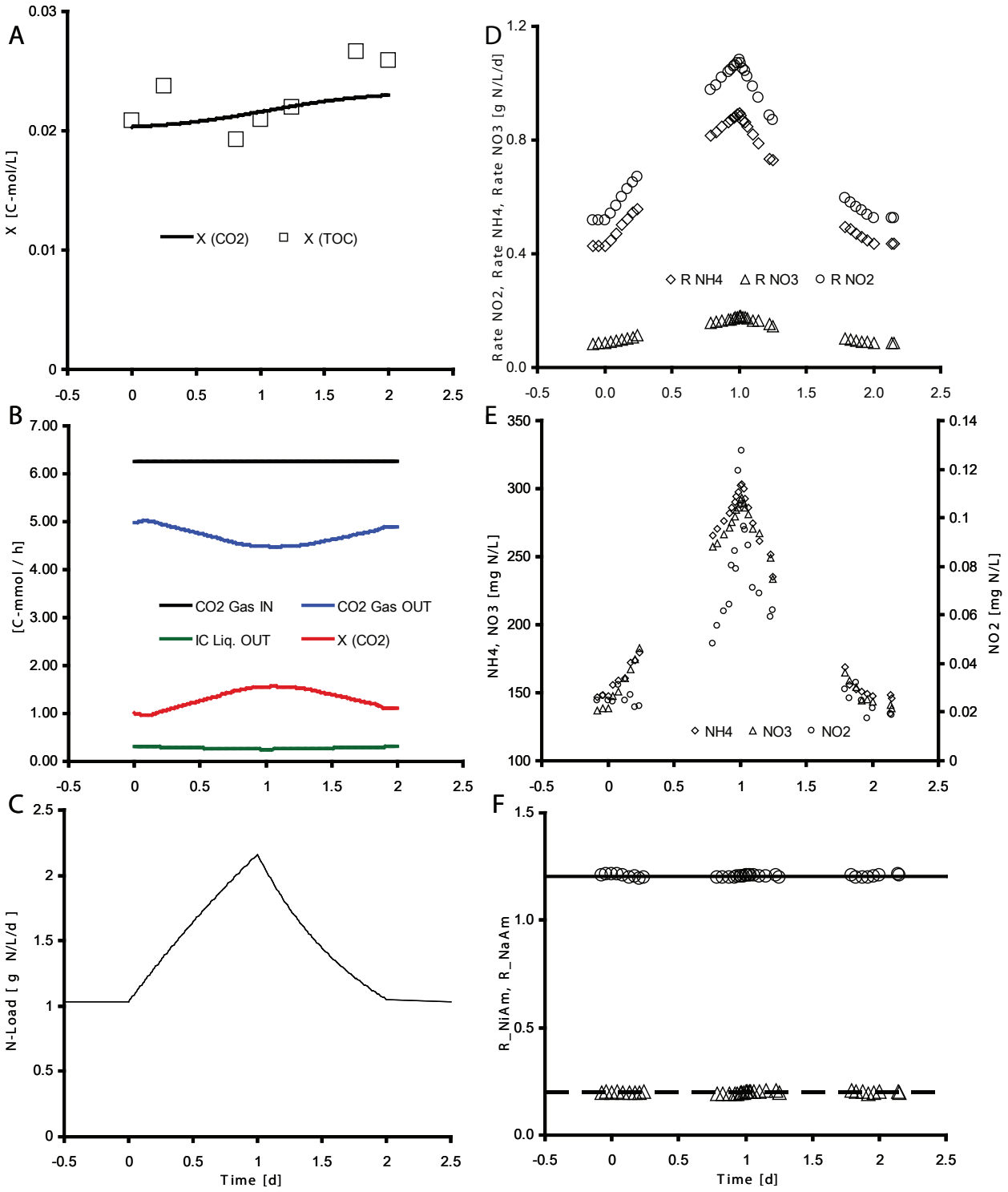


FIG. 3.11

Experiment for kinetic parameter determination (i.e., affinity constant for nitrite). In alphabetical order the graphs show as a function of time the biomass concentration as calculated from RCO2 [X(CO2)] and measured as TOC [X(TOC)] (A); the carbon dioxide entering [CO2 Gas IN] and leaving [CO2 Gas OUT] the system in the gas phase, the inorganic carbon leaving the system in the liquid phase [IC liq. OUT] and the biomass growth rate as calculated from RCO2 [X(CO2)] (B); the imposed total nitrogen loading rate (C); the ammonium [R NH4], nitrite [R NO2] uptake rate and the nitrate [R NO3] production rate calculated from off-line concentration measurements (D); the ammonium [NH4], nitrite [NO2] and nitrate [NO3] concentrations in the reactor as measured off-line (E); the ratio between nitrite and ammonium uptake (R_NiAm, circles) and the ratio between nitrate production and ammonium uptake (R_NaAm, triangles) calculated from off-line concentration measurements and the ammonium and nitrite loading rate (F).

A model mimicking the anammox process and the MBR operations during the experiment was implemented (par. 3.2.8). The maximum specific nitrite uptake rate (q_s^{max}, mg N C-mol^{-1} d^{-1}) used in the model was calculated by the result of a batch test conducted 24 hours before the experiment (par. 3.2.4.3) and the biomass concentration present in the reactor. The nitrite affinity constant of anammox bacteria was calibrated in order to fit the model output. The best data fitting was evaluated by minimizing the sum of squared residuals according to the method of least squares (Fig. 3.13). The nitrite half saturation constant was identified as equal to 35 µg N L^{-1}. Since this nitrite half saturation constant was identified in a suspended culture where mass transfer limitation doesn't act, it can be considered as the intrinsic half saturation constant for anammox bacteria. The values of the (apparent) nitrite half-saturation constant (mg N L^{-1}) estimated by batch test and shown in Figure 3.10, were related to the mean diameter (µm) of the aggregates on the day the batch test was performed (Fig. 3.4). The obtained pairs of values were interpolated with an exponential function in which the constant was set equal to the identified intrinsic half-saturation constant expressed in mg N L^{-1} (0.035) (Fig. 3.14). The so obtained exponential equation (Eq. 3.8) can be used to make a rough estimation of the apparent nitrite half-saturation constant (K_S, mg N L^{-1}) relative to a bacterial aggregate of a certain diameter (D, µm).

(3.8)

$$K_S = 0.035\exp\left(0.0057D\right)$$

From the actual ammonium and carbon dioxide uptake rate the actual yield can be calculated according to Equation (3.1). The actual yield during the NLR dynamic change was 0.071±0.002 C-mol N-mol^{-1} (Fig. 3.15).

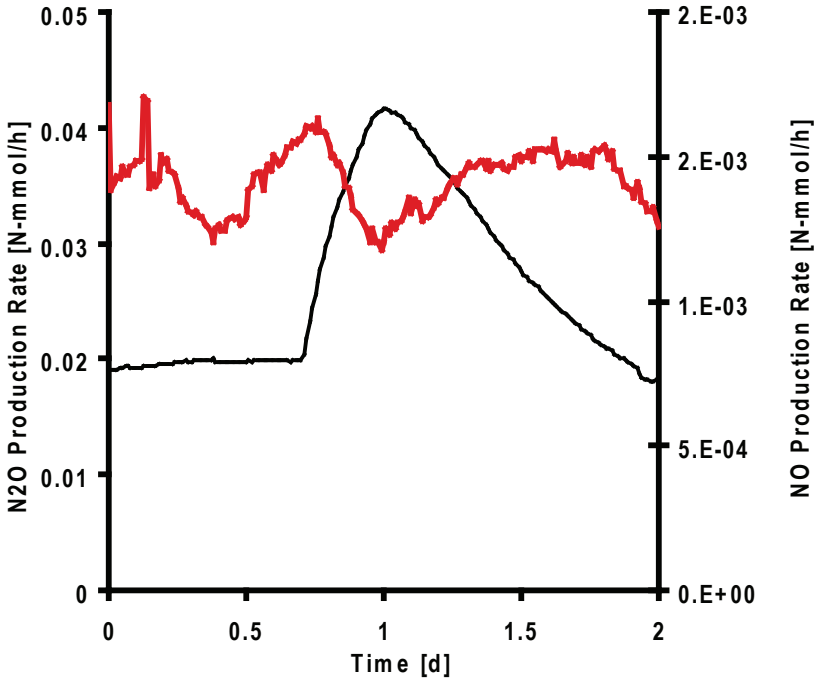


FIG. 3.12
nitric oxide (NO, red, right axis) and nitrous oxide (N_2O, black, left axis) production during the experiment as calculated from on-line measurements.

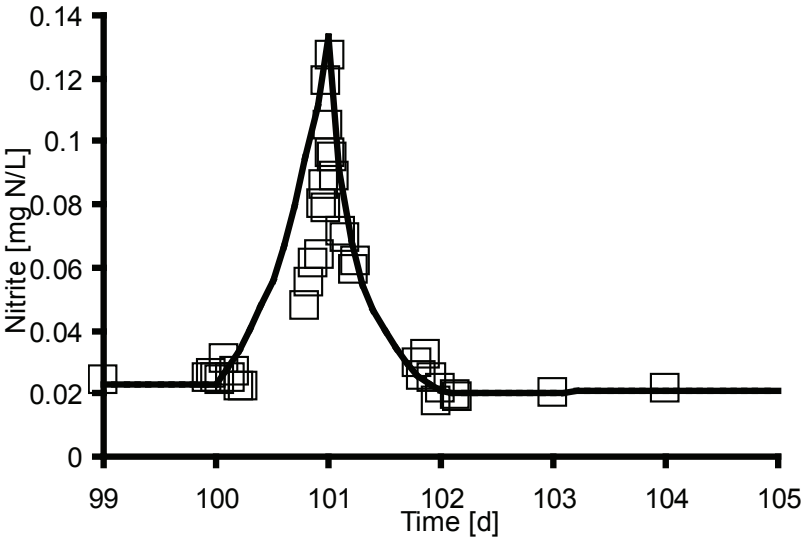


FIG. 3.13
Nitrite concentration as measured during the experiment of the nitrogen loading rate dynamic change (squares) and as simulated by the model. The model was run 100 days before imposing the NLR dynamic change in order to reach the steady state: day 100 in figure represent the experiment starting time.

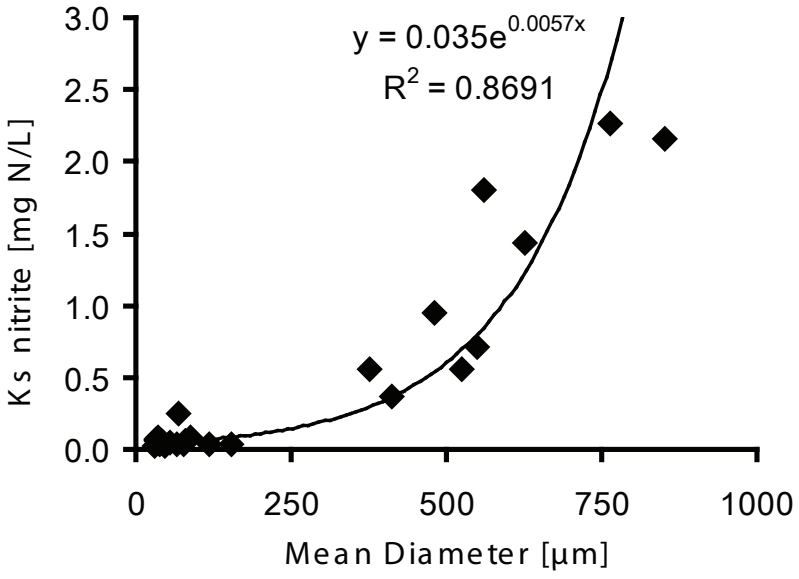


FIG. 3.14
Exponential relation between nitrite half saturation [mg N L^{-1}] constant and aggregate size [µm].

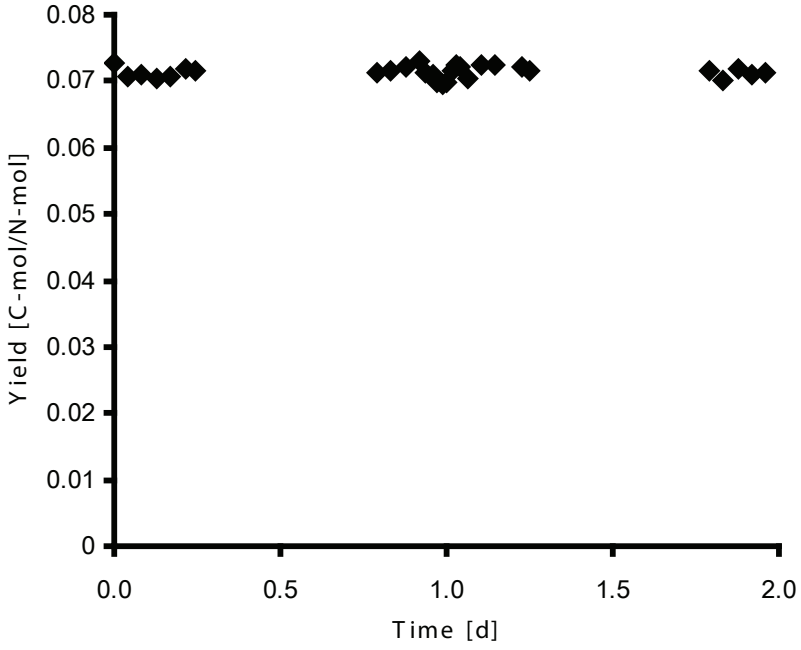


FIG. 3.15
Actual biomass yield as calculated from carbon dioxide and ammonium uptake rate.

3.3.5
Study of the stoichiometry and kinetic (μ^{max})

Biomass yield was shown to be rather constant during the *NLR dynamic change experiment* where NLR increased up to 210% of the nitrogen loading rate at steady state (NLR*, 1 g N L^{-1} d^{-1}). The maximum nitrite uptake rate measured with the in-reactor batch test (as described in par. 3.2.4.3) conducted the day before the NLR started to increase, was equal to 1.273 g N L^{-1} d^{-1}. Since the maximum imposed nitrite loading rate during the experiment was equal to 1.058 g N L^{-1} d^{-1}, we can conclude that the biomass yield was rather constant for an imposed nitrite loading rate ranging from 39.6 up to 83.1% of the maximum nitrite conversion rate of the reactor (nitrite as only limiting substrate). From the obtained yield (0.071 C-mol N-mol^{-1}) and the elemental composition of the biomass, the stoichiometry of the Anammox process was calculated to be:

(3.9)

$$1NH_4^+ + 1{,}146NO_2^- + 0{,}071HCO_3^- + 0{,}057H^+ \rightarrow$$
$$0{,}986N_2 + 0{,}161NO_3^- + 0{,}071CH_{1{,}74}O_{0{,}31}N_{0{,}20}S_{0{,}01}P_{0{,}009} + 2{,}002H_2O$$

(considering ammonium as N-source)

(3.10)

$$1NH_4^+ + 1{,}218NO_2^- + 0{,}071HCO_3^- + 0{,}085H^+ \rightarrow$$
$$1N_2 + 0{,}203NO_3^- + 0{,}071CH_{1{,}74}O_{0{,}31}N_{0{,}20}S_{0{,}01}P_{0{,}009} + 2{,}016H_2O$$

(considering nitrate as N-source)
The traditional Pirt equation (Pirt, 1965) account for substrate uptake for growth independent maintenance purposes:

(3.11)

$$q_s = \frac{1}{Y_{XS}^{\max}}\mu + m_S$$

where q_S is the biomass specific substrate uptake rate, Y_{XS}^{max} the maximum yield of biomass (X) on substrate (S), μ the actual biomass specific growth rate and m_S the maintenance coefficient.

Since the yield was shown to be rather constant and considering the maintenance as negligible, is possible to speculate on the maximum biomass specific growth rate of anammox bacteria (μ^{max}), by realizing that the actual biomass specific growth rate (μ) is equal to the inverse of the SRT (reliably controlled at 12 days). Given the biomass concentration as constant at steady state and throughout the batch-evaluation of the maximum nitrite uptake rate (reasonable assumption given the low growth rate and low yield of anammox bacteria) and considering that the reactor was previously operated under nitrite limitation at steady state, the μ^{max} can be calculated as:

(3.12)

$$\mu^{max} = \frac{q_S^{max}}{q_S SRT} = \frac{Rate_S^{max}}{Rate_S SRT}$$

Given the maximum nitrite uptake rate of 1.273 g N L^{-1} d^{-1} (195 µmol-NH_4^+ g protein^{-1} min^{-1})[1], the actual nitrite uptake rate (equal to the imposed nitrite load at steady state) of 0.504 g N L^{-1} d^{-1} and SRT of 12 days, a maximum biomass specific growth rate of anammox bacteria of 0.21 d^{-1} was calculated. The corresponding doubling time would then be 3.3 days. Even taking into account the decay rate at 35°C reported by Scaglione et al. (2009, 0.0048 d^{-1}), the μ^{max} would be equal to 0.2057 d^{-1}, corresponding to a doubling time of 3.37 days (one hour longer than without considering maintenance).

3.4 DISCUSSION

Oxygen as key-factor to obtain suspended culture

Although the obtainment of aggregates is a logic consequence of enrichment reactors that select on settling ability (like SBRs, gas-lift reactors, etc.), the reversed statement is not necessarily true. Thus, an enrichment system where no selection on settling ability is present (like the MBR), might still lead to the formation of aggregates. Others studies reported indeed the presence of aggregates in MBR systems where the anammox process (Trigo et al., 2006; Wyffels et al., 2004) or nitrification (Wyffels et al., 2003) was employed.

Van der Star et al. (2008) was successful in obtaining free cells enrichment and reported that the trigger was the reduction of calcium and magnesium levels and the addition of small amounts of yeast extract together with other factors like (a) the absence of selective pressure for settling, (b) a high growth rate and (c) low shear stress. The MBR presented in this study met the indications (a), (b) and (c) reported by van der Star and coworkers (2008) throughout the whole experimentation. Bivalent ions (like calcium and magnesium ions) are known flocculation enhancers (Mahoney et al., 1987; Pevere et al., 2007; Sobeck and Higgins, 2002). Moreover, small calcium or magnesium precipitates in the reactor (e.g., hydroxyapatite, calcite) might act as nucleation seeds for the growth of small granules. Nevertheless very low level of calcium and magnesium in the feeding medium (0.038 and 0.24 mM, respectively) were reported in an MBR with granular biomass (Trigo et al., 2006), showing that low levels of bivalent ions are not sufficient to allow growth as free cells. Regarding the dosage of yeast extract, stress because of the absence of a micronutrient could stimulate the production of extracellular polymeric substances (EPS) and therefore the addition of vitamins to the medium could potentially lead to a lower EPS production and aid suspended growth. This study differentiated between the effect of lowering of calcium and magnesium concentration and addition of vitamins, thus modifying the medium composition fed to the anammox culture as described in par. 3.2.2. None of the medium composition applied resulted effective. The oxygen load supplied with the feeding medium resulted to be the key factor for growth as free cells. What was then the reason for the obtainment of suspended biomass by van der Star et al. (2008) and in this study on day 304 (see par. 3.3.1)? The medium composition used in the study of van der Star et al. (2008) contained yeast extract (1 mg L^{-1}). Yeast extract, beyond the vitamin content, is also an organic electron donor (1 mg-yeast L^{-1}≈1.4 mg COD L^{-1}) and as such can supply the aerobic growth of heterotrophic microorganisms. This (even very small) side population growing on yeast extract could have been responsible for oxygen depletion, allowing anammox bacteria to grow as free cells. Before the first time free cells were noticed in this Chapter, an experiment in which the nitrogen load imposed to the reactor was twofold with respect to the steady state N-load was performed. After one week the imposed N-load

1 Given R_NiAm=1.205, 0.45 gVSS L^{-1}, 0.6 g protein $gVSS^{-1}$.

was back to steady state value. During this week the biomass concentration in the reactor increased from 0.45 to about 0.55 g VSS L^{-1} causing therefore a transient increase in the amount of biomass decayed in the unit time. The organic matter resulting from the decay rate could have also in this case allowed the transient growth of a side population of oxygen consuming heterotrophs. Flocs in the MBR enrichment were indeed noticed again within few days. Ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) could also potentially scavenge the oxygen entering the reactor, but in both the free cell enrichments reported in this Chapter and by van der Star et al. (2008) they were not detected, though they were present in the inoculum (Kampschreur et al., 2008). Likely the steady state conditions in these systems were such (free ammonia concentration, short and defined SRT, oxygen concentration below detection limit) that nitrifiers could not maintain themselves in the reactors.

According to these evidences then, oxygen would condition anammox bacteria in two ways: on the one hand a low oxygen concentration (reversibly) inhibits anammox activity completely (Strous et al., 1999); on the other hand an even lower oxygen concentration (below detection limit) force free cells to aggregate in small flocs (diameter of 10-100 μm), but doesn't affect the activity at all. A possible explanation for this peculiar phenomenon could reside in a kind of self-protection strategy of anammox bacteria. The EPS content of anammox biomass (in g EPS $gVSS^{-1}$) was measured to be much lower when growing as free cells than when growing in aggregates, but in both cases these EPS presented a strong affinity for oxygen (Yuemei Lin personal communication). We hypothesize that the EPS secreted by anammox bacteria have the ability to adsorb a small amount of oxygen molecules, preventing them from entering the cell wall and stop the bacterial activity. The oxygen adsorbed on the EPS covering one cell, could then promote the attachment of another cell creating a kind of "cell-to-cell-bond". After several cells aggregated because of this mechanism, the core of the aggregate would be even more protected from oxygen due diffusion limitations. When the oxygen adsorption capacity of EPS is saturated, an increment of the oxygen present in the bulk would then stop the anammox activity. This hypothesized EPS ability would give the anammox bacteria an important advantage in those natural systems where oxygen concentrations are very low but variable (e.g. in oceanic oxygen minimum zones, OMZ). Further research is needed to evaluate the consistency of this fascinating theory. In this Chapter the method to obtain a highly enriched (98±1%) suspended culture of anammox bacteria was shown and for the first time the trigger was identified to be oxygen. We therefore fulfilled a pre-condition necessary to accurately investigate diverse aspects of the molecular biology, biochemistry, ultrastructure, physiology and ecology of anammox bacteria, as well as assessing the impact of their activities on the environment and their applications in wastewater and waste-gas treatment.

Nitrite half-saturation constant

The half saturation constant (or affinity constant, K_s) is an important parameter for the engineering of biological processes as well as for the comprehension of bacterial ecology. In literature only rough estimations of the K_S of anammox bacteria for nitrite were reported: 0.2–3 μM by van der Star et al. (2008) (with ''Kuenenia'') and <5 μM by Strous et al. (1999) (with ''Brocadia''). In this chapter we evaluated K_s by conventional methods (batch tests results and steady state condition) and a dedicated novel method for the accurate determination of this important kinetic parameter was developed. The K_S of free cells anammox bacteria for nitrite was estimated to be 2.5 μM. Furthermore the evidence of a direct link between biomass aggregate size and estimated K_S was shown and an exponential equation to estimate the apparent K_S as function of the aggregate diameter is proposed: the increasing mass transfer limitation associated with aggregates of bigger size led to the estimation of the apparent K_S (higher value than the intrinsic half-saturation constant). Finally the sampling method applied in this study (immediate stop of biological conversion by sudden temperature decrease) allow the accurate measurement of the actual nitrite concentration at steady state enhancing the reliability of the estimated value here reported: in presence of highly active biomass a small (few seconds) delay between the sampling and the stop of the nitrite uptake (i.e., by physical solid/liquid separation by filtration) would lead to K_S underestimation. In (aerated) nitrite-limited systems (such as CANON, OLAND, deammonification), nitrite oxidizing bacteria compete with anammox bacteria for nitrite. The estimated K_S values for nitrite of NOBs vary considerably both between different studies and between different species (12–955 μM, Both et al., 1992; Hunik et al., 1993; Schramm et al., 1999).

In (aerated) nitrite-limited systems NOBs reside in the outer aerobic layer of the biofilm while anammox bacteria occupy the anoxic core.

Due to the required diffusive transport of nitrite through the biofilm from the aerobic to the anoxic region NOBs will observe somewhat higher nitrite concentrations than anammox cells. In the competition for nitrite this can be compensated by a lower (intrinsic) K_S value for anammox bacteria.

This suggests that in aerated nitrite-limited systems the NOB growth can be effectively hindered in two different ways: the competition for nitrite between anammox and NOB, and the competition between AOB and NOB for oxygen. Therefore the maximum level of oxygen under which the system has to be operated can be increased. Given the difficulties of operating a strictly oxygen-limited system in which the oxygen load has to be carefully balanced with influent loading rate, operation with wider margins would be advantageous for process control. A higher affinity for nitrite is a major competitive advantage also in natural systems and thus might be responsible for the relatively large abundance of anammox bacteria in marine systems (Schmid et al., 2007). In this Chapter the K_S for nitrite of anammox bacteria was shown to be lower than the one reported for denitrifiers (4-25 μM, Almeida et al., 1995; Betlach and Tiedje, 1981). Therefore in natural systems anammox bacteria might be able to successfully compete for nitrite also with denitrifying microorganisms.

Nitric oxide and nitrous oxide

Nitric oxide and nitrous oxide emissions from full-scale anammox reactor of 0.003% and 0.6%, respectively (Kampschreur et al., 2008), as well as from full-scale single-stage nitritation-anammox reactor of 0.005 and 1.2%, respectively (Kampschreur et al., 2009, Weissenbacher et al., 2010) have been reported. From lab-scale reactors the range of emissions reported is wider: 0.00025-0.0005% (Strous et al., 1998) and 0.1% (van de Graaf et al., 1997) for NO and 0.03-0.06% (Strous et al., 1998), <0.1% (van de Graaf et al., 1997, Wyffels et al., 2004), <0.01% (Kampschreur et al., 2008). On the other hand batch experiments with physically purified anammox cells did not show any N_2O turnover (Kartal et al., 2007) and has been therefore hypothesized that the low amount of N_2O formed might have been originated by other community members (Kampschreur et al., 2008). Recently Kartal et al. (2010) reported NO conversion coupled to ammonium oxidation and minor (0.6 ppm) N_2O emission from a lab-scale SBR enrichment consisting of about 80% "*Candidatus* Brocadia Fulgida" (Kartal et al., 2007). In this chapter a not previously reported high purity anammox culture presenting 97% 16S rRNA sequence similarity with *Candidatus* Brocadia Fulgida (Kartal et al., 2007) was shown to emit nitric and nitrous oxide at a production rate accounting for the 0.003±0.002 and 0.056±0.02% of the imposed nitrogen load, respectively.

Stoichiometry of anammox process

Since Strous and coworkers (1998) reported for the first time the stoichiometry of the process carried out by anammox bacteria, every scientific study applied that equation in their calculations. As stated by the authors themselves, the stoichiometry was obtained by mass balancing on about 200 days of experimental data and the experimentally estimated 90% retention of growing biomass; the carbon balance was furthermore affected by 50% uncertainty on volatile solids measurements. The correct knowledge of the macro-chemical reaction equation of a biological process is crucial both for scientific research and for every biotechnological application. In this Chapter the availability of a high purity anammox culture actively growing in a controlled system (MBR) enabled for an accurate identification of the anammox macro-chemical reaction equation. In a metabolic system that catalyzes one dominant catabolic and anabolic reaction few information are needed for system identification: the yield of biomass on substrate, the elemental biomass composition and the compounds used as carbon and nitrogen source in the anabolic reaction. In this Chapter the yield of biomass on ammonium ($Y_{X/NH4}$) has been identified to be 0.071 C-mol N-mol^{-1}. The yield was calculated to be about constant during an experiment in which the biomass specific nitrite uptake rate (nitrite as the limiting substrate) increased from 39 to 83% of the maximum biomass specific nitrite uptake rate, indicating that the yield value found was a reliable approximation of the maximum yield. The elemental biomass composition of the enriched high purity anammox culture was identified to be $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.09}$ (biomass molecular weight of 22.14 g C-mol^{-1}). While the strictly autotrophic nature of anammox bacteria (inorganic carbon, IC, as C-source) has been specifically investigated, (Strous et al., 1998, Güven et al. 2005, Kartal et al., 2006), the nitrogen-source utilized is to date unknown. Given the yield and the biomass composition identified in this Chapter and inorganic carbon as C-source, the stoichiometry was calculated both for ammonium and for nitrate as N-source. The R_NiAm and R_NaAm resulted equal to 1.146 and 0.161

with NH_4^+ and 1.218 and 0.203 with NO_3^- as N-source, respectively. Considering the results reported in this Chapter from the long-term reactor operation (days 300-612, 1.214 and 0.209, respectively) and the *dynamic load increase experiment*, where yield and nitrite half-saturation constant were identified (1.205 and 0.200, respectively), we propose nitrate as the actual N-source. The macro-chemical reaction equation of anammox bacteria was therefore identified to be:

(3.13)

$$1NH_4^+ + 1{,}218NO_2^- + 0{,}071HCO_3^- + 0{,}085H^+ \rightarrow$$
$$1N_2 + 0{,}203NO_3^- + 0{,}071CH_{1{,}74}O_{0{,}31}N_{0{,}20}S_{0{,}01}P_{0{,}009} + 2{,}016H_2O$$

The higher values found by Strous et al. (1998) and confirmed in many reports might be due to the (combined effect) of other community member, dissimilatory nitrate reduction to ammonium (DNRA), or to higher extra-cellular polymeric substances (EPS) production rate. Recent results show that anammox bacteria do use nitrate directly and even outcompete heterotrophs for organic acids in presence ammonium (Kartal et al., 2006). In systems with high or even not controlled SRT the decay rate of the bacterial community would provide the electron donor production rate for DNRA. The higher EPS production associated with growth in biofilm, corresponds to higher need of reducing equivalent (provided by nitrite oxidation to nitrate) to fix IC to such organic compounds leading therefore to overestimation of R_NiAm and R_NaAm.

Biomass specific growth rate

In the reactor study presented in this Chapter, with the continuous cultivation at an SRT of 12 days, anammox bacteria were shown to grow at an actual growth rate of 0.083 d^{-1} for more than 580 days (doubling time, $t_d = \ln(2) \cdot SRT = 8.3$ days). Typical doubling times in anammox reactors are 15-30 days (Strous et al., 1998; Fux et al., 2004). Furthermore, in the system used the produced biomass was efficiently harvested since it was obtained separate from the effluent (which was removed via a membrane). Taking into account the maximum conversion capacity of the reactor, the maximum specific growth rate of anammox bacteria was estimated to be 0.21 d^{-1} (t_d = 3.3 days). Tsushima et al. (2007), during the exponential growth phase in shake flasks at 37°C, estimated an anammox bacteria doubling time of 3.6-5.4 days by quantitative polymerase chain reaction (qPCR). In view of the maximum conversion capacity of the reactor (at 38°C), van der Star et al. (2008) estimated a t_d of 5.5-7.5 days and an even lower t_d of 3 days was indicated as possible, after a one-time microscopic observation based on qualitative population estimations. The fastest doubling time (1.8 days) was reported by Isaka et al. (2006), based on the comparison of the number of anammox cells of two different reactors inoculated at the same time under similar conditions and operated at 37°C. From the comparison of the cell numbers at two different point sin time (each measurement in one of those two different reactors) the growth rate was calculated. The validity used by Isaka et al. (2006) is highly questionable and the obtained results are therefore doubtful. The surprisingly fast maximum specific growth rate reported in this Chapter for the first time (in scientific meaningful circumstances) might be due to the growth as free cells (thanks to the strict anaerobiosis maintained in the reactor). The lower production of EPS associated to the growth as free cells, might have resulted in a higher yield of biomass (as active bacteria) on substrate, due to the energy saved in the highly energy-consuming autotrophic carbon fixation for EPS production. Moreover the complete anaerobiosis might avoid any inhibition due to the presence of oxygen. It should be noted that the maximum specific growth rate reported in this chapter was observed at lower temperature (30°C) than those comparable maximum specific growth rates reported in literature (37-38°C).

3.5 CONCLUSIONS

Proper bioprocess design requires detailed kinetic information on the microorganism utilized and the stoichiometry of the process they catalyze. In this chapter an in-depth study of the anammox physiology and kinetics has been carried out. Precondition for accurate and reliable results is the availability of an almost pure and highly active suspended anammox cells culture. The method for growing anammox bacteria as free-cells in high purity was here shown univocally for the first time. Oxygen was found to be the key factor,

forcing anammox bacteria in a membrane bioreactor to aggregate already at a load of 0.7 μmol O_2 L^{-1} d^{-1}. The nitrite half saturation constant for anammox bacteria as free-cells was identified to be equal to 35 μg N L^{-1} with a novel developed method. Nitric oxide and nitrous oxide were shown to be produced during the anammox process accounting for the 0.003 and 0.056% of the imposed nitrogen load respectively. The yield of biomass production on ammonium uptake was calculated to be 0.071 C-mol N-mol^{-1}, while the elemental biomass composition was measured as $CH_{1.74}O_{0.31}N_{0.20}S_{0.01}P_{0.09}$ (22.14 g C-mol^{-1}). From the yield and the elemental biomass composition the macro-chemical reaction equation was identified and validated by long-term reactor operations and dedicated experiment. The anammox culture enriched during this study exhibited an unreported high biomass specific maximum growth rate of 0.21 d^{-1} corresponding to a doubling time of 3.3 days. Further research is however needed in order to confirm the anammox bacteria capability to actively grow at this surprising rate. The MBR system presented in this Chapter represents a powerful tool for both scientific (large availability of almost pure anammox culture) and applicative (faster reactor start-up) purposes.

CHAPTER 4

The effect of nitrite inhibition on the anammox process

Part of this Chapter has been submitted for publication as **Lotti T.**, Van der Star W.R.L., Kleerebezem R., Lubello C., van Loosdrecht M.C.M. **The effect of nitrite inhibition on the anammox process**

Outline

The negative effect of nitrite on anammox activity has been reported widely during the past decade. Although the adverse effect is clear, conflicting reports exist on the level at which it occurs and its reversible/irreversible nature. An in-depth study on nitrite inhibition therefore was performed in which the influence of environmental factors was evaluated. Anammox activity was measured in anammox granules by continuously monitored standardized manometric batch tests extending the interpretation by evaluation of lag times, maximum conversion rates during the tests and substrates/product conversion ratios. The granules were obtained from a one-stage anammox reactor, the dominant anammox organisms belonged to the *Brocadia* type. Direct inhibition levels for nitrite 0.4 g N L^{-1} (IC_{50}) were found, activity recovered fully after removal of the exposure. Conversion in fresh medium after exposure to up to 6 g N L^{-1} nitrite for 24 hours did result in loss of conversion of less than 60%. Presence of ammonium during nitrite exposure resulted in a stronger loss of activity afterwards (50% and 30% at 2 g N L^{-1} nitrite in presence and absence of ammonium respectively), while the presence of oxygen led to a maximum activity reduction of 32% at 2 g N L^{-1} nitrite. The recovery after exposure indicates that the adverse effect of nitrite is reversible and thus inhibitory rather than toxic in nature. Similarities between exposure at three different pH values indicate that nitrite rather than nitrous acid is the actual inhibiting compound.

4.1 INTRODUCTION

The anaerobic ammonium oxidation (anammox) process represents a cost-effective nitrogen removal process for treatment of ammonium-rich wastewater (Fux and Siegrist 2004, Van Dongen et al., 2001), getting rapidly introduced in practice worldwide (van der Star et al., 2007). The responsible microorganisms grow on ammonium with nitrite as electron acceptor resulting in production of dinitrogen gas. The anaerobic and autotrophic nature of these organisms permits significant savings on aeration and does not require organic carbon addition, and generates low excess biomass. The bacteria performing the anammox process form the order “Brocadiales” within the phylum *Planctomycetales* (Jetten et al., 2010) of which the two “candidatus” genera *Brocadia* and *Kuenenia* are the most relevant for wastewater treatment.

One of the most critical aspects in the anammox process stability is nitrite, since it is the electron acceptor in the process and converted by anammox bacteria, but also a potential inhibiting compound. This inhibition is however not consistently reported. It has been reported that nitrite concentrations of 5 and 40 mg N L^{-1} are already severely inhibitive (Wett (2007) and Fux (2003), respectively). Strous (1999), who first reported the adverse effect of nitrite, found a complete but reversible, inhibition of the process at 100 mg N L^{-1}. Other authors reported similar concentrations as detrimental for the anammox process, but indicating the nitrite inhibition either as reversible or irreversible (Cho et al., 2010, Fernàndez et al., 2010, Jetten et al., 2005, Lòpez et al., 2008, Van Dongen et al., 2001). Several researchers report a high tolerance of the anammox bacteria to nitrite (Cho et al., 2010, Dapena-Mora et al., 2007, Egli et al., 2001, Fernàndez et al., 2010) with the highest reported non-inhibitory value reported by (Kimura et al., 2010) (toxicity threshold higher than 300 mg N L^{-1}).

The wide range of observations regarding nitrite which was observed makes it difficult to predict, model or design anammox-based technologies. The non-consistency of the results reported in literature could be due to the different aggregation status of the biomass (biofilm, floc, suspended) and/or to the different anammox taxon used. The authors therefore made an in-depth evaluation of the conditions at which nitrite toxicity occurs (time, presence of oxygen/ammonium), and whether those conditions influenced the severity of the effect. Particular emphasis is laid on the recovery of anammox bacteria after exposure. We differentiate between inhibition, defined as a phenomenon which is reversible and depending on the time of exposure and the concentration of inhibiting compound, and toxicity, that is defined as the irreversible process of activity loss depending on the time of exposure and the concentration of the toxicant.

The use of standardized manometric batch tests was introduced into the anammox toxicity research field by Dapena-Mora (2007) and has proven very effective. We used it (modifying the method to increase accuracy and reliability) therefore as a reproducible platform for our research and extended the interpretation by evaluation of lag times, maximum conversion rates during the tests and substrates/products consumption/ production ratios.

4.2 MATERIALS AND METHODS

4.2.1 Manometric test equipment

The assays were performed in closed bottles equipped with manometric sensors including a data storage system for 360 datapoints (OxiTop Control AN6, WTW, Weilheim, Germany). The system was used previously for evaluation of anammox activity (Scaglione et al., 2009). The manometric devices consisted of 340 mL vials provided with a measuring head with a pressure transducer (sensitivity level 1 hPa). Each vial had two lateral holes closed with a puncturable rubber septum for substrate injections and sampling.

4.2.2 Origin of the biomass

The biomass used originates from the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant (van der Star et al., 2007). The reactor contains granular anammox sludge and treats reject water after partial nitritation in a SHARON reactor. During the experimental period, the anammox reactor was operated at the design volumetric load of 7.1 kg N m^{-3} d^{-1} (van der Star et al., 2007). During 2010 the average reactor conditions where: temperature 34±2.5 ºC, pH 7.2±0.4 and concentrations of nitrogen in effluent were 50±20 mg NH_4^+-N L^{-1}, 15±15 mg NO_2^--N L^{-1}, 95±20 mg NO_3^--N L^{-1}. The biomass was confirmed to consist of a "*Brocadia*" enrichment during the period of the tests by *fluorescence in situ hybridization* (FISH), the sludge hybridized with Amx-820 and not with Kst-157 probes (Schmid et al., 2001).

4.2.3 General procedure for manometric tests

After sampling, the granular sludge was brought under non-aerated conditions to the laboratory (30 minutes travel time) and directly used for the tests. The biomass was washed and re-suspended in a *washing medium*: a medium containing the microelements needed to avoid nutrient limitation (Van De Graaf et al., 1996) as well as 25 mM HEPES buffer (HEPES stands for N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid). The pH value of the medium was set to 7.5 with 0.1 M NaOH or H2SO4. After this, the headspace and liquid phase (200 mL) were sparged with nitrogen gas to obtain anoxic conditions. The bottles were placed in a thermostatic shaker, at 170 rpm and 30 °C until the headspace pressure (rising as a result of the temperature change) had stabilized. Then overpressure was released (by inserting a needle connected to a water-filled vessel to act as a water-lock) and substrates were injected. The *injected solutions* contained $NaNO_2$, $(NH_4)_2SO_4$ and NH_4HCO_3 dissolved in milli-Q water. The initial concentration of ammonium and nitrite was 50 mg N L^{-1} unless mentioned otherwise. To avoid inorganic carbon limitation the initial bicarbonate concentration was set to 32.7 mg L^{-1} while taking into account that part of the inorganic carbon partitioned to the headspace during equilibration. The pressure increase caused by the nitrogen gas production and accumulation in the headspace was automatically measured and recorded during the entire test for subsequent processing. Once the pressure reached a constant value (and all nitrite was assumed to be converted), a liquid sample was taken for chemical analysis (pH, ammonium, nitrite and nitrate).

4.2.4 Preliminary tests

To assess the accuracy and reliability of the method for measurement of the maximum specific anammox activity (MSAA) a set of preliminary assays was performed in duplicate according to *par.4.2.3*. The same test runs were performed with different batches of biomass during the entire experimental period to exclude any effect on changes in biomass composition in the full scale reactor.

— Ammonium nitrite level: Starting concentrations of 40, 50, 60, 70 and 80 mg N/L ammonium nitrite were tested. The initial biomass concentration was 1.0 g VSS L^{-1}. The test was also used to evaluate the nitrogen balance for each experiment.
— Biomass level: Varying levels of biomass (0.2, 0.5, 1.0, 1.5, 2.0, 3.0, 4.0 g VSS/L) were

used for tests with 40 mg NH_4^+-N L^{-1} and 40 mg NO_2^--N L^{-1}as well as 60 mg NH_4^+-N $L^{-1}$and 60 mg NO_2^--N L^{-1}.

— Effect of buffer solution: Biomass was suspended in 5.3 mM phosphate buffer (Dapena-Mora et al., 2007), HEPES (25 mM) buffer or non-buffered medium (the rest of the medium composition was standard as described above). The experiments were performed in triplicate.

4.2.5 ACTIVITY AFTER NITRITE EXPOSURE TESTS

When exposure to a (potentially) inhibitory compound was tested, the procedure described in *par.4.2.3* was preceded by exposure to (ammonium) nitrite (as $NaNO_2$) or to ammonium (as $(NH_4)_2SO_4$) and nitrite for 1 or 24 hours and subsequent washing (Fig. 1, *protocol A*). When toxicity to ammonium nitrite was tested, ammonium and nitrite had equimolar nitrogen concentrations in the exposure medium. Sodium nitrate was added to the exposure medium of a control assay (unexposed biomass) at a concentration of 70 mg NO_3^--N $L^{-1}$to avoid sulphate reduction.

The protocol used in these tests (*protocol A*) is illustrated in Fig. 4.1 and consisted of the following stages:

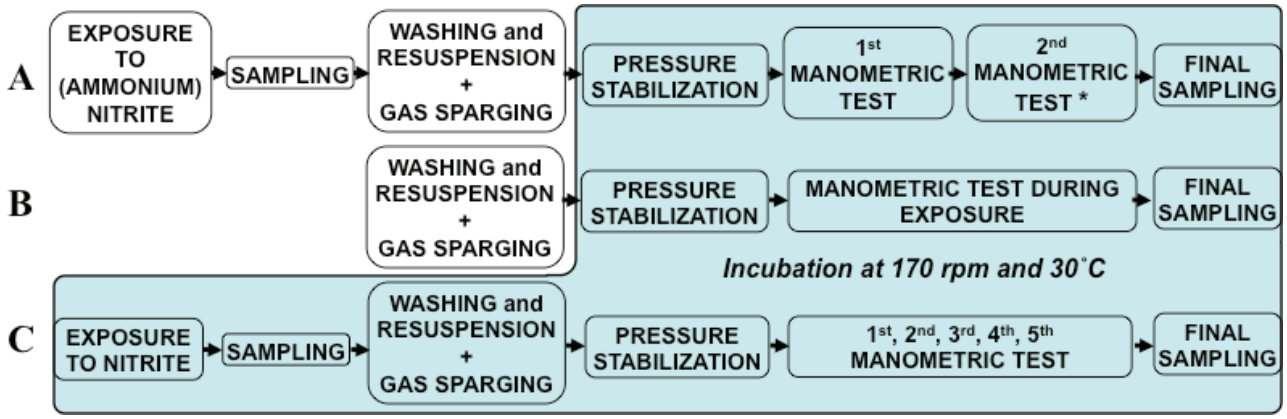
i) Anoxic exposure to various concentrations toxicant for 1, 2 or 24 hours
ii) sampling for determination of pH and measurement of ammonium, nitrite and nitrate concentrations, followed by
iii) washing and resuspension of the biomass in *washing medium*;
iv) sparging to obtain anoxic conditions and transferring the vessels to a thermostatic shaker (170 rpm and 30 °C);
v) waiting for ca 60 min to allow for pressure stabilization followed by removal of overpressure by insertion of a needle connected to a neoprene tube immersed in water;
vi) dosage by injection of a concentrated solution of ammonium, nitrite and inorganic carbon (procedure: see previous paragraph);
vii) second dosage of substrates (only performed when explicitly stated; procedure: see previous paragraph);
viii) sampling for chemical analysis.

4.2.6 NITRITE INHIBITION TESTS

The inhibition of nitrite on anammox activity was tested by *protocol B* (protocol adapted from *protocol A*, Fig. 4.1).

Biomass was prepared in a *washing medium* (see previous paragraph), which also contained 85 mg N L^{-1} ammonium (as NH_4HCO_3). After pressure equilibration, pressure was reduced, 0.5-4 mL nitrite solution was added to achieve the desired starting concentration (Table 4.1) and the manometric test was started. The control assays consisted of manometric tests with 50 mg N L^{-1} ammonium and 50 mg N L^{-1} nitrite.

FIG. 4.1
Adopted experimental procedures: *protocol A*: activity after exposure tests; *protocol B*: nitrite inhibition test; *protocol C*: long term activity after exposure. * Only when explicitly stated, the second manometric test was performed.



4.2.7
Long term effect after exposure

To evaluate the effect of successive injections of non-toxic levels of ammonium nitrite after nitrite exposure, tests were performed using an extension of *protocol A* (*protocol C*) at 24 h of exposure to 1000 mg N L^{-1} nitrite. The control assay was performed by two standard manometric tests without toxicity exposure. The assay was conducted in four replicates. In addition, manometric measurements took place also during the exposure phase (24-hour long manometric test; called *Exposure phase* in *par 4.3.4*). The *suspension medium* also contained 0.1 g N L^{-1} $(NH_4)HCO_3$. A concentrated nitrite solution (prepared dissolving $NaNO_2$ in mill-Q water) was injected in the vessels in order to obtain 1 g N L^{-1} as initial nitrite concentration. After washing, the biomass was suspended in the *suspension medium*, degassed in order to remove oxygen and five successive feedings (called batch *1-5* in *par 4.3.4*) were injected (initial ammonium nitrite concentrations of up to 50 mg N L^{-1}). The first manometric test after washing was conducted 2 hours after the toxicity exposure. Subsequent manometric tests were performed after 8, 23, 27 and 94 hours respectively after the 24 hours toxicity exposure.

4.2.8
Nitrite inhibition test conditions

Inhibition-related tests were either focused on the immediate effect of nitrite (as $NaNO_2$) or on exposure to ammonium (as $(NH_4)_2SO_4$) and nitrite (as $NaNO_2$), or the effect after exposure for a designated time as described above. An overview of the test conditions is shown in Table 4.1.

4.2.9
CALCULATIONS

4.2.9.1
Accuracy of the method

The total amount of dinitrogen gas produced during the test was calculated from the overpressure recorded by the transducer in the gas phase at the end of the assay by using the ideal gas low equation. The absolute change in CO_2 partial pressure due to carbon fixation is less than 4% of the change in N_2 partial pressure. It was therefore neglected in the evaluation. The amount of substrates consumed and product produced was calculated by measuring the concentrations of ammonium, nitrite and nitrate at the beginning and at the end of each essay. The relative error of the method was calculated based on the total nitrogen mass balance considering both liquid and gas phase.

4.2.9.2
Maximum specific anammox activity

From the recorded data of pressure increase in time (see Fig. 4.2, for a typical response; the data shown are relative to one of the *preliminary tests*), the N_2 increase in time was calculated (assuming ideal gas conditions which means for 30˚C a headspace volume equal to 140 mL, 1 hPa equals $5.55 \cdot 10^{-3}$ mmol N_2). A typical response of a manometric test, where nitrogen gas produced [mmol] was calculated through ideal gas law from recorded data [hPa], is shown in Figure 4.2. The nitrogen gas production rate was calculated through linear regression of a set of 20 datapoints (N_2 [mmol] in headspace versus time [min]) corresponding to a time interval of at least 10 minutes and therefore a curve describing the N_2 production rate in time was obtained. The curve describing the N_2 production rate in time typically presented an initial positive slope (reactivation phase, corresponding to phase I in Fig. 4.2), followed by a plateau corresponding to the highest values (corresponding to phase II in Fig. 4.2) and a final negative slope ending on the abscissa (substrate limiting phase, corresponding to phase III in Fig. 4.2). The average of the values on the plateau (identified by $d^2N_2/dt^2=0$) was considered as the maximum anammox activity and expressed as mmol N_2 min^{-1}. Dividing this value by the known amount of biomass present in the bottle at the beginning of the test, the maximum specific anammox activity was calculated and expressed as g N_2-N $(g\ VSS)^{-1}$ d^{-1}. The amount of biomass grown during each manometric test can be calculated by considering the complete consumption of the limiting substrate during the test (50 mg NO_2^--N L^{-1} for all tests, except 85 mg NH_4^+-N L^{-1} for the *immediate*

Table 4.1: Test conditions [a]

Test type	Protocol	Exposed chemical	Exposure concentrations [mg N L^{-1}]	Exposure time [h]	Biomass concentration [g VSS/L]	Number of standard manometric tests after exposure	Other conditions
Inhibition	B	NO_2^-	0, 100, 150, 200, 250, 300, 400, 500, 1000, 3000[b]	-	1.5 ÷ 2	0	presence of 85 mg NH_4^+-N L^{-1}
Activity after exposure	A	NO_2^-	0, 500, 1000, 2000, 6000	1, 24	1.5 ÷ 2	2	
Activity after exposure	C	NO_2^-	1000	24	1.5 ÷ 2	5[c]	presence of 100 mg NH_4^+-N L^{-1}
Activity after exposure	A	NO_2^- + NH_4^+	0, 250, 500, 1000, 2000	1, 24	1.5 ÷ 2	2	
Activity after exposure, Aerobic conditions	A	NO_2^- + NH_4^+	0, 250, 500, 1000, 2000	1, 24	1.5 ÷ 2	2	During toxicity DO (mg L^{-1}): 5.0[d]
pH effect	A	NO_2^-	0, 500, 1000	2	1.5 ÷ 2	1	pH = 6.8, 7.8[e]

[a] All assays were conducted as two replicates according to *protocol A* except when explicitly stated.

[b] For the manometric tests with nitrite starting concentrations of 1 and 3 g N L^{-1}, the activity decreased in time and no maximum could be found; MSAA was considered as the average activity 60 to 80 minutes after the injection.

[c] Assay conducted with four replicates

[d] During the exposure, the biomass was continuously sparged with a mixture of air and nitrogen gas in order to keep a dissolved oxygen concentration (DO) in the bulk of 5 mg O_2 L^{-1}. The air and the nitrogen gas flows were regulated by mass flow controllers at a total rate of ca 50 mL min^{-1}.

[e] The *exposure medium* pH was set to 6.8 and 7.8 respectively with 0.1 M $NaOH/H_2SO_4$, while pH during the manometric tests after exposure was 7.5 as usual. After the exposure and after the manometric test pH was measured to verify it had remained constant.

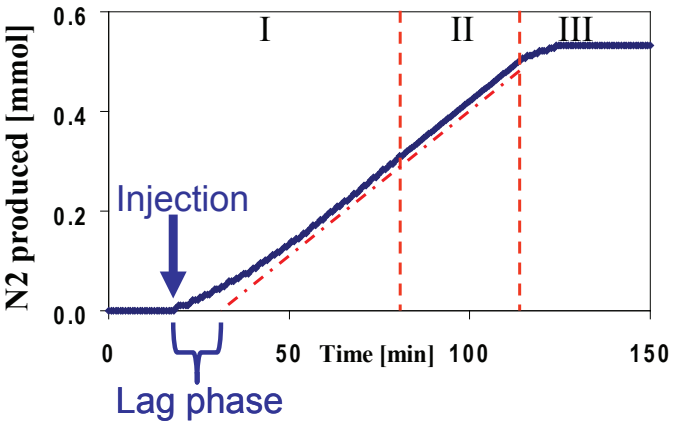


FIG. 4.2
Typical response of a manometric test where nitrogen gas produced [mmol] was calculated through ideal gas law from recorded data [hPa]. The data shown are derived from a *preliminary tests*. The dash dot line is a graphical representation of the calculation to determine the *lag phase*.

inhibition to nitrite test), the average starting biomass concentration (1.5 ÷ 2 g VSS L^{-1}) and the yield of biomass on the limiting substrate (0.09 g VSS g NO_2^--N^{-1} and 0.12 g VSS g NH_4^+-N^{-1} according to Strous et al., 1998). This value corresponds to less than 0.31-0.69% of the initial biomass concentration for *nitrite-limited* and *ammonium-limited* test respectively, indicating that the biomass concentration can be considered constant throughout the test.

4.2.9.3
Substrates/products molar ratio

The molar ratios of substrates (nitrite on ammonium converted) and of product (nitrate produced on ammonium converted) was calculated from N-compound concentrations in the liquid samples before and at the end of the test, according to the following relation:

(4.1)
$$R\_NiAm = \frac{\left[NO_2^-\right]_{START} - \left[NO_2^-\right]_{END}}{\left[NH_4^+\right]_{START} - \left[NH_4^+\right]_{END}}$$

(4.2)
$$R\_NaAm = -\frac{\left[NO_3^-\right]_{START} - \left[NO_3^-\right]_{END}}{\left[NH_4^+\right]_{START} - \left[NH_4^+\right]_{END}}$$

4.2.9.4
Lag phase

In order to quantify the delay between the substrates injection and the occurrence of the maximum specific anammox activity a parameter called *lag phase* was defined. The part of the curve of the nitrogen gas development in time at the moment the maximum specific anammox activity occurred was extrapolated till the abscissa and the *lag phase* was defined as the difference between the time relative to this intercept and the injection time (see Figure 4.2 for a graphical representation of the calculation).

4.2.9.5
Activity percentage

The percentage of activity maintained after the exposure to, or in presence of, inhibitory compounds was calculated with respect to the average of the activities of the control assays (unexposed biomass).

4.2.10
Analytical procedures

Soluble nitrogen compounds were measured via spectrophotometric flow injection analysis (QuickChem 8500 series 2 FIA System, Lachat Instruments, Loveland, Colorado, USA). The methods applied were QuikChem®Methods 10-107-06-5-E for ammonium (range 0.1 to 10.0 mg N L^{-1}, measurement of NH_3 after increasing pH and volatilization) and 10-107-04-1-C for nitrate/nitrite (range 0.01 to 2.0 mg N/L, direct measurement of nitrite, or measurement proceeded by reduction of NO_3^- to NO_2^- to yield the concentration of "NO_3^- +NO_2^-") according to the protocol of the manufacturer. The length of the sample loop of the nitrate/nitrite detection was increased in order to obtain a measurement range from 0.05±0.01 to 10±0.01 mg N L^{-1}. TSS and VSS were determined according to the Standard Methods (APHA 2005).

4.3
RESULTS

4.3.1
Accuracy of the method

A set of preliminary assays was performed in duplicate to assess the accuracy and reliability of the method to estimate the maximum specific anammox activity (MSAA). The tested initial concentrations of ammonium nitrite of 40 to 80 mg N L^{-1} (total nitrogen concentration

of 80 to 160 mg N L^{-1}) were chosen in order to be below the inhibition threshold indicated in literature (Dapena-Mora et al., 2007, Egli et al., 2001, Strous et al., 1999), but high enough to allow for complete reactivation of the biomass before the limited substrate (nitrite) gets depleted. 50 mg NH_4^+-N L^{-1} and 50 mg NO_2^--N L^{-1} (total nitrogen concentration of 100 mg N L^{-1}) were chosen as standard initial concentration for the manometric test. The variations in the MSAA-values identified in ten replicates proved to be less then 2%. The MSAA values were furthermore shown to be independent of the biomass concentration (0.2-4 g VSS L^{-1}) and showed a low (5%) variability. 2 g VSS L^{-1} was choosen as appropriate biomass concentration for further testing. Without a pH-buffer solution the pH increased during the tests to 7.9. Both the HEPES and phosphate buffer enabled a constant pH throughout the experiment. HEPES-buffer (25 mM) was selected as buffer solution in subsequent experiments. There was no indication that these buffers negatively influenced the anammox activity.

The accuracy of the measurements was checked by evaluating the nitrogen mass balance. The nitrogen balance had always less then 5% inaccuracy indicating that the pressure measurement was accurate. The observed *R_NiAm* ratio was 1.35 (±0.07) and the *R_NaAm* ratio was 0.28 (±0.06), indicating a normal growth of the anammox bacteria in the test. Repeated testing with the same biomass was performed. The experiments showed an increase from the first to the second test of 1.4 to 4% and an increase of 2-8% (compared to the first test) in the 3rd and the 4th tests. The second, third and fourth test gave almost the same results as the first test indicating there was no strong positive or negative effect of the test system.

4.3.2
Inhibition of Anammox conversion due to nitrite

Experiments in which MSAA was measured with varying nitrite concentrations (in the presence of 85 mg NH_4^+-N L^{-1}) were performed according to *protocol* B to evaluate the inhibitive effect of nitrite on the conversion process. MSAA decreased with increasing initial (50 and 500 mg NO_2^--N L^{-1}) concentrations (Fig. 4.3). The residual activity in the presence of 1000 and 3000 mg NO_2^--N L^{-1} was 7 and 3% respectively (Fig. 4.3). For inhibition of nitrite a half maximal inhibitory concentration (IC_{50}) of 400 mg N/L was determined.
At initial nitrite concentrations up to 500 mg N L^{-1} the length of the lag phase increased, with a maximum of 70 minutes. For initial nitrite concentrations until 500 mg NO_2^--N/L the *R_NiAm* and *R_NaAm* values (Fig. 4.4) were close to the standard stoichiometric ratios (Strous et al., 1998). At higher nitrite levels the *R_NiAm* ratio increased just as the *R_NaAm* levels 2.97 and 0.75, respectively, at 3000 mg NO_2^--N L^{-1}.

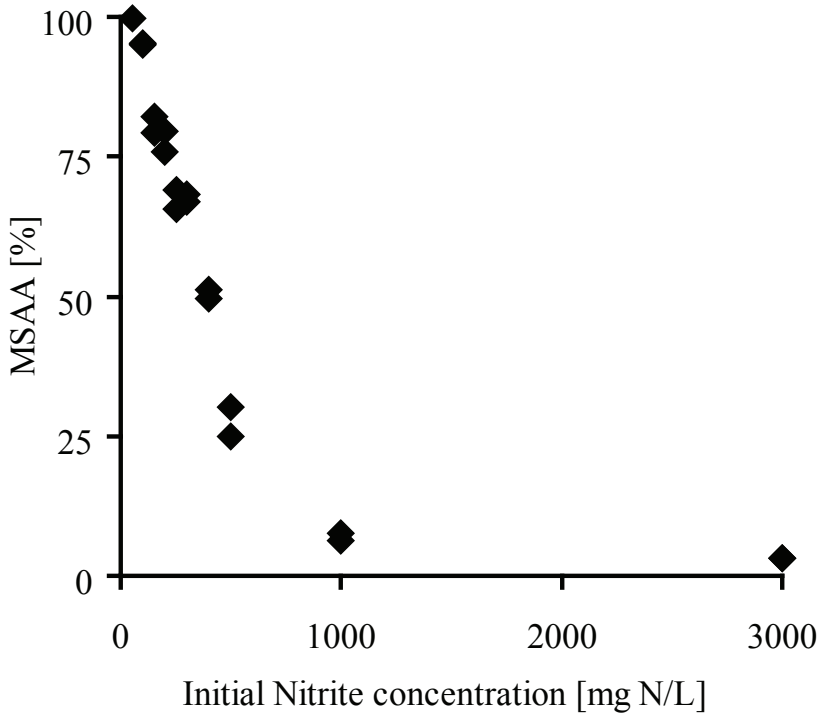


FIG. 4.3
Maximum specific anammox conversion rate (MSAA, as a % of conversion of non-exposed biomass).

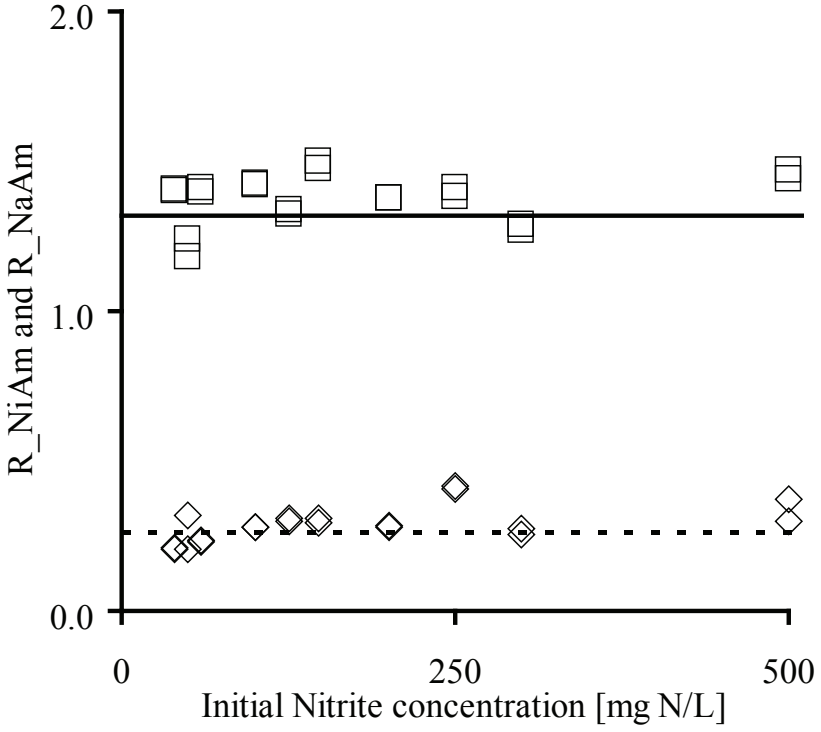


FIG. 4.4
R_NiAm **(squares) and** ***R_NaAm*** **(diamonds) during manometric activity tests at different initial nitrite concentrations; the continuous and dashed lines represent literature values for** ***R_NiAm*** **and** ***R_NaAm*** **for anammox bacteria (Strous et al., 1998).**

4.3.3 Exposure tests

Exposure tests were performed according to *protocol* A and consisted of activity tests under standard (non-toxic) conditions after pre-incubation at potentially toxic concentrations nitrite or ammonium nitrite. After incubation for 1, 2 or 24 hours with nitrite or ammonium nitrite, biomass was washed with washing medium to remove the (possibly) nitrogen compounds. Thereafter activity tests where performed.

4.3.3.1 Exposure to ammonium nitrite

Exposure to concentrations of 250 to 2000 mg N L^{-1} of ammonium nitrite (total nitrogen concentration of 500 to 4000 mg N L^{-1}) did lead to partial degradation of ammonium nitrite during the exposure phase. During 24 hours exposure to an initial concentration of 250 mg N L^{-1} ammonium nitrite, nitrite was fully removed. During the exposure to higher concentrations, the exposure time was not sufficient to convert all the nitrite present (less than 40%). Exposure for 1 hour did lead 15% ammonium nitrite conversion at the lowest concentration of 250 mg N L^{-1}.

The percentage of anammox activity that remained after 1 and 24 hours exposure to the tested substrate concentrations is shown in figure 5, 1A. Exposure to higher concentrations resulted in lower activity values. Concentrations up to 500 mg N/L ammonium nitrite had no significant effect on the activity for both exposure times. After 1 hour exposure at concentrations of 2 g N L^{-1}, the MSAA was reduced by 32%. Exposure for 24 hours to 1 and 2 g N L^{-1} ammonium nitrite resulted in a reduction of MSAA by 50%.

The length of the *lag phase* after the exposure to ammonium nitrite (Fig. 4.5, 2A) ranged from 4 to 12 minutes for concentrations up to 500 mg N/L for both the exposure durations. For higher exposure concentrations, the effect of the exposure duration and concentration increased strongly to 77 minutes after exposure of 24 hours at 2 g N L^{-1}. Lag times after exposure of 1 hour never exceeded 24 minutes.

R_NiAm and *R_NaAm* increased after exposure to higher ammonium nitrite concentrations and also increased upon longer exposure (Fig. 4.5, 3A). Although the nitrate production levels were stable, ammonium removal levels decreased when the biomass had been exposed to higher ammonium nitrite levels (Fig. 4.5, 3A).

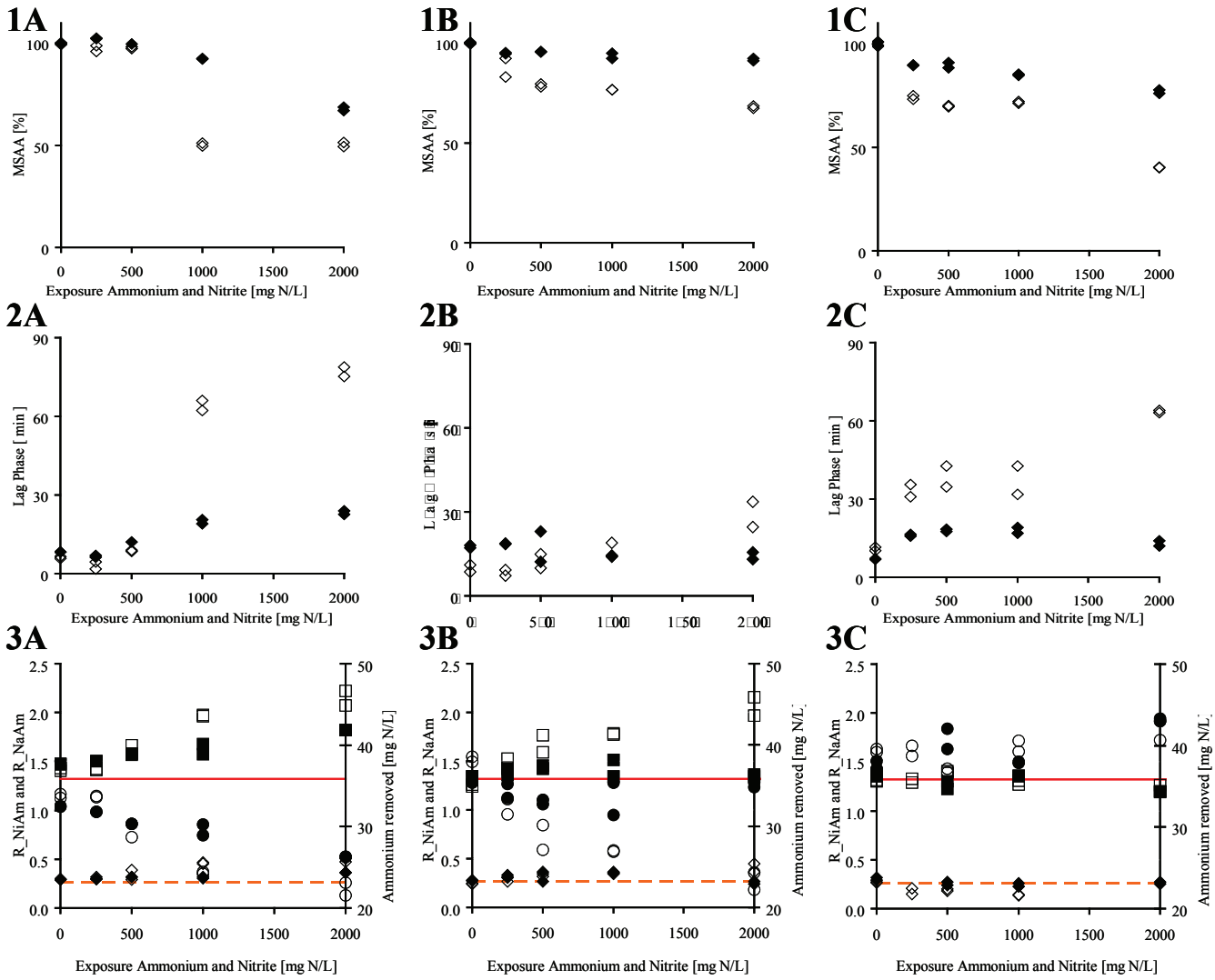


FIG. 4.5

Anammox activity assays after exposure for 1h (closed symbols) and 24h (open symbols) to different concentrations of NH_4NO_2 in anoxic conditions (column A), NH_4NO_2 in aerobic conditions (column B), $NaNO_2$ in anoxic conditions (column C). The concentration on the x-axis corresponds to the nitrite concentration during exposure (mg N/L). Row 1 indicates the maximum specific anammox conversion rate (MSAA, as a % of conversion of non-exposed biomass). Row 2 indicates the length of the *lag phase* (minutes) after exposure. Row 3 indicates: the conversion ratios *R_NiAm* (equation 1, *left y-axis,* ■,□) and *R_NaAm* (equation 2, *left y-axis,* ♦,◊); ammonium removed during standard manometric batch test after exposure (mg N/L, *right y-axis*, ●,○); the continuous and dashed lines represent standard values for *R_NiAm* (1.32) and *R_NaAm* (0.26) (Strous et al., 1998).

4.3.3.1.1
Exposure to ammonium nitrite in an aerobic environment

To assess whether the presence of oxygen has an extra effect on the exposure to ammonium nitrite, activity tests (under anoxic conditions) were conducted after exposure in the presence of oxygen. Preliminary tests in which biomass was exposed to 50 mg NH_4^+-N L^{-1}, 50 mg NO_2^--N L^{-1} and 8 mg O_2 L^{-1} for 24 hours showed no activity loss and *lag phase* values less than 30 minutes during the standard anoxic activity test conducted afterwards (data not shown). Exposure to oxygen inhibited removal of ammonium nitrite during the exposure phase completely. Subsequent standard anoxic activity tests resulted in a reduction of less than 10% in MSAA after 1 hour exposure (Fig. 4.5, 1B). Exposure for 24 hours resulted in higher losses in MSAA after exposure at 1 g N L^{-1} (24%) and 2 g N L^{-1} (32%) ammonium nitrite. The lag phase increased with increasing exposure time but less compared to exposure under anoxic conditions with a maximum lag time of 33 minutes after exposure for 24 hours at 2 g-N L^{-1} ammonium nitrite (Fig. 4.5, 2B). Also after exposure in the presence of oxygen, nitrate production was ca 9.9 mg N L^{-1} (as expected after the conversion of 50 mg N L^{-1} nitrite), but ammonium conversion levels were less than expected (83% after exposure for 1 hour to 2 g-N L^{-1} and 60% after exposure for 24 hour to 2 g N L^{-1}) causing increasing discrepancies with the standard stoichiometric ratios (Fig. 4.5, 3B). During a second test conducted after exposure (both for 1 and 24 hours) the ammonium conversion levels markedly increased (at least 88% of the expected conversion) coming closer to the theoretical biochemical reaction.

4.3.3.2
Exposure to nitrite only

The activity after exposure for 1 or 24 hours with nitrite (in the absence of ammonium) is shown in figure 4.5, 1C. No significant nitrite conversion took place during exposure. At 1 g NO_2^--N L^{-1} the nitrate produced was less than 4 mg N/L, which was similar to a biomass-free control (results not shown). After 1 hour exposure the negative effect of nitrite was limited with a maximum activity reduction of 22% after exposure at 6 g N/L. Exposure for 24 hours resulted in higher losses in MSAA. The effect of 24 hours exposure to 2 g N L^{-1} was similar to exposure to 1 g N L^{-1}. The activity reduction caused by exposure to 6 g N L^{-1} was 60%. A second injection of nitrite and ammonium after exposure resulted in an increase in activity of 6 to 23% (Fig. 4.6) compared to the previous test. The increase was higher at increasing exposure concentration and exposure time for exposure concentrations until 2 g N L^{-1}.

The lag phase in general didn't increase due to nitrite exposure, except for the long term high nitrite concentrations. A second test with this sludge showed a normal lag time again, indicating recovery of the biomass (Fig. 4.5, 2C).

Nitrate production and ammonium consumption in activity tests after exposure to nitrite were 5 to 11 mg N L^{-1} and 37 to 43 mg N L^{-1} respectively, giving a *R_NaAm* ratio between 0.14 and 0.31 (Fig. 4.5, 3C). A decrease in removed ammonium at increasing exposure concentrations was not observed (Fig. 4.5, 3C).

4.3.3.2.1
Effect of pH on exposure to nitrite only

The effect of pH on incubation with nitrite was evaluated by tests at pH 6.8 and 7.8 during two hours nitrite exposure. Under these conditions the MSAA was reduced by 18 and 19% respectively (Fig. 4.7) comparable to the incubation at pH 7.5 (Fig. 4.5, 1C). No significant difference in MSAA during standard tests after exposure for two hour at pH 6.8 and pH 7.8 was observed. As with tests at pH 7.5 (see *par.4.3.3.2*), no nitrite was removed in absence of ammonium during the exposure. Often nitrous acid is indicated as the compound leading to toxicity (Anthonisen et al., 1976). In our tests we cannot see that the nitrous acid concentration is the relevant compound; i.e. the 10 times increasing nitrous acid concentration at pH 6.8 compared to 7.8 at equal total nitrite concentration did not affect the inhibition observed. It rather seems to be the total nitrite concentration which is determining the extent of inhibition.

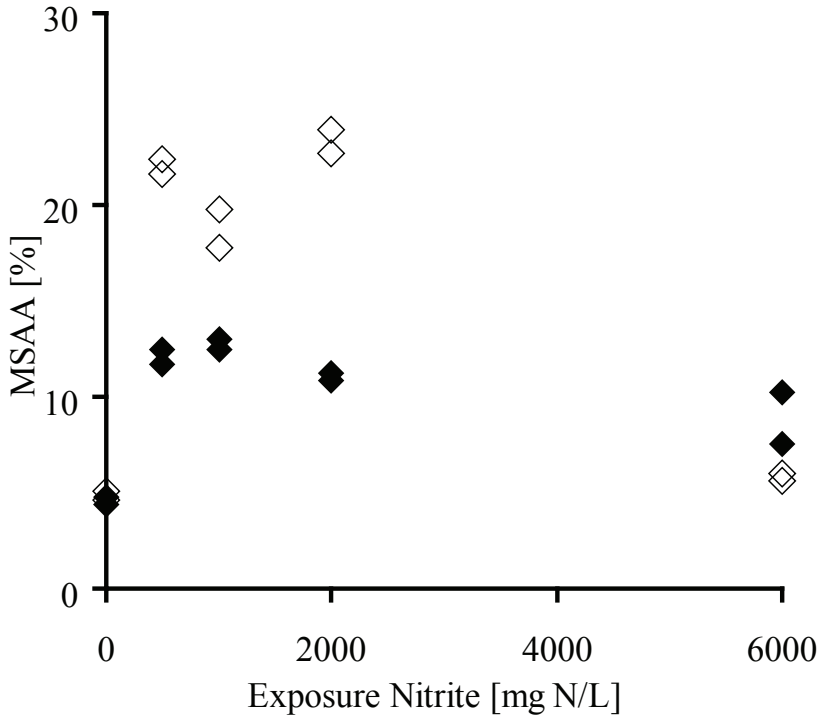


FIG. 4.6
Activity increase in the second manometric test with respect to the first manometric test after exposure for 1h (♦) and 24h (◊) to different concentrations of nitrite.

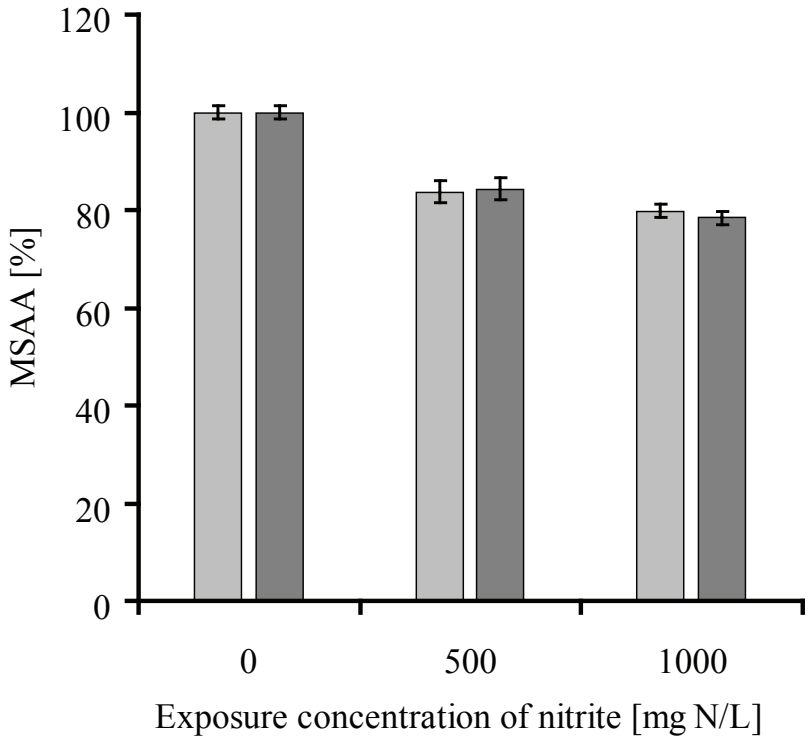


FIG. 4.7
Maximum specific anammox activity (as a % of conversion of non-exposed biomass) after two hours exposure to nitrite at pH 6.8 (light grey) and 7.8 (dark grey). Error bars represent standard deviation.

4.3.4
Long term recovery of AMX activity after nitrite exposure

The reversibility of nitrite inhibition was evaluated after exposure of anammox biomass to 1000 mg NO_2^--N L^{-1} for 24 hours. The anammox activity was followed during this incubation. Biomass was then washed and five successive manometric tests (batch *1-5* tests) were conducted with initial ammonium and nitrite concentration of 50 mg N L^{-1} during the following 98 hours.

Incubation at 1000 mg NO_2^--N L^{-1} resulted in a progressive loss of activity. After 2 hours the activity loss was 93-94% with respect to the control assay. After washing the sludge to remove nitrite activity was regained with a lag phase of ca 20 minutes, while in the successive tests with the same sludge the lag phase was less than 8 minutes. The maximum specific activity increased during the first three consecutive tests conducted after washing of the biomass, while there was negligible difference between the 3rd, 4th and 5th test (Fig. 4.8 and Tab. 4.2). The nitrate to ammonium ratio during these tests remained in the normal range.

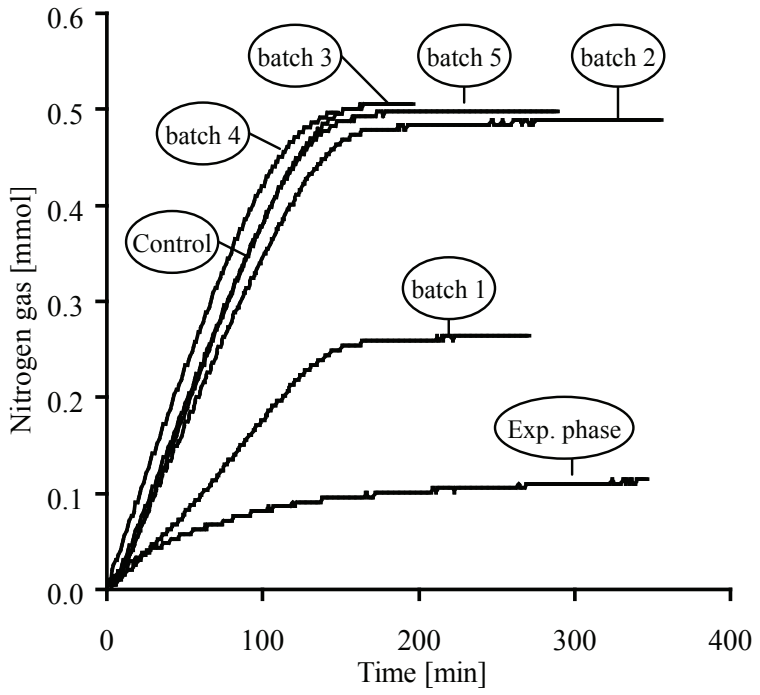


FIG. 4.8
Nitrogen gas production in time during the whole manometric tests. Labels indicate the abbreviation of the corresponding manometric test. Only the first 350 minutes of the 24 hours *Exposure phase* test are shown.

Table 4.2: Manometric test duration, timing and MSAA for the 24 hours exposure to 1 g-N L^{-1} nitrite, successive manometric tests conducted after biomass washing and relative control.

Manometric test	Manometric test duration	MSAA
	[h]	[g N_2-N (g VSS)$^{-1}$ d^{-1}]
Control assay	2	0.458
Exposure phase	24	0.061[a]
batch 1	7	0.209
batch 2	11.5	0.390
batch 3	4	0.430
batch 4	4	0.454
batch 5	4	0.439

[a] This is not the MSAA but the SAA calculated 1h after the injection

4.4 DISCUSSION

1) Suitability and accuracy of the tests

The manometric batch tests used in this work are adaptions of manometric batch tests first developed as standardized tests for biological oxygen demand (BOD) determination and were already successfully adapted to nitrogen production for evaluation of the anammox process by Dapena-Mora et al. (2007) and Caffaz et al. (2008). The approach used in the present work furthermore prevented the possible effect of inorganic carbon limitation (Kimura et al., 2011, Lotti et al., 2009) and pH fluctuation was avoided with a 25 mM HEPES buffer. Furthermore the method applied avoided any disturbances of the process during the batch measurement of the activity. Disturbances that may occur include volume and/or temperature fluctuations due to manual sampling in the case of tracking the substrates concentration or the nitrogen gas production. Together with the high measurement frequency (one measurement per minute), the experimental setup allows for detailed analysis of the development of the activity in time. In a too short test the lag phase may not have ended before (near) depletion of ammonium nitrite and thus lead to underestimation of the maximal activity. The presence of a plateau in the N_2 production rate is a confirmation that the maximum activity was reached during the test. The results showed a high accuracy of the manometric test for the measurement of the maximum specific anammox activity. The tests were very reproducible (<5% variation) and when evaluated with liquid samples the nitrogen balances closed. Clearly the manometric test can be very reliable used for anammox activity evaluation when used in a careful way.

2) Inhibition of the anammox process by nitrite

A wide range of data on nitrite inhibition has been reported (Table A.1 in Appendix) using different determination systems and biomass in different aggregation states. Unlike results obtained from measurements directly within the (laboratory scale) reactor, the results of batch tests outside the reactor environment are relatively consistent. Inhibition appears to be more severe in case of suspended and flocculent biomass (Table A.1 in Appendix) suggesting that the outer layer of the biofilm (biofilm on support/carriers or granular biomass) can act as a protecting shell for the inner core (due to diffusion and, in case of non-complete inhibition, residual nitrite removal rate). This is supported by the studies of Fernàndez et al. (2010) and Cho et al. (2010) who investigated nitrite inhibition using biomass in different aggregation states (Table A.1 in Appendix). Despite differences in (I) dominating anammox taxon, (II) average MSAA and (III) -slight- different experimental conditions, the IC_{50} reported for biofilm systems (400 and 350 mg N L^{-1} for (Cho et al., 2010) and (Fernàndez et al., 2010) respectively) is similar to the value found in this work (400 mg N L^{-1}, Fig. 4.3). 350-400 mg L^{-1} can thus be regarded as an accurate and relatively situation independent value for the IC_{50} in case of biofilm or granular sludge. From nitrite concentration ranges experienced by anammox microorganisms during normal reactor operation (Table A.1 in Appendix), no indications for inhibition-adaption could be inferred.

3) Mode of action of nitrite

In literature the adverse effect of nitrite is referred to as reversible inhibition (e.g. Strous et al., 1999), irreversible inhibition (e.g. Jetten et al., 2005, Van Dongen et al., 2001) and toxicity (e.g. Wett 2007). Reversible inhibition refers to a reversible decrease of the catabolic activity during exposure, while toxicity or “irreversible inhibition” refers to damages to the microorganisms associated with an irreversible decrease of the microbial activity. In order to clarify whether nitrite exposure resulted in reversible inhibition or toxicity, the reversibility of the exposure should be assessed. In the present work it is shown that after nitrite exposure to 1 g N/L for 24 hours the anammox activity can be fully recovered by decreasing nitrite concentration through biomass washing within two days (Fig. 4.8 and Table 4.2) confirming the results obtained by Kimura et al., 2010 with anammox in gel-carriers.

The recovery of anammox bacteria from nitrite inhibition during an exposure of up to 24 hours, contradicts the attribution of reactor failures to nitrite exposure in many reactor studies (e.g. Egli et al., 2001, Lòpez et al., 2008, Table A.1). Furthermore inhibition was reported in an SBR system at concentrations higher than 100 mg NO_2^--N L^{-1} (Strous et al., 1999) and in a long-term experiment irreversible inhibition was reported when nitrite concentration was maintained at 40 mg N L^{-1} over several days (Fux 2003). On the other hand, a full-scale reactor (origin of biomass in this work) was shown to operate at a total nitrogen conversion rate of 7.5 kg N m^{-3} d^{-1} for months at a nitrite concentration of 40 to 80 mg N L^{-1} and was capable of full recovery within a few days after exposure 350 mg NO_2^--N

L^{-1} for more than one day (de Kreuk (2011), personal communication). Furthermore, Abma et al. (2010) reported recently that in a full-scale nitritation-anammox reactor operations took place at typical concentrations of 20 to 30 mg N L^{-1} of nitrite, indicating growth can take place at these values. Thus, while it seems logic to associate a non-working anammox reactor with high nitrite levels, such a level is –at least after short exposure– probably an effect rather than a cause of damaged biomass.

4) Role of nitrous acid

For nitrifiers and denitrifiers, nitrous acid –rather than the dissociated form nitrite– is toxic (Anthonisen et al., 1976). The mode of action is related to the ease of diffusion of nitrous acid over the cell wall, where it dissociates resulting in a drop in pH and loss of the proton motive force. Also for the anammox process, nitrite toxicity is generally regarded to be HNO_2 related (Fernàndez et al., 2010), although there are no anammox related measurements supporting this assumption. Direct toxicity tests at pH 7, 7.4, and 7.8 however indicated that the ion itself (NO_2^-) is the actual inhibitor (Strous et al., 1999). In our exposure tests at 500/1000 mg N L^{-1} nitrite at different pH-values, the remaining activity decreased by only 1.8 to 2.7% (at 500 and 1000 mg N L^{-1} respectively) although the difference in HNO_2 concentration varied by factor 10. Comparable changes in HNO_2 at constant pH a much larger decrease in activity was found. Combining the results of both tests a clear correlation between the decrease in activity and the total nitrite concentration was found. This is a strong indication that the actual inhibitor is nitrite rather than nitrous acid for anammox bacteria and that the toxicity is not due to dissipation of the pmf by HNO_2. It could well be that indeed the ladderane membrane lipids in the anammox cell membrane form such a tight membrane (Sinninghe Damsté et al., 2005) that HNO_2 diffusion is effectively prevented.

5) Effect of exposure time and exposure conditions

The results presented in this work show that increased concentrations and exposure time increase the loss of activity. The joint effect of concentration and exposure time could not be correlated straightforwardly. Inhibition by nitrite also took place in the absence of ammonium and when cells were not catabolically active (Fig. 4.5, 1C). For exposure to concentrations of up to 1 g NO_2^--N L^{-1}, the absence of ammonium during the exposure seemed to have a more severe effect, while the effect was reversed at higher values (Fig. 4.5, 1B, 1C). However even after 24 hours exposure to 6 g NO_2^--N L^{-1} the activity maintained in the first manometric test after biomass washing was still 40% of the control activity and a second feeding leads to an additional increase of about 7% (Fig. 4.6), showing that even such an extreme nitrite level did not seem to completely destroy the anammox metabolism. Anammox bacteria are strictly anoxic and their inhibition by oxygen has been shown (Strous et al., 1997, Van Dongen et al., 2001). This inhibition was demonstrated to be reversible even at concentrations as high as 9 mg L^{-1} (calculated based on "37°C, 1.5 bar and 18% oxygen concentration in the headspace", Egli et al., 2001) and 3.7 mg L^{-1} (based on "32°C, 1 bar(*a*)[1] and 50% air saturation", Strous et al., 1997).

From the results presented in this work, the effect on activity in tests *after* one hour exposure in the presence of 5 mg L^{-1} O_2 was negligible for concentrations up to 2 g N/L of ammonium nitrite (Fig. 4.5, 1B). Oxygen even seemed to decrease the inhibiting effect of substrates resulting in 30% loss of activity in batch tests (compared to a 50% loss after exposure in the absence of oxygen) after exposure for 24 hours to 2 g N L^{-1} ammonium nitrite (Fig. 4.5, 1A, 1B) and in an overall shorter lag phase (Fig. 4.5, 3A, 3B). The results show that when the nitrite exposure concentration is sufficiently low to have actively metabolizing cells (<1 g N L^{-1} of ammonium nitrite in anoxic condition), activity during exposure resulted in higher activity loss than when exposure took place under non metabolizing conditions (presence of oxygen, or absence of ammonium).

Wett (2007) reported that when the anammox process is carried out in presence of oxygen, nitrite is inhibiting already at low concentrations (irreversible toxicity at 50 mg N/L and detrimental effect on the process already at 5 mg N L^{-1}). This does not corresponds to our results. However we could not find an explanation for this difference, except that there might have been a different type of anammox in their culture or it might have lacked the protective effect of biofilm growth in their suspended growth process.

6) Ammonium sorption and conversion ratios

The inhibition effect on general metabolism and biomass synthesis was evaluated based on the decrease of the specific nitrogen gas production rate (which corresponds to

1 (*a*) stands for absolute pressure

catabolic activity) and on the *R_NiAm* and *R_NaAm* ratios, (1.3 and 0.25 respectively), which are indicators for the anabolic activity (nitrite oxidation to nitrate functions as the electron-donating reaction for CO_2 fixation, (Strous et al., 1998)). After exposure to high concentration of ammonium however these ratios varied strongly (Fig. 4.5, 3A, 3B, ascending trend in *R_NiAm* and *R_NaAm* ratios). The results showed that the higher the ammonium exposure (both in aerobic and anoxic conditions) the smaller the amount of ammonium converted while nitrite and nitrate conversions were constant. When the biomass was then used in a second manometric test the amount of ammonium removed was much closer to theoretical values. The increased *R_NiAm* and *R_NaAm* ratios could be taken as an indication of a disturbed metabolism (and thus of concern), but could also be an effect of release of ammonium adsorbed or precipitated as e.g. struvite on the granules during the exposure. Since after the exposure to nitrite (in the absence of ammonium) the ratios are in agreement with theoretical values (Fig. 4.5, 3C) disturbance of the metabolism is unlikely.

An assay (in triplicate) conducted to test the extent of ammonium sorption, confirmed that during 24 hours anoxic exposure in presence of 1 g-N L^{-1} ammonium the granules had an ammonium sorption capacity of 14 to 16 mg N g VSS^{-1} (data not shown). Also (Bassin et al., 2011) reported ammonium sorption capability of anammox granular sludge. Although 70% of this ammonium sorbed was removed during 1 hour washing, the remainder was still present in subsequent activity tests resulting in higher values of the apparent *R_NiAm* and *R_NaAm*. The batch tests also showed that the remaining sorbed ammonium was fully (> 99%) available for the anammox bacteria. An option could be that at these high ammonium concentrations ammonium was accumulated inside the cells. Assuming that the ammonium was accumulated inside the cell and the bacteria accounted for 10% of the exposed biomass volume (10 mL), this would lead to an intracellular ammonium concentration of about 0.9 M after exposure to 1 g N L^{-1} for 24 h. Such a high level is similar to the calculated ammonium concentration of 1 M in *Nitrosomonas europaea* and other aerobic ammonia oxidizing bacteria (Schmidt et al., 2004) and thus might be realistic for anammox bacteria as well.

As due to ammonium sorption (either inside the cells, adsorbed to the EPS matrix or trapped in minerals such as struvite), the use of conversion ratios based on ammonium to characterise anammox activity might be biased. We propose to use the conversion ratio of nitrate produced on nitrite consumed (*R_NaNi*, equation 4.3) as an indicator for the active growth of anammox bacteria (see Fig. A1 in Appendix).

(4.3)

$$R\_NaNi = -\frac{\left[NO_3^-\right]_{START} - \left[NO_3^-\right]_{END}}{\left[NO_2^-\right]_{START} - \left[NO_2^-\right]_{END}}$$

Using the stoichiometry from Strous et al. (1998) *R_NaNi* would amount to 0.20. Note that in complex systems some of the produced nitrate can be converted by denitrification to nitrite or ammonium by regular heterotrophic bacteria or even certain type of anammox bacteria (Kartal et al., 2007).

7) Implications for reactor operation

The results reported in this work show the robustness of the anammox process to inhibition by nitrite as well as to oxygen and that sufficient operational flexibility exists to prevent long term detrimental effects of short-term exposure. High nitrite concentrations may accumulate in anammox bioreactors due to inorganic carbon limitation or failure of the previous process in a treatment scheme, or an unbalance between ammonium oxidation and anammox activity. To minimize the loss in anammox activity operational measures should aim for lowering the reactor nitrite concentration as fast as possible (exposure time has a greater impact than concentration levels). This can be established by diluting the reactor supernatant with nitrite-free medium (usually influent) and this is therefore likely to be an effective strategy as already described by Wilsenach (2006).

From this study as well as from literature data (table A1 in Appendix) biofilm and granular sludge systems seem to be more protected to nitrite inhibition. The tolerance to nitrite enables stable reactor operations at relatively high nitrite concentrations (85 mg N L^{-1} for van der Star et al., 2007) in biofilm systems.

CONCLUSIONS

Despite earlier reports on potential nitrite toxicity for the anammox activity this study shows that the levels of nitrite on which inhibition occurs are rather high (IC_{50} of 0.4 g N L^{-1}), and that biomass relatively quickly (and totally) recovers from high nitrite concentrations. The exposure time to nitrite was identified as an important variable leading to more and more severe inhibition when exposure is prolonged. In several cases where high nitrite is reported as a cause of activity loss, it might well be that instead activity loss had resulted in the high nitrite concentrations observed. It was also again confirmed that anammox bacteria can well recover from aerated periods. Overall our results further underline that the anammox process can be a stable process not prone to temporary adverse effects of oxygen and nitrite in the reactors.

APPENDIX

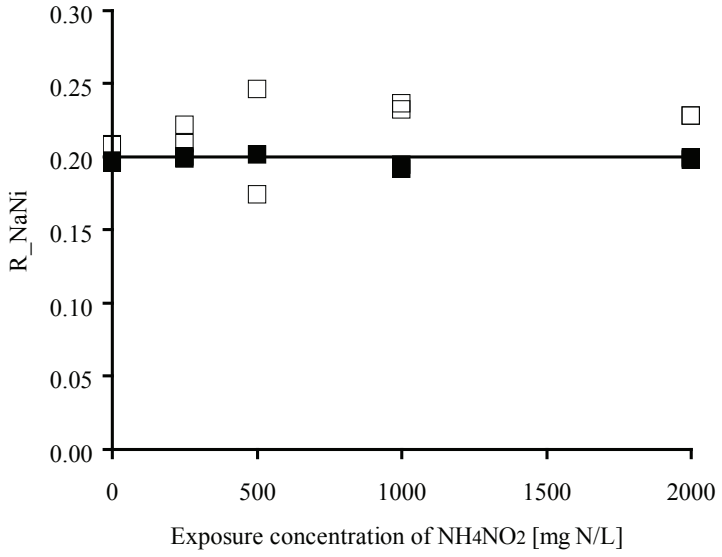


FIG. A.1
***R_NaNi* during manometric test after exposure for 1h (■ and ♦) and 24h (□ and ◊) to different concentrations of ammonium nitrite in anoxic (♦ and ◊) and aerobic (■ and □) conditions; the continuous line represents *R_NaNi* for anammox bacteria (Strous et al., 1998).**

Table A.1: Comparison between data on nitrite inhibition on the anammox process: only those studies where specific tests on nitrite effect have been conducted were included.

Reference	Nitrite level [mg N L^{-1}]	Determination method	Batch (B) or in reactor (R)	Accuracy	Test length	Anammox taxon	Aggregation status	Process configuration[a]	Reactor volume (L)[b]	pH	Normal operation nitrite level [mg N L^{-1}]
(Strous et al., 1999)	100 ("complete inhibition")	NO_2^- conversion rate	B	n.r.	n.r.	*Candidatus* Brocadia anammoxidans	homogenized aggregates	2	10	7, 7.4, 7.8	0÷1.4
(Egli et al., 2001)	180 ("complete inhibition")	NO_2^- conversion rate	B	n.r.	70 h[c]	Kolliken microrganisms[d]	suspended	2	2	7	84
(Fux et al., 2002)	60 ("strong inhibition")	NO_2^- conversion rate	R	n.r.	2 days	Kolliken microrganisms[d]	suspended	2	2500	7.5	n.r.
(Li et al., 2004)	70 ("serious inhibition")	nitrite accumulation	R	n.r.	12 h	n.r.	n.r.	1	3	7.5-8	0÷20[g]
(Fux et al., 2004)	80 (80% loss)	NO_2^- conversion rate	R	n.r.	n.r.	Kolliken microrganisms[d]	biofilm on support [e*]	2	3.5	8.0	18±17
(Jung et al., 2007)	70 (inhibition)	nitrite accumulation	R	n.r.	n.r.	n.r.	granular	2	3.35	7.5-8	0÷35
(Dapena-Mora et al., 2007)	350 (IC_{50})	manometric	B	7±4%	n.r.	*Candidatus* Kuenenia Stuttgartiensis	flocculent	2	1	7.8	0÷15[c]
(Lòpez et al., 2008)	100 (inhibition)	nitrite accumulation	R	n.r.	n.r.	*Candidatus* Brocadia anammoxidans	granular	2	15	7.5-8.2	"close to zero"
(Bettazzi et al., 2010)	>60 (28% reduction at 75)	manometric	B	4.5±3.3%	6-16 h	*Candidatus* Brocadia anammoxidans	flocculent	2	40	n.d.	"close to zero"
(Fernàndez et al., 2010)	≈350 (IC_{50} [c])	manometric	B	n.r.	n.r.	*Candidatus* Kuenenia Stuttgartiensis	biofilm on support [e**]	2	5	7.8	0÷25[c]
(Fernàndez et al., 2010)	≈120 (IC_{50} [c])	manometric	B	n.r.	n.r.	*Candidatus* Kuenenia Stuttgartiensis	flocculent	2	1	7.8	0÷15[c]
(Cho et al., 2010)	≈400 (IC_{50} [c])	NO_2^- conversion rate	B	n.r.	40 h	*Candidatus* Brocadia anammoxidans	granular	2	1.25	n.d.	n.r.
(Cho et al., 2010)	≈230 (IC50[c])	NO_2^- conversion rate	B	n.r.	40 h	*Candidatus* Brocadia anammoxidans	homogenized granules	2	1.25	n.d.	n.r.
(Kimura et al., 2010)	>300 (37% loss at 430)	NO_2^- conversion rate	B	n.r.	1 h	*Candidatus* Brocadia anammoxidans	gel carriers	2	0.5	n.d.	0÷60 [c]
(Oshiki et al., 2011)	224 (IC_{50})	NO_2^- conversion rate	B	n.r.	n.r.	*Candidatus* Brocadia Sinica	flocculent [f]	2	0.8	7.0-7.5	n.r.
present work	400 (IC_{50})	manometric (batch)	B	4.2±0.7	4-8h	*Candidatus* Brocadia anammoxidans	granular	2	70000	7.5	0÷85 [g]

[a] Nitritation and anammox processes in a single reactor (1) or in separate reactors (2)
[b] When experiment are conducted in batch this column reports information on the origin of the biomass used
[c] Implied from figure
[d] The nucleotide sequence of the 16S rRNA gene of the Kölliken anammox organism was deposited in the GenBank database under accession no. AJ250882 (Egli et al., 2001)
[e] PVC as carrier material ([e*]); Zeolite as carrier material ([e**])
[f] Biofilm samples were dispersed by magnetic stirring for 2h (aggragates diameter <100µm)
[g] Reported by (van der Star et al., 2007)
n.r. not reported; n.d. not determined

CHAPTER 5

Inhibition effect of swine wastewater heavy metals and antibiotics on Anammox activity

Part of this Chapter is yet to be submitted for publication as

T. Lotti, M. Cordola, R. Kleerebezem, S. Caffaz, C. Lubello, MCM van Loosdrecht. **Inhibition effect of swine wastewater heavy metals and antibiotics on Anammox activity.**

Outline

The feasibility of anaerobic ammonium oxidation (anammox) process to treat wastewaters containing antibiotics and heavy metals (such as liquid fraction of the anaerobically digested swine manure) was studied in this work. The maximum specific anammox activity (SAA) was evaluated by means of manometric batch tests. The effects of oxytetracycline, sulfathiazole, copper and zinc were studied. The experimental data of the short-term assays were fitted with an inhibition model to identify the half maximal inhibitory concentration (IC_{50}). After 24 hours exposures IC_{50}-values equal to 1.9, 3.9, 650 and 1100 mg L^{-1} were identified for copper, zinc, sulfathiazole and tetracycline respectively. The effect of prolonged exposure (14 days) to oxytetracycline and sulfathiazole was studied by means of repeated batch-assays. Anabolism and catabollism reactions were active during the inhibition tests indicating that anammox bacteria could grow even in the extreme conditions tested. Considering the average concentrations expected in swine wastewaters, the inhibitors studied do not represent a problem for the application of the anammox process.

5.1 INTRODUCTION

Anaerobic digestion is being widely implemented as an efficient process for removal of organic matter from manure allowing the recovery of energy in form of biogas. Digester liquor after anaerobic digestion of manure contains high concentrations of nitrogen and phosphorus as well as heavy metals and pharmaceuticals fed to the livestock. Such a wastewater need to be treated before being discharged to receiving water bodies. Biological nitrogen removal is achieved mostly by complete oxidation to nitrate and subsequent reduction of the nitrate to nitrogen gas under anoxic conditions at the expense of organic carbon (COD). However, COD in digested swine wastewater (after swine manure anaerobic digestion and solid/liquid separation) is not sufficient to remove nitrogen, and therefore external addition of COD is required for complete denitrification, which consequently leads to an increase in the cost of the operation.

Recently, a novel biological nitrogen removal process, anaerobic ammonium oxidation (anammox), which oxidizes ammonium to nitrogen gas with nitrite as electron acceptor under strictly anaerobic conditions, has been introduced (Strous et al., 1998; van der Star et al., 2007). This process is advantageous over most commonly employed nitrification-denitrification processes as no external addition of COD and lower oxygen supply is required combined with a low sludge production. Therefore, manure digester effluent is expected to be a prime application of the anammox technique. Some investigations of the application of anaerobic digestion of swine slurry, following partial nitrification to oxidize ammonium to nitrite and anammox treatment have been reported (Hwang et al., 2005; Yamamoto et al., 2008; Molinuevo et al., 2009), but this treatment chain has not been yet applied in full scale.

Swine wastewater is a mixture of swine urine, excrement and service water and contains high concentrations of nitrogen as well as heavy metals, such as copper and zinc, and veterinary antibiotics. Due to the broad administration of these veterinary antibiotics, to prevent infections, treat diseases as well as growth promoters (Huang et al., 2001), swine wastewater is one of the most complicated agricultural wastewaters with respect to nitrogen removal. Oxytetracycline, belonging to the tetracycline class, is a common antibiotic with a broad range of activity and low cost. Oxytetracycline is administered to livestock animals (including cattle, swine, poultry and fish) and can be found in manure (expressed as mg per kg of dry matter, mg kg DM^{-1}) at concentrations up to 136 mg kg DM^{-1} (Winckler et al., 2003) and at average concentrations of 59 mg kg DM^{-1} (Zhao et al., 2010). Other most commonly found antibiotics in swine manure belong to the sulphonamide class like the cheap and widely used sulfathiazole (Huang et al., 2001). In individual manures peak concentrations up to 235 mg kg DM^{-1} of antibiotics belonging to this class have been detected (Engels 2004). No appreciable sulfonamide antibiotic concentrations (less than 10 mg/kg DM^{-1}) were found in 61 Chinese swine manures (Zhao et al., 2010). Most antibiotics are excreted unmetabolized via swine manure (Mohring et al., 2009). It has been reported that as much as 30-90% can be excreted (Sarmah et al., 2006). Although some researchers have studied the fate of antibiotics in specific environmental compartments, such as soil interstitial water or anaerobic lagoons (Kemper, 2008), and in different biological processes (Arikan et al., 2006), there is little information regarding the effect of antibiotics on Anammox activity (van de Graaf et al., 1995; Fernandez et al., 2009).

Cu and Zn are essential micronutrients for pig metabolism and their feed is supplemented

with these elements, but most of the dietary supply is excreted. Consequently, the slurries contain high concentrations of Cu and Zn (L'Herroux et al., 1997). Considering that during activated sludge process the major portion of Cu and Zn occurred in solid fraction (Suzuki et al., 2010) and despite the fact that during anaerobic digestion process most of the mineral elements are again present in the solid fraction (86% trapped within particles between 3 and 25 µm, Marcato et al., 2008), a relevant concentration of these metals is still present in the digestate (about 1.5 mg L^{-1} according to Vanotti et al., 2007).

Aim of this study was to evaluate the effect of elevated concentrations of veterinary anitobiotics and heavy metals on the activity of anammox bacteria.

5.2 MATERIALS AND METHODS

5.2.1 Specific Anammox Activity test

According to the procedure illustrated in *par.4.3.2*, batch experiments were performed to evaluate the maximum specific anammox activity (MSAA). The assays were performed in closed bottles equipped with online manometric sensors (OxiTop Control System; WTW, OxiTop Control AN6, Weilheim, Germany). Each vessel (340 mL) has two lateral holes sealed with a puncturable rubber septum for substrate and inhibitor injections and supernatant sampling. The procedure is based on the measurement along time of the overpressure (hPa) generated by the nitrogen gas production (N_2) in closed vials. The nitrogen gas production rate was calculated from the headspace overpressure measurements using the ideal gas law equation.

5.2.2 Experimental Set-up

Short-term tests

The biomass was washed and re-suspended in a medium containing 25 mM HEPES buffer to maintain pH 7.5 throughout the test. The headspace and liquid phase (200 mL) was flushed with nitrogen gas to guarantee anoxic conditions. The vials were placed in a thermostatic shaker, at 180 rpm and 30 °C. After headspace pressure stabilization was established, the pressure was reduced to the atmospheric level by inserting a needle connected to a water-filled bottle which acts as a water-lock; then substrates were added by spiked injections through the puncturable septum. The injected solutions were prepared with $NaNO_2$, $(NH_4)_2SO_4$ and NH_4HCO_3 (bicarbonate was added to avoid inorganic-carbon limitation during the test) dissolved in milly-Q water. In the experiments performed the starting concentration was 50 mg N/L ammonium nitrite. When the anammox reaction stopped (no more N_2 production) liquid samples were taken for chemical analysis. The inhibitory effect of copper, zinc, oxytetracycline and sulfathiazole was evaluated.

Long-term tests

The experimental set-up of the long-term effect of exposure to oxytetracycline and sulfathiazole tests was the same as in the short-term tests described above, except for (**i**) the number of manometric tests conducted, (**ii**) the addition of antibiotics at the end of each manometric test, (**iii**) an additional term in the calculation of the SAA:

(**i**) Eleven manometric tests were conducted one per day (five days per week);
(**ii**) At the end of each manometric test a liquid sample (3 mL) was taken for chemical analysis as described for the short-term tests. The oxytetracycline and sulfathiazole amount (mg) withdrawn with this liquid sample were reintroduced in the vessel through injection of 3 mL of a solution appositely prepared (containing Hepes, 25 mM, as well). The liquid volume and composition (antibiotics and Hepes) was therefore about constant in each of the eleven tests;
(**iii**) In the calculation of the maximum specific anammox activity relative to successive injections, the biomass growth was taken into account considering that the nitrate produced was coupled with the carbon dioxide assimilated (0.254 C-mol/NO_3^--mol according to Strous et al., 1998).

5.2.3 Inoculum

The reactor was inoculated with granular anammox biomass, originating from the full-scale anammox reactor of Dokhaven-Sluisjesdijk wastewater treatment plant in Rotterdam, The Netherlands (van der Star et al., 2007). The anaerobic digester supernatant treatment process consists of partial nitritation in a SHARON reactor, followed by anammox process performed in a 70 m^3 gas-lift reactor. During the tests performed in presence of heavy metals, the initial biomass concentration in the vessels was 0.74 gVSS L^{-1} and its SAA was 0.68±0.04 g N_2-N g VSS^{-1} d^{-1}. When the effect of antibiotics was studied, the initial biomass concentration was 2.1 gVSS L^{-1} and its SAA was 0.69±0.04 g N_2-N g VSS^{-1} d^{-1}.

5.2.4 Analytical methods

Soluble nitrogen compounds were measured via spectrophotometric flow injection analysis (QuickChem 8500 series 2 FIA System, Lachat Instruments, Loveland, Colorado, USA). The methods applied were QuikChem®Methods 10-107-06-5-E for ammonium (range 0.1 to 10.0 mg N/L, measurement of NH_3 after increasing pH and evaporation) and 10-107-04-1-C for nitrate/nitrite (range 0.01 to 2.0 mg N/L, direct measurement of nitrite, or measurement preceded by reduction of NO_3^- to NO_2^- to yield the concentration of "NO_3^- +NO_2^-") according to the protocol of the manufacturer. The length of the sample loop of the nitrate/nitrite detection was increased in order to obtain a measurement range from 0.05 to 10 mg N/L. Soluble copper (Cu^{2+}) and soluble zinc (Zn^{2+}) concentration in the liquid bulk were detected using commercial test kits according to the protocol of the manufacturer (brand: Dr.Lange test kits, Hach-Lange GmbH, Düsseldorf, DE, kits LCK329 and LCK529 for copper and LCK360 for zinc) and determined on a designated spectrophotometer (DR 2800). All the samples were filtered at 0.45 µm before analysis. Concentrations of Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS) were determined according to Standard Methods (APHA 2005).

5.2.5 Evaluation of IC_{50}

The half maximal inhibitory concentration, IC_{50}, is the concentration of the tested compound, which corresponds to 50% activity compared to a non-inhibited assay (control). The maximum specific removal rate experimental data were fitted with an inhibition model to identify the IC_{50} (mg inhibitor L^{-1}):

(5.1)

$$Rate = \frac{Rate_{max}}{\left(1 + \frac{I}{IC_{50}}\right)}$$

where Rate is the specific N_2 production rate (g-N_2-N $gVSS^{-1}$ d^{-1}) in the presence of inhibitor, $Rate_{max}$ is the maximum specific N_2 production rate and I is the concentration of the inhibitor (mg L^{-1}). The best fit was evaluated by the method of least squares.

5.3 RESULTS AND DISCUSSION

5.3.1 Short-term effects

Copper and Zinc

Presence of increasing concentrations and prolonged exposure to Copper and Zinc leads to a decreasing specific anammox activity (SAA) (Fig. 5.1). The major part of the inhibition occurs within 8 hours exposure for both copper and zinc. In a rough evaluation both metals have a similar inhibition. For copper there seemed to be a clear exposure effect (activity declined with increasing exposure time). For Zinc such an effect was not clear from the results. In order to facilitate the comparison between the effects of the two

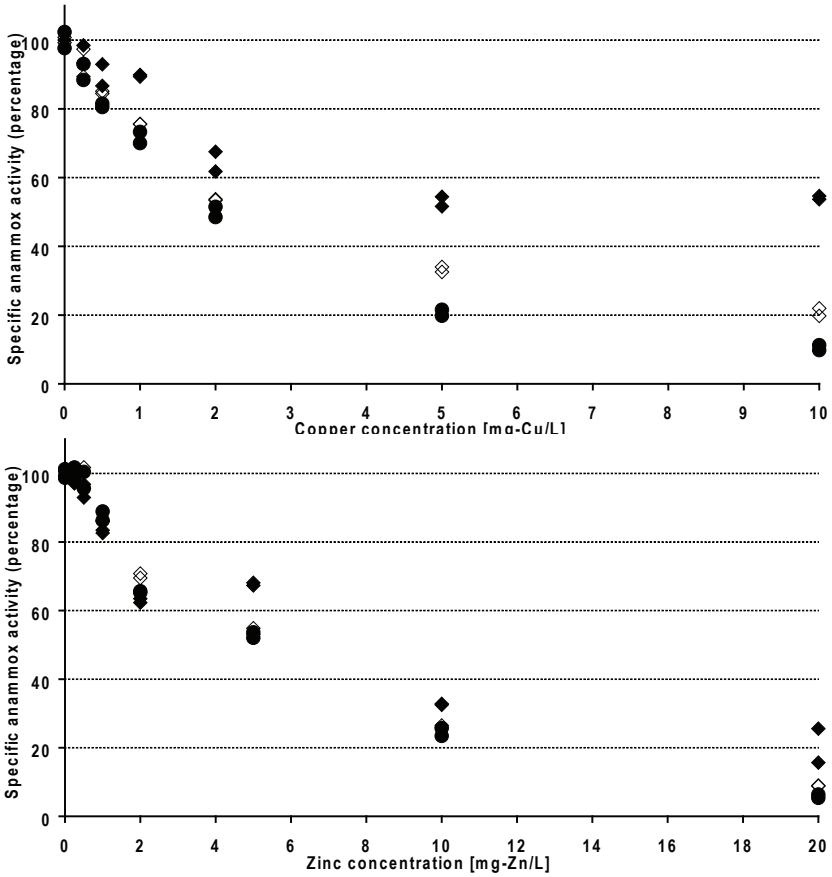


FIG. 5.1
Effect of Copper (upper graph) and Zinc (lower graph) on the specific anammox activity expressed as percentage activity relative to the unexposed culture. Activity evaluated after exposure for 1h (close diamonds), 8h (open diamonds) and 24h (circles).

metals investigated, the corresponding IC_{50} after different exposure times were calculated (Tab. 5.1).

The overall inhibitory potential was higher for copper than for zinc. After 24 hours exposure at 1 mg L^{-1}, a realistic concentration for the anaerobic supernatant from pig manure digestion (Vanotti et al., 2007), the activity decrease was about 25% and 15% for copper and zinc respectively. The effect of exposure time was also strong for inhibition to copper as shown in table 1. During the exposure to both metals however the observed stoichiometry was not affected, resulting in regular nitrate production, and therefore regular growth (see Chapter 3). Since in literature there is no information regarding heavy metals inhibition of anammox activity, the results presented in this study can be only compared with the results found for other microorganisms. Grunditz et al. (1998) reported zinc as a more significant inhibitor than copper for pure culture of nitrifying and nitrite reducing bacteria. Çeçen et al. (2010) showed a higher inhibition effect on nitrification for copper than for zinc in short term batch tests reporting an IC_{50} of about 7 mg L^{-1} for both metals. Madoni et al. (1999) reported 49% and 22% inhibition on activated sludge oxygen uptake rate (OUR) and ammonium uptake rate (AUR) respectively, after 24 hours exposure to 1 mg Cu L^{-1}, while 55% and 43% inhibition on OUR and AUR respectively were observed after 24 hours exposition to 20 mg Zn L^{-1}. The fraction of Cu and Zn adsorbed on the solid ratio and therefore not measured back at the end of the 24 hours exposure was below 8% of the starting concentration without any evident difference between the two metals (Fig. 5.2). The little amount adsorbed on biomass support the validity of our results since the metal soluble fraction is the inhibiting one. Mercato et al. (2008) reported that most of the Cu and Zn (86%) trapped in digested pig slurry was within particles between 3 and 25 μm and less than 2% was trapped within particles larger than 250 μm due to their low specific surface area. This agrees with the fact that the diameter of the granules employed in the experimentation was 1-2 mm.

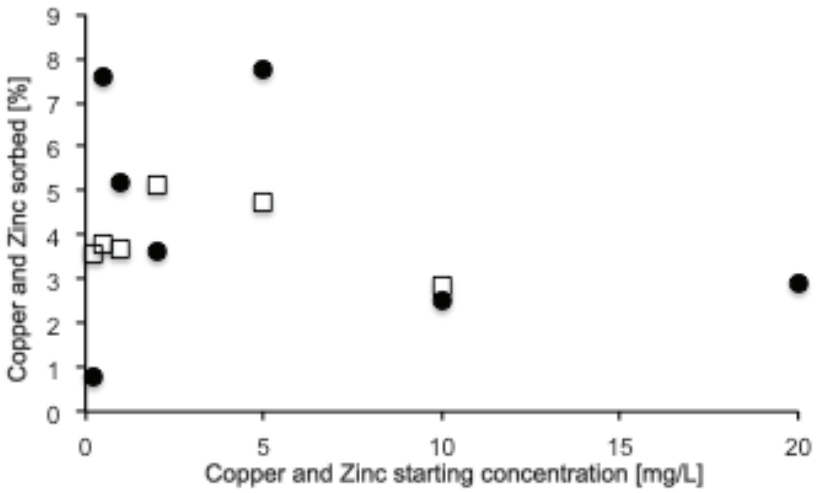


FIG. 5.2
Copper (squares) and Zinc (circles) adsorbed on solid fraction during 24 hours exposure as percentage of the starting concentration (mg L^{-1}).

Table 5.1: IC_{50} for copper and zinc inhibitory effect on specific anammox activity as function of the exposure time.

	Exposure duration [hour]		
	1	8	24
Copper IC_{50} [mg Cu L^{-1}]	5.0	2.6	1.9
Zinc IC_{50} [mg Zn L^{-1}]	4.5	4.2	3.9

Oxytetracycline and Sulfathiazole

The effect of exposure to oxytetracycline and sulfathiazole on the specific anammox activity increased with higher concentrations and longer exposure time (Fig. 5.3). In presence of oxytetracycline, during the first 8 hours there was no appreciable inhibition, after instead, the activity decreased. A higher concentration leads to decreasing activities. The inhibitory effect of sulfathiazole at concentrations higher than 250 mg L^{-1} was already significant 2 hours after the test started and increased continuously according to increasing exposure duration at concentrations higher than 100 mg L^{-1}. A negligible loss in activity was calculated at concentrations smaller than 250 mg L^{-1} of both antibiotics. The estimated SAA obtained showed that the inhibitory effects of sulfathiazole were higher than those resulting from oxytetracycline. The IC_{50} evaluated after 24 hours exposure was indeed smaller for sulfathiazole (650 mg L^{-1}) than for oxytetracycline (1100 mg L^{-1}).

The inhibitory effect of tetracycline hydrochloride (an antibiotic belonging to the same class of oxytetracycline and similar to the latter in structure and properties) on anammox activity was reported having an IC_{50} equal to about 210 mg L^{-1}, and this value also increased with exposure time (Fernandez et al. (2009). The stronger effect reported by these authors could be related to the three times lower specific anammox activity of the biomass used (0.25±0.01 gN_2-N $gVSS^{-1}$ L^{-1}). Campos et al. (2001) reported 50% activity inhibition at 250 mg L^{-1} of oxytetracycline during continuous operation of a nitrifying bioreactor. The effect of this class of antibiotics is however very different according to the microorganism studied and results from literature are often contradictory. In anaerobic digestion and anaerobic lagoons the methane production decrease was reported to be 27% at 3.1 mg oxytetracycline/L (Arikan et al., 2006), or negligible up to 250 mg oxytetracycline L^{-1} (Lallai et al., 2002), 42% at 25 mg L^{-1} of chlortetracycline (Loftin et al., 2005; same author reported no inhibition effects in the case of oxytetracycline) and 45% at 10 mg L^{-1} of oxytetracycline and chlortetracycline together (Alvarez et al., 2010). The inhibitory effect of sulfathiazole on microorganisms applied for wastewater treatment is not extensively studied in literature, but few useful results for comparison can be found. The methane production in anaerobic lagoons was reported to decrease of 14% at 25 mg sulfathiazole L^{-1} (Loftin et al., 2005). Baran et al. (2006) reported IC_{50} 16 mg sulfathiazole L^{-1} for the green alga Chlorella Vulgaris.

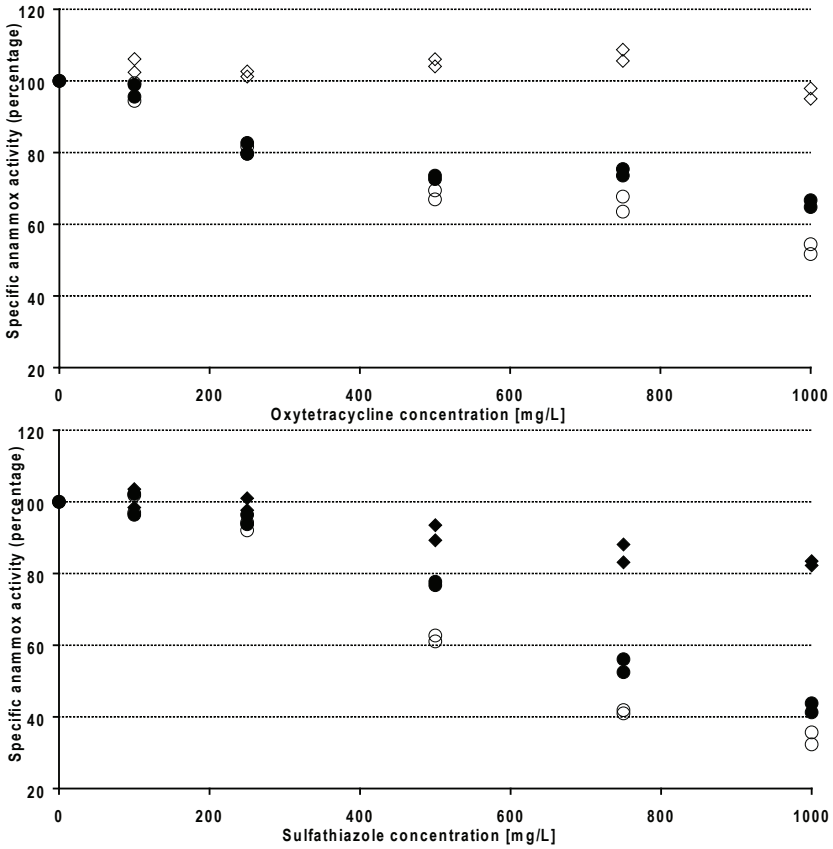


FIG. 5.3
Effect of oxytetracycline (upper graph) and sulfathiazole (lower graph) on the specific anammox activity expressed as percentage activity relative to the unexposed culture. Activity evaluated after exposure for 1h (open diamonds), 8h (close diamonds), 24h (close circles) and 30h (open circles).

5.3.2 Long-term effect of exposure to Oxytetracycline and Sulfathiazole

The effect of prolonged exposition to oxytetracycline and sulfathiazole on the anammox activity was evaluated by repeated batch assays conducted for 14 days at regular intervals of 24 hours (Fig. 5.4). In presence of 100 mg oxytetracycline L^{-1} the activity was about constant for the first seven days while afterwards decreased till 75% of the control activity at the end of the experiment.

At 500 mg oxytetracycline L^{-1} the activity decreased till 50% at day 14, a similar effect was reached after 24 hours exposure to 1000 mg oxytetracycline L^{-1} (Fig. 5.3). A 40% decrease in activity was reported in the continuous operation of an anammox bioreactor after tetracycline hydrochloride was fed for 30 days at a concentration of 10 mg L^{-1} (Fernandez et al. 2009). In the case of prolonged exposure of sulfathiazole a stronger inhibitory effect than oxytetracycline was observed. After 14 days in presence of sulfathiazole the anammox activity decreased till 50% (at 100 mg L^{-1}) and 28% (at 500 mg L^{-1}) relative to the unexposed culture.

The nitrate produced (which is coupled to the carbon dioxide reduction for growth of anammox bacteria) during each batch test was calculated in order to evaluate the anabolic or anabolic and catabolic nature of the antibiotics inhibition. The production/consumption ratios between the nitrogen compounds involved in the anammox metabolism were also evaluated (nitrite on ammonium consumed and nitrate produced on ammonium consumed). No evident trend in nitrate production or remarkable discrepancy from the anammox stoichiometry reported by Strous et al. (1998) was found (data not shown). This indicates that both anabolism and catabolism reactions were active during the inhibition tests and therefore that, even at high antibiotics concentrations, anammox was still able to grow.

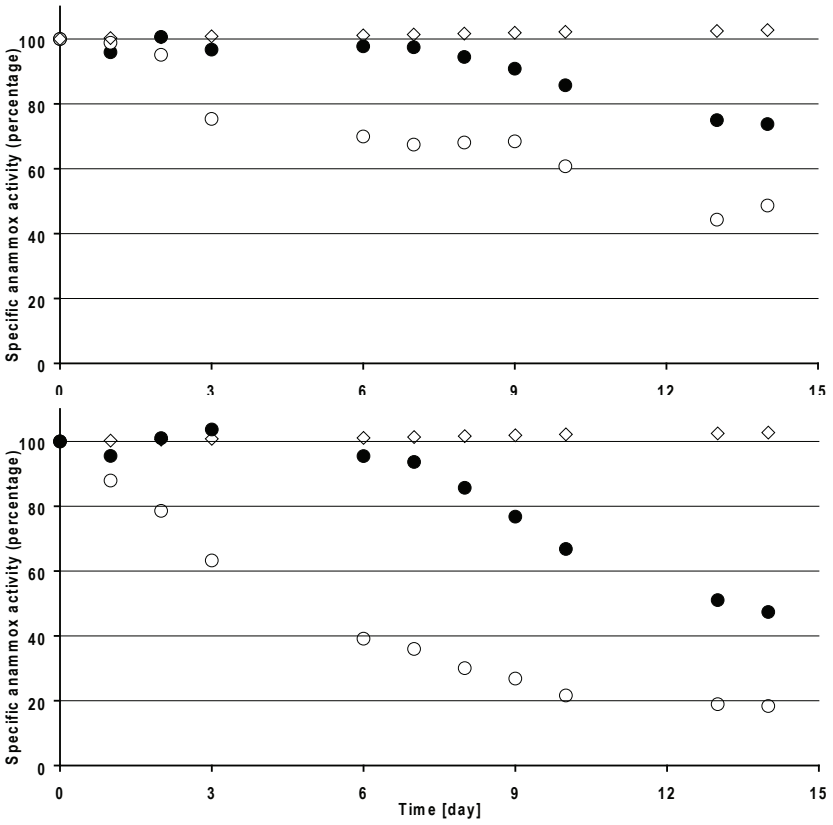


FIG. 5.4
Influence of oxytetracycline (upper graph) and sulfathiazole (lower graph) on the specific anammox activity expressed as percentage activity relative to the unexposed culture (diamonds) during a batch assays repeated for 14 days. Oxytetracycline and sulfathiazole concentrations tested were 100 (close circles) and 500 mg L^{-1} (open circles).

5.4 CONCLUSIONS

Presence of increasing concentrations and prolonged exposure to Copper and Zinc leads to a decreasing specific anammox activity (SAA). For copper there seemed to be a clear exposure time effect (activity declined with increasing exposure time) while for Zinc such an effect was not clear from the results. After 24 hours exposure the evaluated IC_{50} were 1.9 and 3.9 mg L^{-1} for copper and zinc respectively. The inhibiting effect of exposure to oxytetracycline and sulfathiazole on the specific anammox activity increased with higher 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 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% relative to the unexposed culture, respectively. No evident trend in nitrate production nor remarkable discrepancy from the accepted anammox stoichiometry was found, indicating that both anabolism and catabollism reactions were active during the inhibition tests. Considering the average concentrations expected in swine wastewaters (after swine manure anaerobic digestion and solid/liquid separation), 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 higher biomass concentration in the reactor.

CHAPTER 6

Nitrogen removal from swine manure digestate in a lab-scale anammox gas-lift suspension reactor: design and operations

6.1 INTRODUCTION

The application of anaerobic digestion to livestock manure and the energy recovery related to the subsequent methane production, has recently attracted more and more attention (Angenent et al., 2002; Hill et al., 2000; Sanchez et al., 2005). The supernatant resulting from the solid/liquid separation of the anaerobic digestion effluent (digestate or digester liquor) is characterized by high nitrogen (resulting from the mineralization of organic nitrogen), high phosphorus (part of the P-content of the untreated manure which is not adsorbed on the solid fraction) and low COD content (the remaining COD not converted to biogas during anaerobic digestion). The digestate is usually either recycled as liquid fertilizer or further treated by advanced treatment. The use of advanced treatment of anaerobic digester liquor involves investment and high operational costs, which determines that digester liquor is mostly utilized as fertilizer. However, due to the high concentration of nitrogen remaining in the liquor, its excessive use as fertilizer can cause groundwater pollution and therefore recently government regulations are becoming stricter about the amount of nitrogen spreadable on land. Therefore, it is important to find an alternative way to treat digester liquor efficiently to make the use of methane fermentation of livestock manure in an environmentally safe manner. Nitrification–denitrification is generally used for nitrogen removal from digester liquor. When applying this process, a large external dosage of oxygen, alkalinity for nitrification and organic electron donor such as methanol for denitrification is required. The autotrophic nitrogen removal represents a more sustainable and cost effective alternative due to the savings in aeration, chemical dosage and sludge production (van Dongen et al., 2001; Sliekers et al., 2002; Fux and Siegrist 2004). The partial nitritation–anammox process is best suited for the treatment of wastewaters containing high concentrations of ammonium and can be then applied to sewage sludge digester liquor, livestock wastewater, landfill leachate, and power plant wastewater (van Dongen et al., 2001). Recently, several studies on the application of this process to livestock wastewaters has been reported in literature. Tests at lab-scale have been conducted in the USA (Vanotti et al., 2006; Szogi et al., 2007), in Korea, (Dong and Tollner, 2003; Ahn et al., 2004; Choi et al., 2004), in Japan (Yamamoto et al. 2008) and in Northern Europe (Molinuevo et al., 2009). Hwang et al. (2005) tested a combined SHARON-anammox process at lab-scale. In the Anammox reactor (loading rate of 1.36 kg soluble-N m^{-3} d^{-1}) the volumetric and biomass-specific nitrogen removal rate were 0.72 kg soluble-N m^{-3} d^{-1} and 0.44 kg soluble-N $kgVSS^{-1}$ day^{-1}, respectively. Furukawa et al. (2009) demonstrated that a PN reactor using nitrifying activated sludge entrapped in a polyethylene glycol (PEG) gel carrier and an anammox reactor using anammox sludge entrapped in the PEG gel carrier could be applied to livestock manure digester liquor.

A part from a high variability among different breeding conditions and seasonal fluctuations, the excess of biodegradable organic carbon (Molinuevo et al., 2009) and the content of antibiotics and heavy metals (Chapter 5) could represent an issue for anammox process application to piggery wastewaters. Molinuevo et al. (2009) reported denitrification become the dominant process after increasing the fraction of digester liquor up to 12%v/v in the influent (corresponding to 242 mg COD L^{-1}). No details are given about the biodegradable fraction of the COD content of the influent. Yamamoto et al. (2008) tested the combination of partial nitritation and anammox process in cascade (diluting 1:4 the real wastewater). The anammox volumetric nitrogen removal rate (NRR) in this conditions, was reported to decrease to 0.22 kg N m^{-3} d^{-1}, corresponding to 10-20% of the NRR obtained with synthetic influent. In a following research Yamamoto et al. (2011) reported a relatively high anammox nitrogen removal rate of 2.0 kg N m^{-3} d^{-1} (at a nitrogen loading rate, NLR, of 2.2 kg N m^{-3} d^{-1}) treating filtered and diluted (7-10 times dilution) partially nitritated piggery digestate. Quiao et al. (2010) reported a lab-scale partial nitritation reactor combined to a granular anammox reactor treating the liquid fraction of digested piggery wastewater. At a minimum influent dilution of 1.5 times, the anammox reactor was capable of a nitrogen removal rate of 3.1 kg N m^{-3} d^{-1} at a nitrogen loading rate of 4.1 kg N m^{-3} d^{-1}.

Most of the references confirm the feasibility of anammox process application to piggery wastewaters but, to our knowledge, no publication is available which report direct treatment of real wastewaters without any dilution. Moreover, all the results reported were obtained in lab-scale reactors and no information is available at larger scale. The literature results are limited to piggery wastewaters and no results are available on treatment of digestates originating from the anaerobic digestion of wastewaters with mixed composition (piggery manure, poultry manure, energy crops).

Biofilm reactors are used in situations wherein the reactor capacity obtained by using freely suspended organisms is limited by the biomass concentration and hydraulic residence time. This can be the case either for slow-growing organisms (e.g. nitrifiers, methanogens), whose growth in suspension requires long residence times, or for diluted feed streams (often present in waste-water treatment processes), in which only a very low biomass concentration can be achieved without biomass retention. In these cases, biofilms are an effective solution to successfully retain biomass in the reactors and to improve the volumetric conversion capacity. According to van Loosdrecht and Heijnen (1993) biofilm reactors are overall not particularly useful when fast-growing organisms (i.e. with a maximum specific growth rate, μ, of 0.1 h^{-1}) or concentrated feed streams are used (e.g. in industrial fermentation processes). In these situations, sufficient biomass will be formed to metabolize the substrate with relatively short residence times without the need for any form of retention; it is the oxygen supply to the liquid phase, not the biomass concentration, which is often the limiting factor. For this reason, in the majority of industrial fermentation processes where high substrate concentrations are used, biofilm formation is either unnecessary or even disadvantageous, and the range of applications of immobilized-cell systems in industry is mainly limited to wastewater treatment processes (Godia and Sola 1995; Tomaszek and Grabas 1998). Biofilms are extensively used in environmental biotechnology because biofilm reactors can be operated at high biomass concentrations to treat the large volumes of dilute aqueous solutions that are typical of industrial and municipal wastewaters without the need for separating the biomass and the treated effluent. Although the use of biofilms overcomes limitations caused by a low reactor-biomass concentration, for high reactor capacities, a new bottleneck has to be considered because the delivery of poorly soluble substrates (e.g. oxygen) to the biofilm surface might become limiting. Systems with static biofilms (e.g. trickling filters) have small specific biofilm surface areas (typically less than 300 m^2 biofilm m^{-3} reactor) available for substrate transport and reaction, and thus a limited reactor capacity (the oxygen-transfer rate is typically less than 3 kg m^{-3} d^{-1} for trickling filters). Therefore, static biofilm reactors can be useful if the biomass retention and not the mass transfer is the main requirement, for example, when large volumes of liquid with very low substrate concentrations have to be treated (e.g. the removal of xenobiotics from ground water). For more concentrated streams, the enlargement of the biofilm specific surface area can lead to a substantial reduction in the reactor volume and the area requirements of the process. A dramatic increase in biofilm surface area can be obtained by growing biofilms as small particles. The choice of the optimal particle size is a compromise between the conversion rate and the particle sedimentation rate. If the particles become too small (i.e. their settling velocity is too small), the process might again be limited by the biomass concentration that is achievable in the reactor, as for cell suspensions. Gravity separation can be enhanced by growing the biomass in the form of dense spherical aggregates. These aggregates can either form spontaneously as large, dense granules (Lettinga et al., 1980; Beun at al., 1999), or attached to suspended carriers (particle-supported biofilms, Tijhuis et al., 1994). The physical and structural properties of particle-supported biofilms and granules are similar, as are their hydrodynamic, mass transfer and reaction characteristics. Therefore, particle-supported biofilms and granules can be considered as a single category. An extensive review of the applications of biofilm reactors in environmental biotechnology, particularly for the treatment of waste water, has been presented by Nicolella et al. (2000).

The main reactor types that are applicable for the suspension of particle-based biofilms are the upflow sludge blanket (USB), bioflim fluidized bed (BFB), expanded granular sludge blanket (EGSB), biofilm airlift suspension (BAS) and internal circulation (IC) reactors. In USB, BFB and EGSB reactors, the particles are kept fluidized by an upward liquid flow. In BAS reactors, a suspension is obtained by pumping air into the system, and in IC reactors, the gas produced in the system drives the circulation and mixing of the liquid and solids in an airlift-like reactor. The USB (Lettinga et al., 1980) is an upflow, fluidized bed reactor containing granules and no bed media, developed in the late 1970s for the anaerobic treatment of low-strength wastes. Suspended solids in the influent, which accumulates in the reactors, pose a major problem for the operation of the USB and reduce the reactor capacity; to overcome this limitation, the BFB concept was developed (Heijnen et al., 1989). In BFB reactors, the liquid to be treated is pumped through a bed of small media (typically sand with a particle size of 0.2–0.8 mm) covered by biofilms at a sufficient velocity to cause fluidization. Owing to the presence of heavy carriers, the BFB can be operated at a higher fluidizing velocity than the USB, and the accumulation of inert material is strongly reduced. Because of particle segregation and low liquid and particle shear on biofilms, the control of biofilm thickness and structure is difficult in BFB reactors and the elutriation

of particles as a result of biofilm overgrowth is a major problem in this system. Better control of biofilm thickness and structure is possible in BAS reactors, where thin, dense biofilms can be easily maintained. The EGSB and the IC reactors are the most recent evolutions of the USB concept. Biothane Biobed EGSB (Lourens and Zoetemeyer 1992) and Paques IC (Vellinga 1986) systems operate at much higher liquid upflow velocities than the conventional USB, thus eliminating limitations related to the accumulation of inert material.

Since the first observation of anammox conversion in an autotrophic denitrification reactor, with a sulfide-limiting loading for the denitrification process in the presence of ammonium (Mulder et al., 1995), anammox-based processes for wastewater treatment have been developed and in recent years the first application have occurred. In 2002 the first full-scale anammox reactor has been taken into operation at the sludge treatment plant of Dokhaven-Sluisjesdijk WWTP, Rotterdam, NL. The process, now fully operational (Abma et al., 2007), takes place in an IC reactor fed with partially nitritated sludge liquor from an adjusted SHARON process (Mulder et al., 2001; Van Dongen et al., 2001).

For the study reported in this Chapter a lab-scale reactor had to be designed. The particle-based biofilm reactor technology appeared the logic choice given the necessity of biomass retention and the attractive advantages (e.g. small footprint, biological reaction and solid-liquid separation in one single reactor) of this relatively novel technology. Cause of its simple design and its previous application in other studies on the anammox process (Sliekers et al., 2003; Dapena-Mora et al., 2004; Arrojo et al., 2008) the biofilm airlift suspension reactor was chosen as the reactor type to be used in this study. In case of anaerobic processes is anyway more correct to refer to this reactor configuration as gas-lift instead of airlift for obvious reasons. A biofilm gas-lift suspension reactor (BGS) was therefore designed and constructed. In this Chapter the design procedure of the BGS reactor as well as the treatment of swine manure digestion effluent by the anammox process will be illustrated.

6.2 MATERIALS AND METHODS

6.2.1 Biofilm airlift suspension reactor design

Notation

A	cross-sectional area of reactor, riser (R) or downcomer (D), m^2
D	column diameter, m
d	particle diameter, m
g	gravitational constant, m s^{-2}
H	column height effective (e) for circulation, m
K	friction (f) coefficient
m	draught tube cross-sectional area fraction (=A_R/A)
p	pressure drop (Δ) caused by gravity (g) or friction (f), Pa
v_L	circulation velocity, m s^{-1}
v_{Gs}	superficial gas velocity based on total cross-sectional area, m s^{-1}
v_{SP}	swarm velocity of particles, m s^{-1}
ε	volume fraction of gas (G), liquid (L) or solid (S) compared to dispersion, overall or in riser (R) or downcomer (D)
ρ	average density in riser (R) or downcomer (D) or density of gas (G), liquid (L) or solid (S), kg m^{-3}
ξ	fraction of contribution of top section to fraction coefficient

Introduction

Three-phase gas-lift (airlift) loop reactors are used in many chemical and biochemical industrial processes of interest. Their advantages are in the intimate contact between gas, liquid and solid phases and the controlled mixing. Three-phase airlift suspension reactors are proposed as an alternative to slurry bubble columns with suspended particles. In comparison with bubble columns the minimum gas velocity needed for complete suspension is reported to be less in an airlift system (Heck and Onken, 1987; Becker *et al.,* 1990). In comparison with mechanically stirred systems shear rates are less, what makes the airlift system attractive for processes with particles that are sensitive to shear, such as biofilm particles. The BAS reactor (Fig. 6.1) consists of an airlift column with a three-phase separator on the top. Air is introduced into the riser section of the airlift and drives the circulation of the gas, liquid and solid (biofilm) phase throughout the reactor. The BAS technology was originally developed for the aerobic purification of anaerobically treated industrial wastewaters (Heijnen et al., 1990, 1993). In anaerobic processes (e.g. anaerobic digestion, anammox), the circulation in the reactor is induced by recirculating the gas produced by biochemical reactions (biogas). In the study presented in this Chapter the biogas (i.e. nitrogen gas) produced in the anammox process was not enough to maintain the biofilm particles (granules) in suspension and was therefore integrated by externally added mixture of N_2 and CO_2 (95 and 5%, respectively).

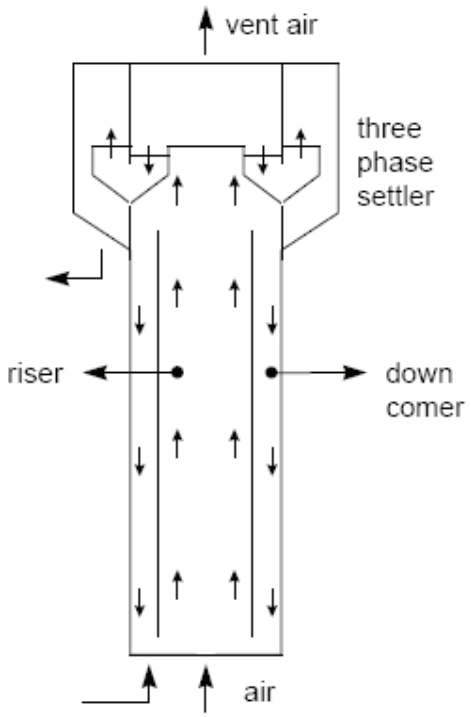


FIG. 6.1
schematic representation of biofilm airlift suspension reactor (BAS)

The liquid circulation velocity is the crucial parameter in the design of the BAS reactor because it determines the following processes:

— *Liquid mixing.* 95% homogeneity is achieved within seven circulations (Van der Lans, 1985).
— *Bubble recirculation.* Depending on the liquid circulation velocity different flow regimes with respect to gas bubble entrainment into the downcomer will exist. In processes where substrates (e.g. oxygen) have to be transfer from the gas phase to the liquid/solid phase, the liquid circulation velocity should be sufficient to ensure entrainment of bubbles into the downcomer and from the downcomer into the riser again. This is desired to achieve maximal aeration (gas hold-up).
— *Solids suspension.* There exists a critical minimal gas supply rate below which the liquid circulation velocity is insufficient to maintain a stable particle suspension. The liquid circulation velocity should at least be well above the settling velocity of the particles.

Knowledge of the liquid circulation rate is therefore of obvious importance. Reactor geometry (height, diameter, draught tube dimensions), gas supply rate and solids loading and properties will all determine the liquid circulation velocity and therewith mixing, gas hold-up and solids suspension.

Observed flow regimes in three-phase airlift reactors

The presence of suspended solids decreases the driving force for liquid circulation in comparison with a two-phase system by changing the average densities in riser and downcomer. Theoretically, for a three-phase system that is fully in steady state there exist a minimal gas supply rate for which the driving force for circulation becomes zero. In practice, the critical value is even higher than this theoretical minimal value as a result of fluctuations in the circulation velocity due to the interaction between dynamic variations in average densities in riser and downcomer (Van der Lans, 1985). It is obvious that in order to bring the solids in suspension at start-up the applied gas flow rate must be above this critical value. Moreover, at start-up, the settled particles form an extra flow resistance, necessitating a much higher gas flow rate (Hysteresis effect, Heck and Oncken, 1987). Three solid flow regimes are reported in literature (Douek et al., 1994) for three-phase airlift systems. These are, with increasing gas flow rate, packed bed (settled solids), fluidized bed (no solids recirculation) and circulated bed or complete suspension regime. Although depending on reactor geometry, solids loading and particle characteristics, in general the minimal gas supply rate for bubble entrainment into the downcomer is above the minimal

gas supply rate for the complete solids suspension regime. Based on observations at different scales (10^{-3}-10^{2} m^3), in three-phase systems, the following regimes have been noted with increasing air velocity (Fig. 6.2):

No gas entrainment (regime I)

This occurs at low gas velocities when the liquid circulation velocity is insufficient to entrain bubbles into the downcomer. The downcomer liquid velocity is nowhere higher than the bubble rise velocity.

Gas entrainment but no gas recirculation (regime II)

When the actual liquid velocity in the downcomer becomes about equal to the (downcomer) bubble swarm slip velocity, air is entrained into the downcomer. Sometimes a lower bubble free zone and an upper zone with bubbles is observed. The bubbles appear to remain stationary (e.g. Livingston and Zhang, 1993) or the gas entrainment rate (downflow) equals the bubble escape rate (upflow) (e.g. Choi and Lee, 1990). However, often an axial bubble distribution (e.g. Douek *et al.,* 1994) and an axial bubble size distribution is present. A minor gas recirculation rate may occur as a result of entrainment of tiny bubbles into the riser. In literature (e.g. Chisti, 1989) this regime is therefore not always clearly distinguished from the following regime. Complete solids suspension is commonly but not always present.

Complete gas recirculation (regime III)

All the air bubbles that are entrained into the downcomer are carried all the way down along the downcomer and into the riser again. There is no axial bubble size distribution in the downcomer any more, apart from a relatively small compression effect. The liquid velocity in the downcomer is significantly higher than the bubble swarm velocity, which leads to a large downward gas flow rate in the downcomer. Therefore, the riser gas flow rate, combining the sparged air and the recirculated air, is significantly higher than the injection flow rate. Again complete solids suspension is common, but incomplete suspension cannot be excluded.

The importance of the first two regimes (I and II) decreases with scale and it is difficult to operate in the regime of complete gas circulation (regime III) at laboratory scale. It's obvious that the gas flow rate to be supplied in case of regime III is higher than in case of regime II and I. Since our reactor is at lab-scale and there's no need to operate in regime III (the gas phase is used just for mixing and not to bring substrate like in aerobic process) our aim is the design and construction of a BAS reactor operating in regime I.

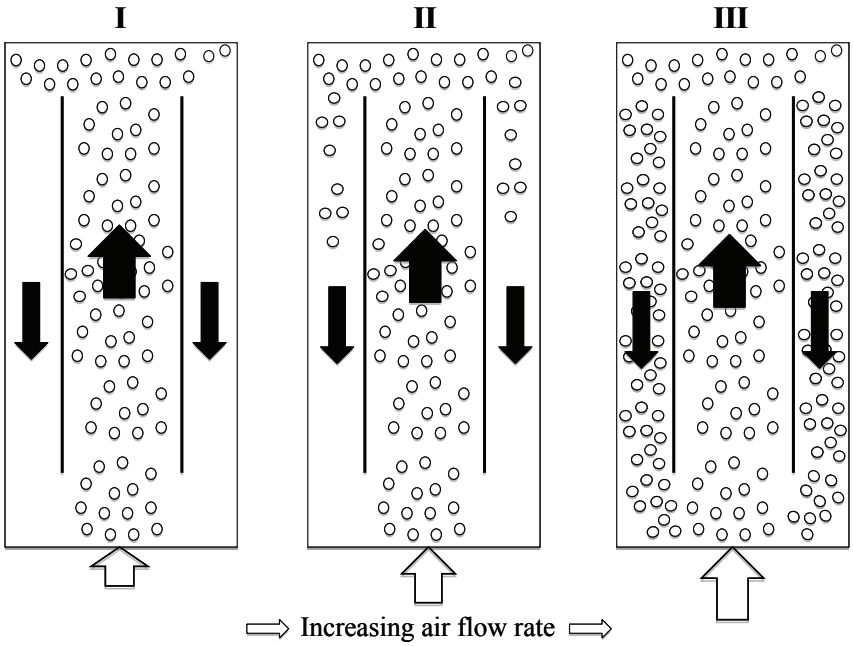


FIG. 6.2
The three observed gas flow regimes within the complete solids suspension regime: low gas velocity: no gas carry-over (I); transition regime: gas entrainment into downcomer but no gas recirculation (II); complete gas recirculation (III).

Design Criteria

Modeling of the hydrodynamics is essentially different for the three regimes aforementioned. Literature reports about gas-liquid airlift systems are abundant now. For regime I, an extensive review was given by Joshi et al. (1990). Chisti (1989) uses a simple approach to incorporate regime II. To model the gas recirculation regime (III), an extra expression is needed to describe the effect of the increased riser gas velocity due to gas recirculation (Heijnen et al., 1997).

Neglecting accumulation of momentum, a simple force balance holds for an airlift reactor:

gravity force = friction force

Expressed in pressure terms,

(6.1) $$\Delta p_g = \Delta p_f$$

An airlift reactor consists of two connected vertical channels, a riser and downcomer with effective (draught tube) height H_e. The driving force of motion is

(6.2) $$\Delta p_g = \rho_D g H_e - \rho_R g H_e$$

The difference in weight of contents of downcomer and riser causes a circulation flow. The expression for the (average) density in a three-phase (gas, liquid, solid, GLS) system is

(6.3) $$\rho = \varepsilon_G \rho_G + \varepsilon_L \rho_L + \varepsilon_S \rho_S$$

in which the gas term is negligible and

(6.4) $$\varepsilon_L = 1 - \varepsilon_G - \varepsilon_S$$

The driving head in a three-phase system may now be written as

(6.5) $$\frac{\Delta p_g}{g H_e} = \rho_D - \rho_R = \left(\varepsilon_{GR} - \varepsilon_{GD}\right)\rho_L - \left(\varepsilon_{SR} - \varepsilon_{SD}\right)\left(\rho_S - \rho_L\right)$$

The equation shows that if the solids hold-up in the riser is larger than in the downcomer, the presence of solids lowers the driving force.

The frictional force in its most simple form is represented by

(6.6) $$\Delta p_f = K_f \cdot \frac{1}{2}\rho v_L^2$$

The friction coefficient K_f depends on the circulation velocity v_L, but was demonstrated to be constant for two phase airlift systems (Van der Lans, 1985; Verlaan et al., 1986). It is a parameter that lumps all frictional effects such as wall friction, flow reversal at top and bottom and the loss due to the presence of a sparger, but it is a constant characteristic for a given apparatus. The use of the liquid density ρ_L for the density is reasonable in three-phase system but it's a simplification. The friction coefficient K_f can be calculated from the reactor geometry as (Heijnen et al., 1997)

(6.7) $$K_f = 2.5\left(\frac{1}{m} + \xi\frac{m}{(1-m)^2}\right) + 1.6\left(\frac{0.007}{m^2} + \frac{0.017}{(1-m)^2}\right)\frac{H_e}{D}$$

where m represents the draught tube cross-sectional area fraction between riser and reactor ($m=A_R/A$) and ξ represents the contribution of the top part of the reactor to the friction due to flow reversal (ξ=1, for usual BAS design).

Expression (6.7) is very interesting since it depends only on geometry and not on scale. The value of the friction coefficient can be determined experimentally simply by measuring circulation velocity and riser gas fraction without solids loading, provided there is no bubble carryover into the downcomer. In view of the restricted accuracy of the prediction this should always be done for comparison after construction of the column (Heijnen et al., 1997). In this Chapter the value of the friction coefficient was only predicted with Equation (6.7).

The simplest expression for the liquid circulation velocity v_L follows then from Eqations (6.5) and (6.6):

(6.8)
$$v_L^2 = \frac{2gH_e}{K_f}\left[\left(\varepsilon_{GR}-\varepsilon_{GD}\right)-\left(\varepsilon_{SR}-\varepsilon_{SD}\right)\left(\frac{\rho_S}{\rho_L}-1\right)\right]$$

Expressions to predict the differences in gas and solid hold-ups between riser and downcomer are now essential to be able to calculate the circulation velocity v_L. In regime I (ε_{GD}=0) the riser can be approximated to behave as a bubble column. An expression for the gas hold-up in bubble columns, such as the simple correlation for aqueous systems operated in the heterogeneous regime

(6.9)
$$\varepsilon_G = 0.6 v_{GS}^{0.7}$$

(Heijnen and Van't Riet, 1984) may therefore be used to predict the overall gas hold-up for regime I without downcomer gas flow rate. Equation (6.9) was demonstrated to be a reasonable prediction of the riser gas hold-up without gas in the downcomer in case of low liquid velocities (with $0.006<v_{GS}<0.06$ m s^{-1} based on riser cross sectional area) (Van der Lans, 1985). For the solid phase the same approach may be used. The solid distribution is not the result of suspension but of entrainment and is therefore axially homogeneous. Since there is no injection flow rate of solids this results in the simple expression

(6.10)
$$\varepsilon_{SR}-\varepsilon_{SD} = 2\varepsilon_S\frac{v_{SP}}{v_L}$$

where v_{SP} is the particle swarm velocity (solid slip velocity) and ε_S is the average solids hold-up which follows from the amount of solids added to the column and is therefore a known input parameter, provided that the suspension is complete and homogeneous and the system is batch. The swarm velocity of particles (v_{SP}) is defined as the difference between the single particle terminal velocity (v_T) and the circulation velocity (v_L). The single particle terminal velocity can be calculated as

(6.11)
$$v_T = \sqrt{\frac{4}{3}\frac{g}{C_D}\left(\frac{\rho_S-\rho_L}{\rho_L}\right)d}$$

where C_D is the drag coefficient and d is the particle diameter

The relationship valid for fluidization of rigid particles, readily available in the chemical-engineering literature (Eq. 6.11), have to be modified to take into account the effect of the biofilm layer. In particular the drag coefficient can be calculated as

(6.12)
$$\left(C_D\right)_{biofilm-particle} = 1.6\cdot\left(C_D\right)_{clean-particle}$$

Using Equation (6.9) for the riser gas hold-up, the circulation velocity for regime I follows then from Equations (6.8), (6.10) and ε_{GD}=0 as

(6.13)
$$v_L = \left(\frac{4gH_e}{K_f}\right)^{1/3}\cdot\left[0.3v_L\left(\frac{v_{Gs}}{m}\right)^{0.7}-\varepsilon_S v_{SP}\left(\frac{\rho_S}{\rho_L}-1\right)\right]^{1/3}$$

However, the equation to calculate the circulation velocity for regime I is not explicit and an iteration procedure is needed.

In order to maintain stable operations in a biofilm airlift suspension reactor the granules (self-aggregating biofilm) morphology needs to be smooth (applicability of Eq. (6.12)), dense (ρ_S used in Eq. (6.11) and (6.13)) and constant in time. In fact in these systems, biofilm growth alters particle size, apparent density, shape and roughness, and all these three factors habve a strong influence on the hydrodynamic behavior of the reactors. In the reactor study reported by Sliekers et al. (2003) the gas was sparged from the bottom of the reactor at a maximum gas flow of 200 mL min^{-1} for fluidization of the biomass. Arrojo et al. (2008) found a correlation between the superficial gas upflow velocity applied in an airlift SBR and anammox activity, suggesting that a riser superficial gas upflow velocity higher

than 10.6 cm min^{-1} (gas flow rate equal to 300 mL min^{-1}) may be negatively affecting. The limit for the superficial gas upflow velocity of 10.5 cm min^{-1} was considered during the design of the reactor presented in this Chapter.

6.2.2 Inoculum

See paragraph 3.2.1.

6.2.3 Analytical methods

Nitrogen soluble compounds and chemical oxygen demand (COD) were measured using commercial test kits according to the protocol of the manufacturer (brand: Dr.Lange test kits, Hach-Lange GmbH, Düsseldorf, DE, kits LCK 302, 303, 340, 341, 342, 414, 514, 614) and determined on a designated spectrophotometer (DR 5000). Soluble COD was measured after sample filtration (0.2 µm). TSS and VSS were determined according to the Standard Methods (APHA 2005).

6.2.4 Medium composition

The medium fed to the biofilm gas-lift suspension (BGS) reactor, consisted in a combination (in different proportions) of digester liquor originating from piggery wastewater anaerobic digestion diluted and synthetic medium. The digestate was taken from a piggery farm (20000 pigs) in Lombardy, Italy, and was sieved (mash size 0.8 mm) and stored at -20°C before utilization. The digestate composition is reported in Table 6.1. The synthetic medium was prepared according to Chapter 3. After the combination of synthetic medium with rising volumes of digestate (v/v% of digestate on synthetic medium 0, 10, 25, 50, 75%) the nitrite and ammonium content was adjusted to 1180±140 and 980±170 mg N L^{-1}, respectively by $(NH_4)_2SO_4$ and $NaNO_2$ addition. The experimental days in which the different medium compositions were applied are illustrated in Table 6.2.

Table 6.1: digestate composition

pH		8.0-8.4
Conductivity	mS cm^{-1}	7.3-8.5
Total Suspended Solid	gTSS L^{-1}	0.55±0.2
Tot-COD	mg L^{-1}	1845±1200
soluble-COD Tot-COD^{-1}	%	57±17
BOD_5 Tot-COD^{-1}	%	17±3
Ammonium	mg N L^{-1}	20-200
Nitrite	mg N L^{-1}	150-350
Nitrate	mg N L^{-1}	0-20
Total Kjeldahl Nitrogen (TKN)	mg N L^{-1}	20-200

Table 6.2: Volumetric ratio (as percentage) between digestate and synthetic medium used in different time periods.

Days	% (v/v)
1-5	0
6-9	10
10-17	25
18-24	50
25-33	75
34-42	100

6.2.5
Reactor operation and setup

A schematic representation of the biofilm gas-lift suspension reactor is depicted in Figure 6.3. The reactor was continuously fed with the medium described in par. 6.2.4 at a flow rate of 3±1 L d^{-1} by means of a peristaltic pump (HRT=2-3 d). The liquid volume was controlled at 7 L by a fix outlet installed in the top part of the reactor (at the top of the three phase separator): the discharge tube connected to the outlet was inserted in a vessel filled with effluent acting as a water lock. The SRT was not actively controlled, but from effluent particulate concentration was estimated to be about 50 days. The effluent was discharged by gravity. The reactor was equipped with on-line sensors for the measurement and data logging of pressure (P, mbar), temperature (T, C), oxidation/reduction potential (ORP, mV), dissolved oxygen (DO, saturation percentage) and pH. The temperature was controlled at 35°C by means of water jacket. pH was not controlled but was monitored on-line to be stable between 7.2 and 7.8 throughout the experimentation. The pressure inside the system was ensured by means of a water lock positioned at the gas exit at 8-10 mbar and was monitored throughout the experimentation by a pressure transducer installed on the reactor lead (Leo Record Pressure Data Logger, SensorONE Ltd, UK). The gas phase, recycled by means of a recirculation pump, was composed by the nitrogen gas produced during anammox reaction and by an externally added gas mixture of N_2 and CO_2 (95 and 5%, respectively) and was supplied in the bottom of the reactor by a porous sparger (positioned below the riser) at a flow rate of 0.2 L min^{-1}. The addition of the externally dosed N_2/CO_2 mixture was regulated by a pneumatic electrovalve to maintain the reactor at an overpressure of 8-10 mbar. A 5 L vessel was connected to the gas line in order to act as a gas buffer and maintain overpressure in the reactor during the flow rate fluctuation of the gas recycle pump.

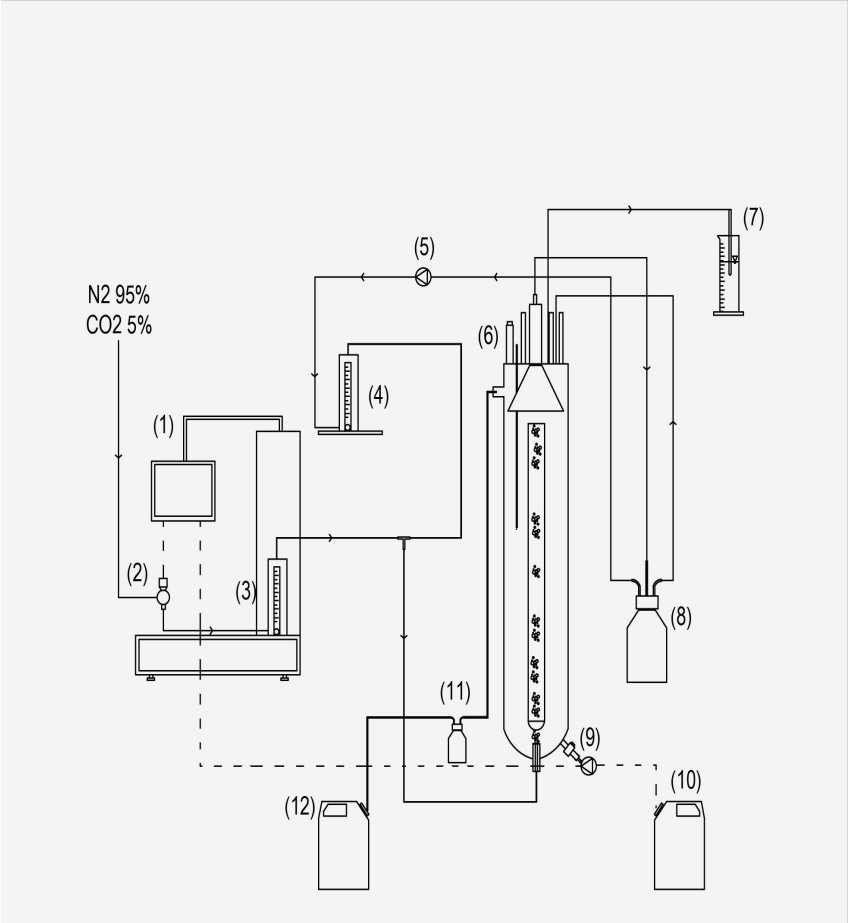


FIG. 6.3
schematic representation of the biofilm gas-lift suspension reactor. The setup consisted of: PC for data logging (1), electrovalve (2), rotometers (3,4), gas recycle pump (5), on-line sensors (pressure, temperature, ORP, pH, dissolved oxygen meter, 6), water lock connected to gas outlet (7), gas buffer vessel (8), feeding (peristaltic) pump (9), feeding medium tank (10), water lock of the liquid effluent discharge line (11), liquid effluent tank (12). Continuous lines represent gas and liquid tubing lines. Dashed lines represent the PC remote control of (2) and (9).

6.2.6
Batch test

The maximum activity of the anammox biomass present in the reactor was evaluated by means of two different procedures: batch test in which the nitrite concentration was followed in time and manometric batch test in which the nitrogen gas production was followed in time. In both procedures the influent pump was stopped and a solution containing ammonium 13 M (as $(NH_4)_2SO_4$) and nitrite 10 M (as $NaNO_2$) was injected into the reactor (starting ammonium and nitrite concentrations 2 mM and 2.6 mM, respectively). In the first procedure, the maximum volumetric nitrite removal rate ($Rate_{NO2}^{max}$, g NO_2^--N L^{-1} d^{-1}) was estimated by the (negative) slope of the decreasing nitrite concentration in time. In the second procedure manometric measurements were conducted in the reactor to determine the maximum N_2 production rate ($Rate^{max}_{N2}$). Before conducting the experiment the reactor was sealed in all its components in order to obtain a batch system. Pressure increase in time due to nitrogen gas production by the anammox reaction was recorded by a pressure transducer positioned in the reactor lead. Pressure data were then elaborated as reported in paragraph 4.2.9. From the obtained maximum volumetric N_2 production rate, the maximum volumetric nitrite removal rate was calculated according to the anammox reaction stoichiometry.

6.3
RESULTS AND DISCUSSION

6.3.1
Reactor design and validation

According to the criteria illustrated in par. 6.2.1 the reactor geometry was designed to be operated according to the following (flexible) operational parameters:

— Working volume 7 L; three-phase separator volume 1.5 L; headspace volume 1 L
— Flow regime I: no gas entrainment in the downcomer (to minimize external gas dosage)
— Granules average feret diameter of 0.8-1.5 mm retained in the system
— Complete biofilm suspension
— H_e/D ratio in the range 6-10
— Liquid flow rate entering the reactor equal to 3±1 L d^{-1} (wide operational window concerning the applied load)
— Superficial gas upflow velocity < 10.5 cm min^{-1} (maximum allowed shear stress)

The biofilm gas-lift suspension reactor, built according to the features described above, is shown in Figure 6.4.

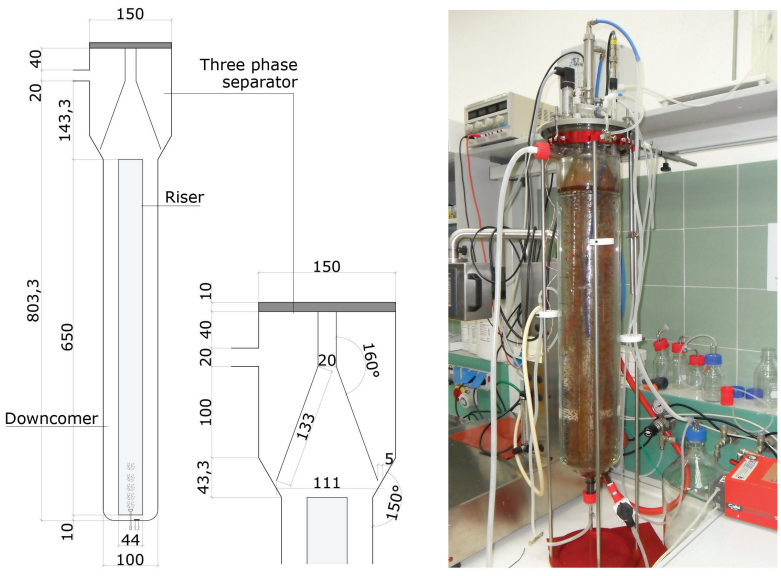


FIG. 6.4
Anammox biofilm gas-lift suspension reactor.

A lithium chloride (LiCl) tracer test was conducted in order to verify whether the liquid phase in the reactor was completely mixed and to verify the completely mixed volume. Lithium chloride was inserted at the bottom part of the reactor as spike addition of a concentrated LiCl solution (2 g L^{-1}). Liquid samples were taken at regular time intervals and lithium concentrations were measured by ion chromatography (DX-120, Dionex S.p.A, Milano, Italy). The test was conducted in absence of biomass and starting with the reactor filled with tap water. A tap water flow of 0.1 L min^{-1} was continuously pumped into the reactor by means of a peristaltic pump. Given that lithium is not converted in the reactor (tracer) and hypothesizing a completely mixed volume *V*, the following mass balance holds:

(6.14)
$$\frac{dC_{Li}}{dt}V = Q \cdot C^{IN}{}_{Li} - Q \cdot C_{Li}^{OUT}$$

where C_{Li} (mg L^{-1}) is the lithium concentration in the reactor, or in the influent entering the reactor (IN), or in the effluent leaving the reactor (OUT); *Q* is the applied flow rate (L min^{-1}); *t* is the time (min). Since the influent contained no lithium Equation (6.14) can be written as

(6.15)
$$\frac{dC_{Li}}{dt} = -\frac{Q}{V}C_{Li}^{OUT}$$

Integrating between $C(t_0)$ and C(t) and between $t=t_0$ and t=t, the equation of the lithium concentration in time can be identified:

(6.16)
$$C_{Li}(t) = C_{Li}(t_0)\exp\left(-\frac{t}{HRT}\right)$$

where $HRT=V\,Q^{-1}$ is the hydraulic retention time (min). Equation (6.16) represents the theoretical concentration decrease in time in an ideally mixed reactor of volume *V*. The experimental data were interpolated with an exponential equation like Eq. (6.16) changing the value of *V* and $C_{Li}(t_0)$. The best fitting was evaluated by means of the least-squares method (Fig. 6.5).

The best fitting was found for *V*=6.95 L and $C_{Li}(t_0)$=2.8 mg L^{-1}. Since the model mimic the theoretical tracer concentration in time, the hypothesis of completely mixed volume V can be considered correct. The reactor was therefore properly designed concerning the liquid mixing feature required. The slightly smaller working volume identified with the tracer test (6.95 vs 7 L) may be due to the three-phase separator volume (1.5 L) which is obviously less intensively mixed than the lower part of the reactor.

6.3.2 Reactor operation

The gas-lift biofilm reactor was operated under nitrite limitation throughout the experimentation. The influent flow rate was increased (decreased) on daily basis when nitrite concentration was found to be lower (higher) than 1-2 mg N L^{-1}. The average ammonium and nitrite concentration in the influent medium were 980±170 and 1180±140, respectively (Fig. 6.6). During the experimentation the imposed loading rate of ammonium and nitrite was equal to 390±220 and 470±290 mg N L^{-1} d^{-1}, respectively, while the ammonium and nitrite removal rate were 350±200 and 470±280 mg N L^{-1} d^{-1}, respectively. The imposed loading rate and the removal rate of ammonium and nitrite are shown in Figure 6.7.

The digestate proportion (v/v) in the influent medium was increased stepwise after 5-7 days of stable conversion. Nitrite consumption was almost complete (>99.7%) throughout the experimentation (Fig. 6.8). Ammonium removal efficiency was also about 90% with a slight negative trend when digestate proportion was increased till 50% of the influent medium. Total nitrogen removal efficiency was constantly above 87% and was stable at about 89% when undiluted digestate was fed to the reactor. In Table 6.3 the average removal efficiency relative to rising digestate content in the feeding medium is presented.

In the anammox reactor (treating the effluent of a SHARON reactor) reported by Hwang et al. (2005) the volumetric nitrogen removal rate reached 0.72 g soluble-N L^{-1} d^{-1}. The

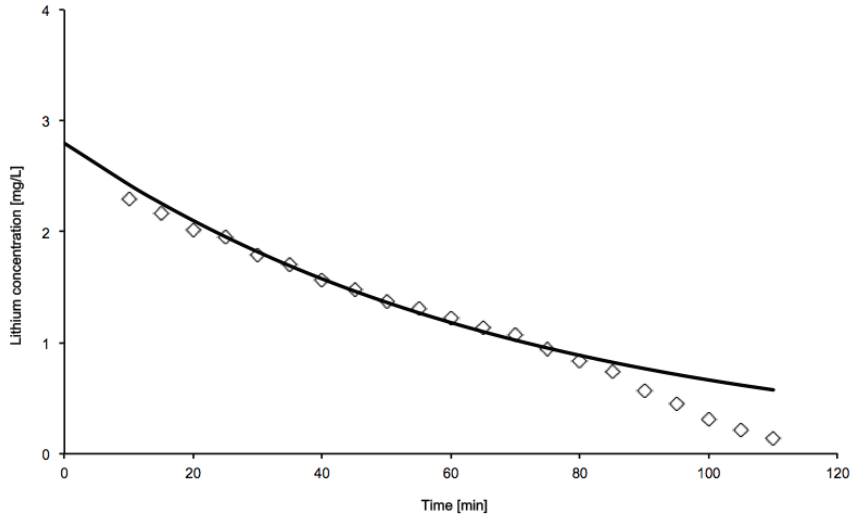


FIG. 6.5
Lithium (tracer) concentration in time: experimental data (diamonds) and theoretical behavior (V=6.95 L, $C_{Li}(t_0)$=2.8 mg L^{-1}) according to Eq. (6.16) (line).

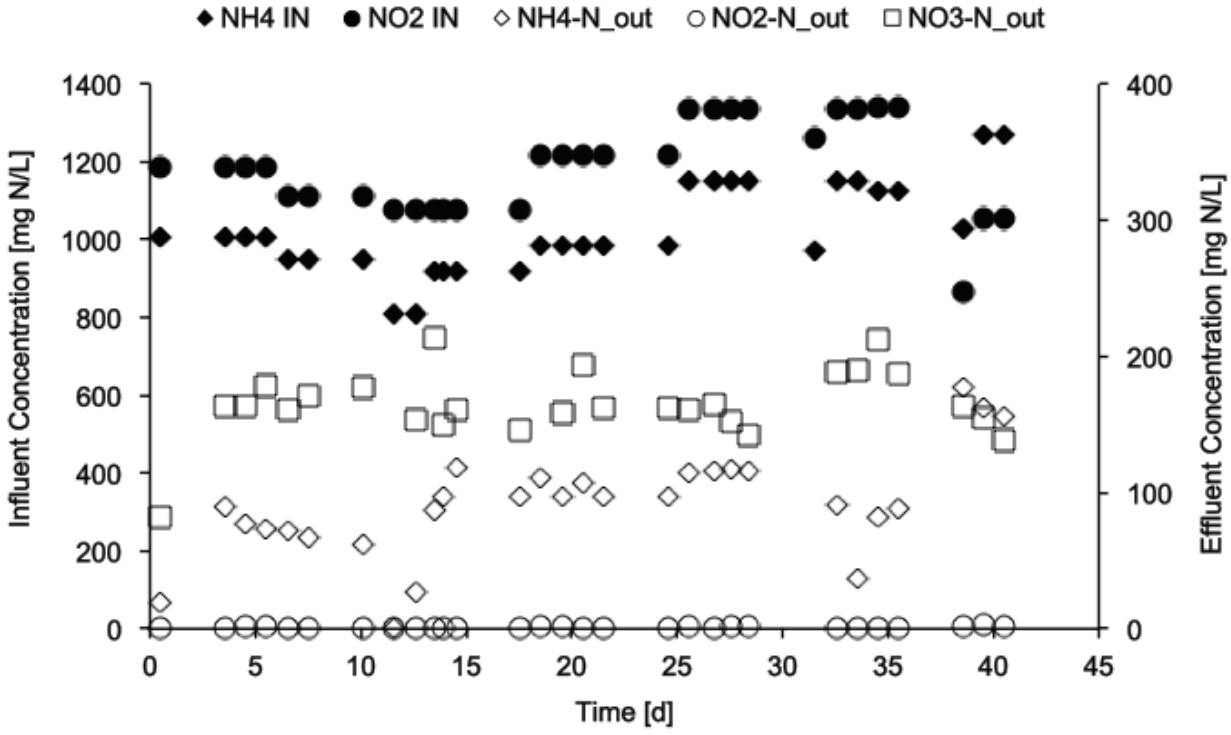


FIG. 6.6
influent (closed symbol) and effluent (open symbol) concentration of ammonium (diamonds), nitrite (circles) and nitrate (squares) in time.

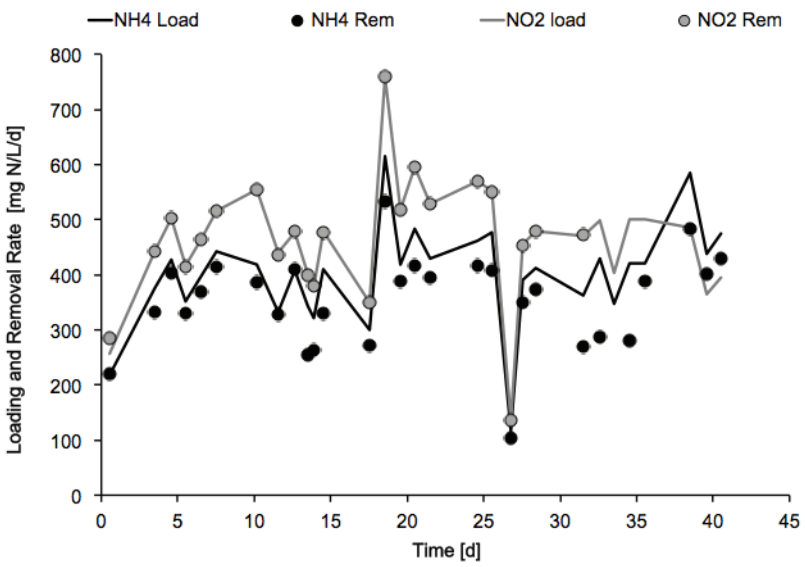


FIGURE 6.7
Nitrogen imposed loading rate (line) and removal rate (marker) [mg N L^{-1} d^{-1}] of ammonium (black) and nitrite (grey) in time [d].

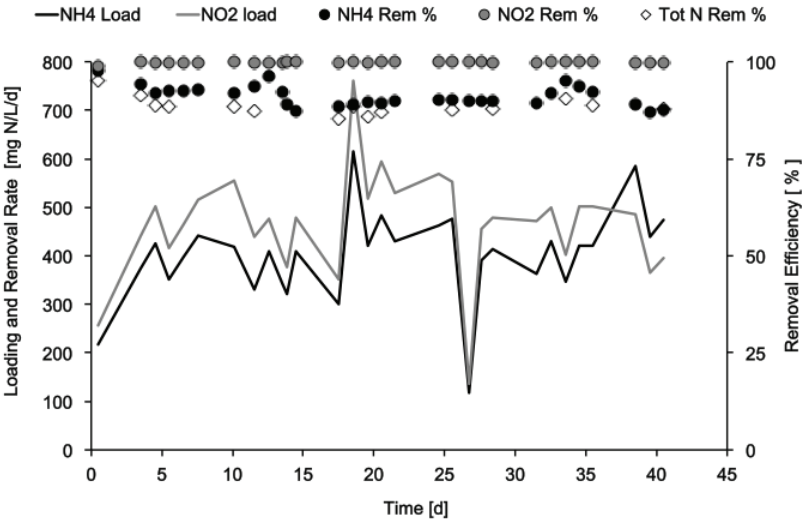


FIG. 6.8
Nitrogen imposed loading rate (line, left axis) [mg N L^{-1} d^{-1}] and removal efficiency (marker, right axis) [%] of ammonium (black) and nitrite (grey) in time [d]. Open diamonds represents the removal efficiency of total nitrogen.

anammox volumetric nitrogen removal rate (NRR) reported by Yamamoto et al. (2008) was 0.22 kg N m^{-3} d^{-1} when treating the effluent of a partial nitritation reactor fed with diluted (4 times) swine digestate. In a following research Yamamoto et al. (2011) reported a relatively high anammox nitrogen removal rate of 2.0 g N L^{-1} d^{-1} (at a nitrogen loading rate, NLR, of 2.2 g N L^{-1} d^{-1}) treating filtered and diluted (7-10 times) partially nitritated piggery digestate. Quiao et al. (2010) reported an anammox NRR of 3.1 kg N m^{-3} d^{-1} when treating a diluted (> 1.5 times) and partially nitritated liquid fraction of digested piggery wastewater.

Table 6.3: Nitrogen removal efficiencies relative to time periods in which the feeding medium contained a constant digestate volumetric percentage.

Days, d	Digestate, v/v %	Nitrogen Removal Efficiency, %		
		Tot N	Ammonium	Nitrite
1-5	0	90.7	93.8	99.8
6-9	10	87.8	92.3	99.9
10-17	25	87.9	91.8	99.8
18-24	50	87.5	89.8	99.9
25-33	75	89.2	90.5	99.9
34-42	100	89.0	90.8	99.9

The ratio between nitrite and ammonium removal rate (R_NiAm) and the ratio between nitrate production and ammonium removal rate (R_NaAm) varied during the experimentation (Fig. 6.9). A negative trend of R_NiAm can be noticed for digestate volumetric percentage in the medium higher than 75% (since day 25). Same trend can be noticed for the R_NaAm especially when the reactor was fed with undiluted digestate (since day 34). This is not surprising when considering the COD content of the digestate (about 300 mg COD L^{-1} as BOD_5, par. 6.2.4). The increment of digestate percentage in the feeding medium corresponds to an increment of COD concentration in the medium itself and therefore of COD-loading rate. When the reactor was fed with undiluted digestate, the COD removal rate was about 100 mg COD L^{-1} d^{-1}, at a COD loading rate of about 290 mg COD L^{-1} d^{-1}. Assuming that the electron content of the removed COD was used to reduce nitrate to nitrite in the typical denitrifying metabolic pathway or even in the DNRA (dissimilatory nitrate reduction to ammonium) pathway, the observed COD removal rate would correspond to a nitrate reduction rate to nitrite of 90 mg N L^{-1} d^{-1}. The heterotrophic reduction of nitrate to nitrite would lead to underestimation of the actual R_NiAm and R_NaAm. Since recent results showed that anammox bacteria use nitrate directly and even outcompete heterotrophs for organic acids in presence ammonium (Kartal et al., 2006) DNRA can't be excluded. Molinuevo et al. (2009) reported that denitrification become the dominant process after increasing the fraction of digester liquor up to 12%v/v in the influent (corresponding to 242 mg COD L^{-1}), but no details are given about the biodegradable fraction of the COD content of the influent. In this Chapter, even if it's not possible to quantify the denitrification activity, we can certainly exclude that denitrifiction contributed significantly to the nitrogen removal: less than 5% total nitrogen entering the system could be removed by heterotrophic denitrification (about 40 mg N_2-N L^{-1} d^{-1} production rate) according to COD and nitrogen mass balances. Hwang et al. (2005) reported an average (on about two months data) R_NiAm of 2.13, but they speculated that denitrification accounted only for 6.4 mg N L^{-1} d^{-1} (at a loading rate of 1.36 g N L^{-1} d^{-1}) according to COD mass-balance.

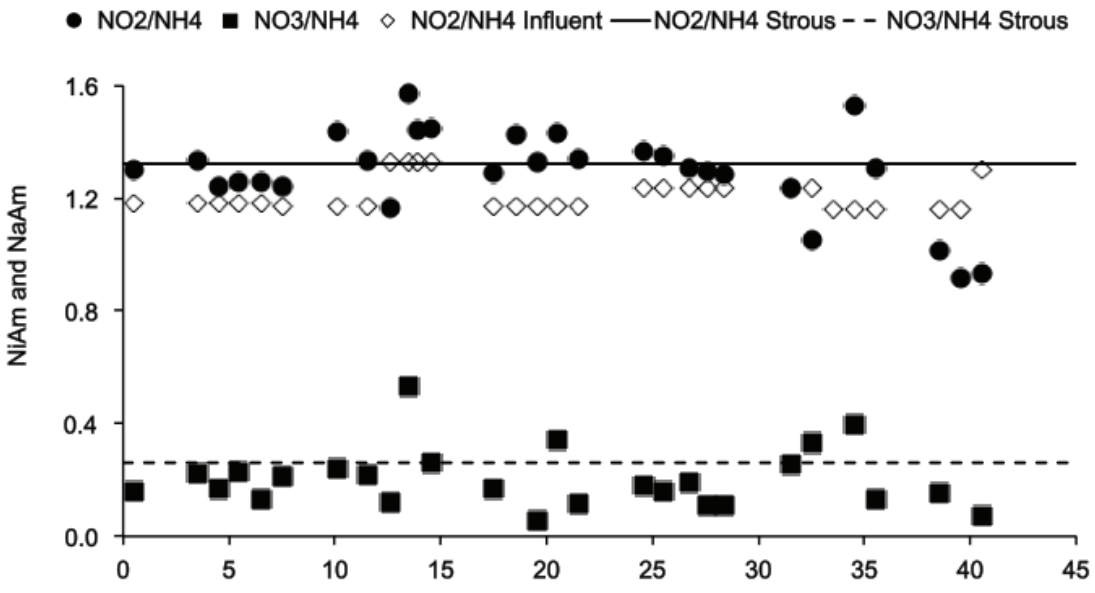


FIG. 6.9
ratio between nitrite and ammonium removal rate (R_NiAm) during reactor operation (closed circles) and in the influent (open diamonds); ratio between nitrate production and ammonium removal rate (R_NaAm, closed squares). The continuous and dashed line represents R_NiAm and R_NaAm as reported by Strous et al., 1998, respectively.

6.3.3
Maximum activity measurement

The maximum volumetric nitrite removal rate (MNRR) was evaluated in the reactor in seven occasions during the experimentation in order to monitor the reactor performance and adjust the imposed nitrite-loading rate. The batch tests were carried out following two different procedures as described in par. 6.2.6. The results (with both procedures) are shown in Table 6.4, where day zero stands for the day in which the reactor was inoculated. On day 0 the reactor was inoculated with an amount of biomass in excess relative to the amount necessary to treat the imposed N-load that was calculated from specific maximum activity assays. It's therefore not surprising that the maximum volumetric nitrite removal rate decreases (about 40%) between day 0 and day 4, due the wash out of excess biomass. During the rest of the experimentation the MNRR was about constant and decreased of 10% passing from 75 to 100% (v/v) of digestate in the influent. The two tests conducted on day 21 and 22 with the different procedures showed 10% discrepancy in the obtained result. The fact that in the manometric procedure there's no necessity for sampling and analytical measurements, is a significant advantage that makes this technique cheaper, less invasive and easier to operate. Since with biofilm systems is very hard to take a biomass mixed sample in a reproducible way, biomass specific activities are not reported in this Chapter. The evidence of these difficulties makes also unreliable the evaluation of maximum activity in small batch tests, taking part of the mixed liquor out of the reactor. For the same reason the biomass specific activity evaluation conducted out of the reactor is hardly comparable with the reactor performance since the actual biomass concentration inside the reactor is known only approximately. The possibility to monitor the maximum volumetric activity directly in the reactor minimizing process disturbances, costs and working time makes the presented rector setup a valuable research tool for the study of the anammox process.

Table 6.4: maximum volumetric nitrite removal rate (MNRR) estimated from the decrease of nitrite concentration in time (C) and from the increase of the reactor headspace pressure in time caused by nitrogen gas production (M). MNRR values are expressed as g N L^{-1} d^{-1}.

Day d	Digestate % (v/v)	Procedure	MNRR g N L^{-1} d^{-1}
0	0	C	1.46
4	0	M	0.85
13	25	M	0.88
21	50	M	1.04
22	50	C	0.90
26	75	M	1.00
39	100	M	0.89

To our knowledge it's the first time that an anammox reactor was shown to treat the undiluted digestate originating from swine wastewater anaerobic digestion at an average nitrogen removal rate of 850±280 mg N L^{-1} d^{-1} and with 89% total nitrogen removal efficiency. In this study though, an absolute steady state (stable conversion for a period longer than three times the SRT) was never reached, but stable conversion was observed for periods of few days in several occasions. Even if the results shown in this Chapter would suggest the feasibility of nitrogen removal from swine digestate with the anammox process, longer experimentation is necessary for reliable evaluations.

6.4 CONCLUSIONS

The feasibility of the treatment of the liquid fraction of digested swine wastewater was tested in this Chapter. A biofilm gas-lift suspension anammox reactor was specifically designed. The digestate was added to the medium fed to the reactor in increasing proportion and undiluted digestate was treated since 34 days after the start-up. A nitrogen removal rate of 850±280 mg N L^{-1} d^{-1}, with 89% total nitrogen removal efficiency was shown. The in-reactor measurement of the maximum anammox activity represents an advantageous technique for the monitoring of process performance. In this Chapter the anammox process was shown to effectively remove nitrogen from a digestate originating from the anaerobic digestion of swine manure. However, given the variable composition of this kind of waste in terms of COD, heavy metals and antibiotics, it's necessary to perform stable reactor operations over a longer period of time, before assessing that anammox bacteria can successfully treat swine wastewaters.

SUMMARY

Nowadays, environmental consciousness and new EU regulations stimulate the search for cost-effective nitrogen removal techniques. A very attractive biological treatment is the fully autotrophic nitrogen removal. In this technique partial nitritation by AOBs is followed by anaerobic ammonium oxidation via the anammox process, resulting in the production of dinitrogen gas. In this system the anaerobic ammonium oxidation (anammox), carried out by different microorganism related to the Plantomycetes, forms the limiting step. A further insight in the physiology and kinetics of these bacteria would allow a more efficient use of their precious metabolic features.

Low-Tech Fed-Batch Operation

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 very important both for research and applicative purposes. Enrichment from environmental sludge samples is a way to fulfill this need. Usually for this enrichment, a sequencing batch reactor (SBR) is used. However, in Chapter 2 of this thesis the efficacy of the low-tech fed-batch was tested. The conclusion of the experimental campaign on the enrichment of anammox biomass taken from Italian sludge samples was that with the fed-batch procedure, an anammox enrichment with activity of 0.44 mg $N\text{-}NH_4^+$ L^{-1} h^{-1} was achieved after about 110-130 days, sufficient to inoculate a continuously operated anammox reactor.

Oxygen as a Trigger for Anammox Growth as Free-Cells

Research groups around the world have been elaborately studying numerous and divers aspects of anammox bacteria. Among these topics are the physiology of the anammox bacteria and their applications in wastewater. The accuracy and reliability of all of these researches depends on a necessary precondition, namely the availability of a highly enriched suspended culture of anammox bacteria.

Regarding this precondition, in Chapter 3 an in-depth study was done on the anammox physiology and kinetics. The method for growing high purity anammox bacteria as free-cells was shown here univocally for the first time. Oxygen was found to be the key factor, forcing anammox bacteria in a membrane bioreactor to aggregate at lower loads. The (intrinsic) nitrite half-saturation constant for anammox bacteria as free-cells was identified to be equal to 35 µg N L^{-1}. From the yield and the elemental biomass composition the macro-chemical reaction equation was identified and validated by long-term reactor operations and dedicated experiment. The anammox culture enriched during this study exhibited an unreported high biomass specific maximum growth rate of 21 d^{-1} corresponding to a doubling time of 3.3 days. Because of the large availability of an almost pure anammox culture and the possibility to a faster reactor start-up time, the MBR system presented in Chapter 3 represents a powerful tool for both scientific and applicative purposes.

No Nitrite Nightmare

Despite earlier reports on potential toxicity of nitrite for anammox activity, the study in Chapter 4 shows that the levels of nitrite on which inhibition of anammox activity occurs are rather high (IC_{50} of 0.4 g N L^{-1}), and that biomass relatively quickly and entirely recovers from high nitrite concentrations. The time of exposure to nitrite was identified as an important factor, leading to increased and more severe inhibition as exposure was prolonged. However, in several cases with decreased anammox activity, high nitrite concentrations were reported as a cause for the activity loss, while that might be an incorrect induction. When anammox activity decreases for unknown or external reasons, the nitrite level in the reactor rises accordingly, as the anammox are unable to process the substrate. It was also confirmed once more that anammox activity can well recover from aerated periods, and therefore the results in Chapter 4 further underline that the anammox process can be a stable one not prone to temporary adverse effects of oxygen and nitrite in the reactors.

Swines are Fine!

In the field of livestock wastewaters the development of anaerobic digestion is hindered by difficulties connected with the nitrogen removal from the produced digestate. The application of the anammox process can possibly provide a cost-effective and environmental friendly solution for the treatment of the digestate, making the production of biogas from manure more attractive.

Since swine wastewater is commonly afloat with unwanted substances like heavy metals and antibiotics, their effect on anammox activity has to be evaluated. The study in Chapter 5 shows that increasing concentrations and prolonged exposure to heavy metals (copper and zinc) and antibiotics (oxytetracycline and sulfathiazole) lead to a decreasing specific anammox activity (SAA).

However, nitrate production stayed consistent and no remarkable discrepancy from the accepted anammox stoichiometry was found, indicating that both anabolism and catabolism reactions were active during the exposure to the inhibitors. Considering the average concentrations expected in swine wastewaters (after swine manure anaerobic digestion and solid/liquid separation), 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 higher biomass concentration in the reactor.

Then in Chapter 6 the feasibility of nitrogen removal by the anammox process from the swine manure digestate was tested. For this process a custom-made biofilm gas-lift suspension anammox reactor was specifically designed. In this reactor the digestate was added to the medium fed to the anammox bacteria with increasing proportions. From the 34th day on, an undiluted digestate was fed and an 89% of total nitrogen removal efficiency was shown. The in-reactor measurement performed of the maximum anammox activity represents an advantageous technique for the monitoring of process performance.

In this study the anammox process was shown to effectively remove nitrogen from a digestate originating from the anaerobic digestion of swine manure. However, given the variable compositions of such waste in terms of COD, heavy metals and antibiotics, it’s necessary to perform stable reactor operations over a longer period of time, before explicitly can be stated that anammox bacteria can successfully treat swine wastewaters.

REFERENCES

Ahn, Y.H., I.S. Hwang, K.S. Min (2004). ANAMMOX and partial denitritation in anaerobic nitrogen removal from piggery waste. Water Science and Technology 49: 145-153.

Álvarez, J.A., L. Otero, J.M. Lema, F. Omil (2010). The effect and fate of antibiotics during the anaerobic digestion of pig manure. Bioresource Technology 101(22): 8581-8586.

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

Angenent, L.T., S. Sung, L. Raskin (2002). Methanogenic population dynamics during startup of a full-scale anaerobic sequencing batch reactor treating swine waste. Water Research 36(18): 4648-4654.

Anthonisen, A.C., R.C. Loehr, T.B.S. Prakasam, E.G. Srinath (1976). Inhibition of nitrification by ammonia and nitrous acid. Journal of the Water Pollution Control Federation 48(5): 835-852.

APHA (2005). Standard methods for the examination of water and wastewater. A.P.H. Association. Washington, DC, USA, American Water Works Association (AWWA) & Wat. Env. Fed. (WEF).

Arikan, O.A., L.J. Sikora, W. Mulbry, S.U. Khan, C. Rice, G.D. Foster (2006). The fate and effect of oxytetracycline during the anaerobic digestion of manure from therapeutically treated calves. Process Biochemistry 41(7): 1637-1643.

Arrojo, B., M. Figueroa, A. Mosquera-Corral, J.L. Campos, R. Mendez (2008). Influence of gas flow-induced shear stress on the operation of the Anammox process in a SBR. Chemosphere 72(11): 1687-1693.

Baran, W., J. Sochacka, W. Wardas (2006). Toxicity and biodegradability of sulfonamides and products of their photocatalytic degradation in aqueous solutions. Chemosphere 65(8): 1295-1299.

Bassin, J.P., M. Pronk, R. Kraan, R. Kleerebezem, M.C.M. van Loosdrecht (2011). Ammonium adsorption in aerobic granular sludge, activated sludge and anammox granules. Water Research 45: 5257-5265.

Bettazzi, E., S. Caffaz, C. Vannini, C. Lubello (2010). Nitrite inhibition and intermediates effects on Anammox bacteria: A batch-scale experimental study. Process Biochemistry 45(4): 573-580.

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

Caffaz, S., E. Bettazzi, D. Scaglione, C. Lubello (2008). An integrated approach in a municipal WWTP: Anaerobic codigestion of sludge with organic waste and nutrient removal from supernatant. Water Science and Technology. 58: 669-676.

Campos, J.L., J.M. Garrido, R. Méndez, J.M. Lema (2001). Effect of two broad-spectrum antibiotics on activity and stability of continuous nitrifying system. Applied Biochemistry and Biotechnology - Part A Enzyme Engineering and Biotechnology 95(1): 1-10.

Çeçen, F., N. Semerci, A.G. Geyik (2010). Inhibitory effects of Cu, Zn, Ni and Co on nitrification and relevance of speciation. Journal of Chemical Technology and Biotechnology 85(4): 520-528.

Chamchoi, N., S. Nitisoravut (2007). Anammox enrichment from different conventional sludges. Chemosphere 66(11): 2225-2232.

Chisti, Y. (1989). Airlift Bioreactors. New York, Elsevier Applied Science.

Cho, S., Y. Takahashi, N. Fujii, Y. Yamada, H. Satoh, S. Okabe (2010). Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. Chemosphere 78(9): 1129-1135.

Choi, E., Y. Eum, K.I. Gil, S.W. Oa (2004). High strength nitrogen removal from nightsoil and piggery wastes. Water Science and Technology 49(5-6): 97-104.

Choi, K.H., W.K. Lee (1990). Recirculation and flow structures of gas in downcomer section of a concentric cylindrical airlift reactor. Journal of Chemical Technology and Biotechnology 48(1): 81-95.

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

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

Dapena-Mora, A., S.W.H. Van Hulle, J.L. Campos, R. Méndez, P.A. Vanrolleghem, M. Jetten (2004). Enrichment of Anammox biomass from municipal activated sludge: Experimental and modelling results. Journal of Chemical Technology and Biotechnology 79(12): 1421-1428.

Dong, X., E.W. Tollner (2003). Evaluation of Anammox and denitrification during anaerobic digestion of poultry manure. Bioresource Technology 86(2): 139-145.

Douek, R.S., A.G. Livingston, A.C. Johansson, G.F. Hewitt (1994). Hydrodynamics of an external-loop three-phase airlift (TPAL) reactor. Chemical Engineering Science 49(22): 3719-3737.

Egli, K., U. Fanger, P.J.J. Alvarez, H. Siegrist, J.R. Van der Meer, A.J.B. Zehnder (2001). Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Archives of Microbiology 175(3): 198-207.

Engels, H. (2004). Behavior of tetracyclines and sulfonamides in ausgewhlten Wirtschaftsdnger and grounds opposite. Göttingen, Germany, Georg-August-Universität Göttingen. Ph.D.

Fernández, I., J. Dosta, C. Fajardo, J.L. Campos, A. Mosquera-Corral, R. Méndez (2010). Short- and long-term effects of ammonium and nitrite on the Anammox process. Journal of Environmental Management 1: 1-5.

Fernández, I., A. Mosquera-Corral, J.L. Campos, R. Méndez (2009). Operation of an Anammox SBR in the presence of two broad-spectrum antibiotics. Process Biochemistry 44(4): 494-498.

Ferris, M.J., G. Muyzer, D.M. Ward (1996). Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. Applied and Environmental Microbiology 62(2): 340-346.

Furukawa, K., Y. Inatomi, S. Qiao, L. Quan, T. Yamamoto, K. Isaka, T. Sumino (2009). Innovative treatment system for digester liquor using anammox process. Bioresource Technology 100(22): 5437-5443.

Fux, C. (2003). Biological nitrogen elimination of ammonium-rich sludge digester liquids. Zurich, Switzerland, ETH Zurich, Switzerland. Ph.D. thesis.

Fux, C., M. Boehler, P. Huber, I. Brunner, H. Siegrist (2002). Biological treatment of ammonium-rich wastewater by partial nitritation and subsequent anaerobic ammonium oxidation (anammox) in a pilot plant. Journal of Biotechnology 99(3): 295-306.

Fux, C., V. Marchesi, I. Brunner, H. Siegrist (2004). Anaerobic ammonium oxidation of ammonium-rich waste streams in fixed-bed reactors. Water Science and Technology. 49: 77-82.

Fux, C., H. Siegrist (2004). Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: Environmental and economical considerations. Water Science and Technology. 50: 19-26.

Godia, F., C. Sola (1995). Fluidized-bed bioreactors. Biotechnology Progress 11(5): 479-497.

Grunditz, C., L. Gumaelius, G. Dalhammar (1998). Comparison of inhibition assays using nitrogen removing bacteria: Application to industrial wastewater. Water Research 32(10): 2995-3000.

Heck, J., U. Onken (1987). Hysteresis effects in suspended solid particles in bubble columns with and without draft tube. Chemical Engineering Science 42(5): 1211-1212.

Heijnen, J.J., J. Hols, R.G.J.M. Van Der Lans, H.L.J.M. Van Leeuwen, A. Mulder, R. Weltevrede (1997). A simple hydrodynamic model for the liquid circulation velocity in a full-scale two- and three-phase internal airlift reactor operating in the gas recirculation regime. Chemical Engineering Science 52(15): 2527-2540.

Heijnen, J.J., A. Mulder, W. Enger, F. Hoeks (1989). Review on the application of anaerobic fluidized bed reactors in waste-water treatment. Chemical Engineering Journal 41(3): B37-B50.

Heijnen, J.J., K. Van't Riet (1984). Mass transfer, mixing and heat transfer phenomena in low viscosity bubble column reactors. The Chemical Engineering Journal 28(2): B21-B42.

Heijnen, J.J., M.C.M. van Loosdrecht, R. Mulder, R. Weltevrede, A. Mulder (1993). Development and scale-up of an aerobic biofilm air-lift suspension reactor. Water Science and Technology 27(5-6): 253-261.

Heijnen, S.J., A. Mulder, R. Weltevrede, P.H. Hols, H.L.J.M. van Leeuwen (1990). Large-scale anaerobic/aerobic treatment of complex industrial wastewater using Immobilized biomass in fluidized bed and air-lift suspension reactors. Chemical Engineering and Technology 13(3): 202-208.

Hill, D.T., J.P. Bolte (2000). Methane production from low solid concentration liquid swine waste using conventional anaerobic fermentation. Bioresource Technology 74(3): 241-247.

Huang C.H., Renewm J.E., Pinkstonm K., Sedlakm D.L. (2001) Occurrence and fate of antibiotic compounds in municipal wastewater and animal waste', 74th Water Environment Federation Annual Conference & Exposition (WEFTEC), Atlanta, GA, 686-697(12).

Hwang, I.S., K.S. Min, E. Choi, Z. Yun (2005). Nitrogen removal from piggery waste using the combined SHARON and ANAMMOX process. Water Science and Technology 52(10-11): 487-494.

Hwang, I.S., K.S. Min, E. Choi, Z. Yun (2006). Resource recovery and nitrogen removal from piggery waste using the combined anaerobic processes. Water Science and Technology 54(8): 229-236.

Jetten, M.S.M., I. Cirpus, B. Kartal, L. Van Niftrik, K.T. Van De Pas-Schoonen, O. Sliekers, S. Haaijer, W. Van Der Star, M. Schmid, J. Van De Vossenberg, I. Schmidt, H. Harhangi, M. Van Loosdrecht, J.G. Kuenen, H. Op Den Camp, M. Strous (2005). 1994-2004: 10 Years of research on the anaerobic oxidation of ammonium. Biochemical Society Transactions 33(1): 119-123.

Jetten, M.S.M., H.J.M. Op Den Camp, J.G. Kuenen, M. Strous (2010). Family I. "Candidatus Brocadiaceae" fam. nov. in Bergey's Manual of Systematic Bacteriology. K.N. R., S.J. T., B.D. R.et al (eds). New York, Springer. 4: 596-602.

Jones, R.M., P. Dold, I. Takàcs, K. Chapman, B. Wett, S. Murthy, M. O'Shaughnessy (2007). Simulation for operation and control of reject water treatment processes. WEFTEC, San Diego.

Joshi, J.B., V.V. Ranade, S.D. Gharat, S.S. Lele (1990). Sparged loop reactors. Canadian Journal of Chemical Engineering 68(5): 705-741.

Jung, J.Y., S.H. Kang, Y.C. Chung, D.H. Ahn (2007). Factors affecting the activity of anammox bacteria during start up in the continuous culture reactor. Water Science and Technology 55: 459-468.

Kartal, B., M.M.M. Kuypers, G. Lavik, J. Schalk, H.J.M. Op Den Camp, M.S.M. Jetten, M. Strous (2007). Anammox bacteria disguised as denitrifiers: Nitrate reduction to dinitrogen gas via nitrite and ammonium. Environmental Microbiology 9(3): 635-642.

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

Kartal, B., L. Van Niftrik, J. Rattray, J.L.C.M. Van De Vossenberg, M.C. Schmid, J. Sinninghe DamstÃ©, M.S.M. Jetten, M. Strous (2008). Candidatus 'Brocadia fulgida': An autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiology Ecology 63(1): 46-55.

Kemper, N., H. Fárber, D. Skutlarek, J. Krieter (2008). Analysis of antibiotic residues in liquid manure and leachate of dairy farms in Northern Germany. Agricultural Water Management 95(11): 1288-1292.

Kieling, D.D., V. Reginatto, W. Schmidell, D. Travers, R.J. Menes, H.M. Soares (2007). Sludge wash-out as strategy for Anammox process start-up. Process Biochemistry 42(12): 1579-1585.

Kimura, Y., K. Isaka, F. Kazama (2011). Effects of inorganic carbon limitation on anaerobic ammonium oxidation (anammox) activity. Bioresource Technology 102(6): 4390-4394.

Kimura, Y., K. Isaka, F. Kazama, T. Sumino (2010). Effects of nitrite inhibition on anaerobic ammonium oxidation. Applied Microbiology and Biotechnology 86(1): 359-365.

L'Herroux, L., S. Le Roux, P. Appriou, J. Martinez (1997). Behaviour of metals following intensive pig slurry applications to a natural field treatment process in Brittany (France). Environmental Pollution 97(1-2): 119-130.

Lallai, A., G. Mura, N. Onnis (2002). The effects of certain antibiotics on biogas production in the anaerobic digestion of pig waste slurry. Bioresource Technology 82(2): 205-208.

Lettinga, G., A.F.M. Van Velsen, S.W. Hobma, W.J. De Zeeuw, A. Klapwijk (1980). Use of the upflow sludge blanket reactor concept for biological wastewater treatment especially for anaerobic treatment. Biotechnol. Bioeng. 22: 699-734.

Li, X., G. Zen, K.H. Rosenwinkel, S. Kunst, D. Weichgrebe, A. Cornelius, Q. Yang (2004). Start up of deammonification process in one single SBR system. Water Science and Technology. 50: 1-8.

Liao, D., X. Li, Q. Yang, G. Zeng, L. Guo, X. Yue (2008). Effect of inorganic carbon on anaerobic ammonium oxidation enriched in sequencing batch reactor. Journal of Environmental Sciences 20(8): 940-944.

Livingston, A.G., S.F. Zhang (1993). Hydrodynamic behaviour of three-phase (gas-liquid-solid) airlift reactors. Chemical Engineering Science 48(9): 1641-1654.

Loftin, K.A., C. Henny, C.D. Adams, R. Surampali, M.R. Mormile (2005). Inhibition of microbial metabolism in anaerobic lagoons by selected sulfonamides, tetracyclines, lincomycin, and tylosin tartrate. Environmental Toxicology and Chemistry 24(4): 782-788.

Lòpez, H., S. Puig, R. Ganigué, M. Ruscalleda, M.D. Balaguer, J. Colprim (2008). Start-up and enrichment of a granular anammox SBR to treat high nitrogen load wastewaters. Journal of Chemical Technology and Biotechnology 83(3): 233-241.

Lotti, T., S. Caffaz, E. Bettazzi, C. Vannini, C. Lubello (2009). Modelling simultaneous anaerobic ammonium oxidizing and heterotrophic bacteria growth in a sequencing batch reactor. IWA 2nd specialized conference on nutrient managment in wastewater treatment processes.

Lourens, P., R.J. Zoetemeyer (1992). Fluidized bed process. U. Patent. USA. 5 116 505.

Madoni, P., D. Davoli, L. Guglielmi (1999). Response of sOUR and AUR to heavy metal contamination in activated sludge. Water Research 33(10): 2459-2464.

Marcato, C.E., E. Pinelli, P. Pouech, P. Winterton, M. Guiresse (2008). Particle size and metal distributions in anaerobically digested pig slurry. Bioresource Technology 99(7): 2340-2348.

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

Mohring, S.A.I., I. Strzysch, M.R. Fernandes, T.K. Kiffmeyer, J. Tuerk, G. Hamscher (2009). Degradation and elimination of various sulfonamides during anaerobic fermentation: A promising step on the way to sustainable pharmacy? Environmental Science and Technology 43(7): 2569-2574.

Molinuevo, B., M.C. Garcia, D. Karakashev, I. Angelidaki (2009). Anammox for ammonia removal from pig manure effluents: Effect of organic matter content on process performance. Bioresource Technology 100(7): 2171-2175.

Neef, A., R. Amann, H. Schlesner, K.H. Schleifer (1998). Monitoring a widespread bacterial group: In situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144(12): 3257-3266.

Nicolella, C., M.C.M. Van Loosdrecht, J.J. Heijnen (2000). Wastewater treatment with particulate biofilm reactors. Journal of Biotechnology 80(1): 1-33.

Noophan, P., S. Sripiboon, M. Damrongsri, J. Munakata-Marr (2009). Anaerobic ammonium oxidation by Nitrosomonas spp. and anammox bacteria in a sequencing batch reactor. Journal of Environmental Management 90(2): 967-972.

Oshiki, M., M. Shimokawa, N. Fujii, H. Satoh, S. Okabe (2011). Physiological characteristics of the anaerobic ammonium-oxidizing bacterium '*Candidatus* Brocadia sinica'. Microbiology 157(6): 1706-1713.

Pynaert, K., B.F. Smets, D. Beheydt, W. Verstraete (2004). Start-up of Autotrophic Nitrogen Removal Reactors via Sequential Biocatalyst Addition. Environmental Science and Technology 38(4): 1228-1235.

Qiao, S., T. Yamamoto, M. Misaka, K. Isaka, T. Sumino, Z. Bhatti, K. Furukawa (2009). High-rate nitrogen removal from livestock manure digester liquor by combined partial nitritation-anammox process. Biodegradation 21(1): 11-20.

Rothrock Jr, M.J., M.B. Vanotti, A.A. Szögi, M.C.G. Gonzalez, T. Fujii (2011). Long-term preservation of anammox bacteria. Applied Microbiology and Biotechnology 92(1): 147-157.

Rottenberg, H. (1990). Decoupling of oxidative phosphorylation and photophosphorylation. Biochimica et Biophysica Acta - Bioenergetics 1018(1): 1-17.

Sánchez-Melsió, A., J. Cáliz, M.D. Balaguer, J. Colprim, X. Vila (2009). Development of batch-culture enrichment coupled to molecular detection for screening of natural and man-made environments in search of anammox bacteria for N-removal bioreactors systems. Chemosphere 75(2): 169-179.

Sánchez, E., R. Borja, L. Travieso, A. Martín, M.F. Colmenarejo (2005). Effect of organic loading rate on the stability, operational parameters and performance of a secondary upflow anaerobic sludge bed reactor treating piggery waste. Bioresource Technology 96(3): 335-344.

Sarmah, A.K., M.T. Meyer, A.B.A. Boxall (2006). A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. Chemosphere 65(5): 725-759.

Scaglione, D., S. Caffaz, E. Bettazzi, C. Lubello (2009). Experimental determination of Anammox decay coefficient. Journal of Chemical Technology and Biotechnology 84(8): 1250-1254.

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

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

Schmid, M., K. Walsh, R. Webb, W.I.C. Rijpstra, K. Van De Pas-Schoonen, M.J. Verbruggen, T. Hill, B. Moffett, J. Fuerst, S. Schouten, J.S.S. Damstel , J. Harris, P. Shaw, M. Jetten, M. Strous (2003). Candidatus "Scalindua brodae", sp. nov., Candidatus "Scalindua wagneri", sp. nov., Two New Species of Anaerobic Ammonium Oxidizing Bacteria. Systematic and Applied Microbiology 26(4): 529-538.

Schmidt, I., C. Look, E. Bock, M.S.M. Jetten (2004). Ammonium and hydroxylamine uptake and accumulation in Nitrosomonas. Microbiology 150(5): 1405-1412.

Sin, G., D. Kaelin, M.J. Kampschreur, I. TakÃ¡cs, B. Wett, K.V. Gernaey, L. Rieger, H. Siegrist, M.C.M. Van Loosdrecht (2008). Modelling nitrite in wastewater treatment systems: A discussion of different modelling concepts. Water Science and Technology. 58: 1155-1171.

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

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

Sliekers, A.O., K.A. Third, W. Abma, J.G. Kuenen, M.S.M. Jetten (2003). CANON and Anammox in a gas-lift reactor. FEMS Microbiology Letters 218(2): 339-344.

Strous, M. (2000). Microbiology of anaerobic ammonium oxidation. Delft, Delft University of Technology, The Netherlands.

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

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

Strous, M., E. Van Gerven, J.G. Kuenen, M. Jetten (1997). Effects of aerobic and microaerobic conditions on anaerobic ammonium- oxidizing (anammox) sludge. Applied and Environmental Microbiology 63(6): 2446-2448.

Suzuki, K., M. Waki, T. Yasuda, Y. Fukumoto, K. Kuroda, T. Sakai, N. Suzuki, R. Suzuki, K. Matsuba (2010). Distribution of phosphorus, copper and zinc in activated sludge treatment process of swine wastewater. Bioresource Technology 101(23): 9399-9404.

Szogi, A.A., M.B. Vanotti, M.C. Garcia Gonzalez, A. Kunz (2007). Development of anammox process for animal waste treatment: Experiences in the USA. ASABE - Proceedings of the International Symposium on Air Quality and Waste Management for Agriculture.

Tang, C.J., P. Zheng, L. Zhang, J.W. Chen, Q. Mahmood, X.G. Chen, B.L. Hu, C.H. Wang, Y. Yu (2010). Enrichment features of anammox consortia from methanogenic granules loaded with high organic and methanol contents. Chemosphere 79(6): 613-619.

Third, K.A., J. Paxman, M. Schmid, M. Strous, M.S.M. Jetten, R. Cord-Ruwisch (2005). Enrichment of anammox from activated sludge and its application in the CANON process. Microbial Ecology 49(2): 236-244.

Tijhuis, L., M.C.M. Van Loosdrecht, J.J. Heijnen (1994). Formation and growth of heterotrophic aerobic biofilms on small suspended particles in airlift reactors. Biotechnology and Bioengineering 44(5): 595-608.

Tomaszek, J.A. and Grabas, M. (1998) Biofilm reactors: a new form of wastewater treatment. In Chemistry for the protection of the environment (Pawlowski et al., ed.), pp. 105–116, Plenum Press

Tsushima, I., T. Kindaichi, S. Okabe (2007). Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. Water Research 41(4): 785-794.

Van De Graaf, A.A., P. De Bruijn, L.A. Robertson, M.S.M. Jetten, J.G. Kuenen (1996). Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142(8): 2187-2196.

Van De Graaf, A.A., P. De Bruijn, L.A. Robertson, M.S.M. Jetten, J.G. Kuenen (1997). Metabolic pathway of anaerobic ammonium oxidation on the basis of 15N studies in a fluidized bed reactor. Microbiology 143(7): 2415-2421.

Van de Graaf, A.A., A. Mulder, P. De Bruijn, M.S.M. Jetten, L.A. Robertson, J.G. Kuenen (1995). Anaerobic oxidation of ammonium is a biologically mediated process. Applied and Environmental Microbiology 61(4): 1246-1251.

Van Der Lans, R.G.J.M. (1985). Hydrodynamics of a bubble column loop reactor. Delft, The Netherlands, Delft University of Technology. Ph.D.

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

Van der Star, W.R.L., W.R. Abma, B. Kartal, M.C.M. Van Loosdrecht (2011). Application of the anammox process. in Nitrification. B.B. Ward, D.J. Arp and M.G. Klotz (eds). Washington, DC, ASM Press: 237-263.

Van Der Star, W.R.L., A.I. Miclea, U.G.J.M. Van Dongen, G. Muyzer, C. Picioreanu, M.C.M. Van Loosdrecht (2008). The membrane bioreactor: A novel tool to grow anammox bacteria as free cells. Biotechnology and Bioengineering 101(2): 286-294.

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

Van Niftrik, L., W.J.C. Geerts, E.G. Van Donselaar, B.M. Humbel, R.I. Webb, J.A. Fuerst, A.J. Verkleij, M.S.M. Jetten, M. Strous (2008). Linking ultrastructure and function in four genera of anaerobic ammonium-oxidizing bacteria: Cell plan, glycogen storage, and localization of cytochrome c proteins. Journal of Bacteriology 190(2): 708-717.

Vanotti M.B., Szogi A.A., Kunz A., Gonzalez M. (2006). Development of anaerobic ammonium oxidation (ANAMMOX) technology using immobilized biomass from swine manure. In: Petersen, S.O., editor. Proceedings of 12th Recycling of Agricultural Municipal and Industrial Residues (RAMIRAN), September 9-12, 2006,Aarhus, Denmark. p. 143-146

Vanotti, M.B., A.A. Szogi, P.G. Hunt, P.D. Millner, F.J. Humenik (2007). Development of environmentally superior treatment system to replace anaerobic swine lagoons in the USA. Bioresource Technology 98(17): 3184-3194.

Vellinga, S.H.J. (1986). Anaerobic purification equipment for wastewater. U. Patent. USA. 4 609 460.

Verlaan, P., J. Tramper, K. Van't Reit, K.C.H.A.M. Luyben (1986). A hydrodynamic model for an airlift-loop bioreactor with external loop. The Chemical Engineering Journal 33(2): B43-B53.

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

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

Wilsenach, J.A. (2006). Treatment of source separated urine and its effects on wastewater systems. Department of Biotechnology. Delft, Delft University of Technology, The Netherlands.

Winckler C., Engels H., Hund-Rinke K., Luckow T., Simon M., Steffens G. (2003). Verhalten von Tetracyclinen und anderen Veterinarantibiotika in Wirtschaftsdunger und Boden. UFOPLAN 200 73 248, Berlin.

World Health Organization (1991). International Program On Chemical Safety, Health and Safety Guide No. 56, Hydrazine, Health And Safety Guide. Geneve 1991.

Yamamoto, T., K. Takaki, T. Koyama, K. Furukawa (2008). Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by Anammox. Bioresource Technology 99(14): 6419-6425.

Zhao, L., Y.H. Dong, H. Wang (2010). Residues of veterinary antibiotics in manures from feedlot livestock in eight provinces of China. Science of the Total Environment 408(5): 1069-1075.

ACKNOWLEDGEMENTS

This thesis is the goal of a long travel lasted three years. I walked on this unexplored land with a lot of effort and hardship, but thanks to the support of my family, my friends and colleagues, I kept on walking. Without them, this travel would have maybe started, but wouldn't surely have led me here.

I would like to thank Simone for introducing me to the marvelous world of bacteria and for sharing with me a big dream. I thank Claudio for just the right amount of freedom and guidance throughout my work, and for taking care of me in the dark periods. Robbert, without you these years in Delft would have been much more difficult. Our "fresh-air" moments were the most pleasant scientific discussions I could have. Mark, I thank you for the chance you gave me and for your ability to guide me and help me at any time and from everywhere around the world. Wouter, what to say? You took a massive amount of with impossible experiments and ideas in which I got lost, and made a real story out of it; in music, I'd say you gave me the LA. Dmitry, it has been a great pleasure for me to meet you. Before knowing you, I had just a vague idea of what being a brilliant scientist means. I thank Christian for making everything look easy and doable and for his love for Italian wine. Marco e Giulia adopted me as a child; they fed me, supported, host me, given me relaxing time. Marco, you are a friend a the best colleague ever, and you were close to me in the worst experience I ever had. I thank Cecilia for the marvelous moments we spent together and for being always herself. I thank Marione for the fruitful, but relaxed discussions and for the contagious passion he put in everything he does. Udo, Delft without you wouldn't be the same, even if it surely be healthier. I thank you for your friendship and for your help with nasty FISH, but I'll never forgive you for that crappy noisy motorbike you made me buy. Rob and Dirk, thanks for forgiving me for all the mess I do in the lab and for protecting me from Lesley. Essengul for the ugliest hair cut ever experienced. Janine for her contagious free spirit. I thank Michele, Marjolein, Bibi, Simone, Jan-Willem, Cora, Beppe, Emanuele, Elena for their help in the lab and more. A special thanks to all the guys from the Kluyver, always ready for jokes and complain with me about Italian politics. Ben, thank for guiding me as a child to the world of RNA sequences. Jan, thanks for staying in front of the damned FIA till Friday night. Elena for allowing me to play with her precious creatures. Riccardo, Giulio and Cecilia for considering me part of the group even if I'm always away. Matthijs, Jelmer, Shiva, Elena, Jang, Marlies and the other guys that make my life nicer in the lab. Merle for the fruitful discussions and for waiting patiently for the holy-model. I thank my housemates Javier, Luis and Coco for playing drums, percussions and trombone in the middle of the night and making our house a cozy mess. The guys from Publiacqua for showing interest and help me with my strange red bacteria. I thank l'Rocchi cause he made possible to build the gas-lift without asking anything back. I thank Jacqueline for the amazing contribution to this thesis and for bringing nice warm food to the lab with Jacob. To Duccio, Giacomino, Andrea and all the Mathenessenweg gang for the nights in Rotterdam. Also thanks to my cute anammox that even when I try to kill them lough on me. The last is for my Italian friends, anywhere they live. They were always present. Special thanks to Michelino, the only American guy who talks in English with an Italian accent and is able to prepare more than two hundreds spectacular ravioli home-made; many thanks also for the night spent together making the layout of this thesis.

Il ringraziamento finale va alla mia famiglia. A Chiara, Antonio e le mie bellissime nipotine Giulia e Matilde, il cui unico zio è emigrato proprio quando cominciava a piacergli. Alla mia cara nonna che dice, dice e poi è sempre lì. Infine i'babbo e la mamma, che mi hanno sopportato e supportato in ogni circostanza con un amore immenso e che, come sempre, hanno fatto da parafulmini a tutte le mie insoddisfazioni e ai miei momenti di follia. Grazie, questa la dedico a voi.

LIST OF PUBLICATIONS

CONFERENCE PROCEEDINGS

Lotti T., Caffaz S., Bettazzi E., Vannini C., Lubello C. (2009) Modelling of anaerobic ammonia oxidizing and heterotrophic bacteria growth an a sequencing batch reactor. Proceedings IWA 2nd Specialized Conference Nutrient Management in Wastewater Treatment Processes. Krakow (Poland) 6-9 September 2009

Bettazzi E., Caffaz S., **Lotti T.**, Lubello C. (2009) Nitrogen removal from high loaded wastewater in Florence district: an experimental study. Proceedings IWA 2nd Specialized Conference Nutrient Management in Wastewater Treatment Processes. Krakow (Poland) 6-9 September 2009

Scaglione D., **Lotti T.**, Ficara E., Caffaz S., Canziani R., Lubello C., Malpei F. (2010) Anammox enrichment in conventional sludge samples via a simple semi-batch procedure with activity measures. Proceedings IWA World Water Congress 2010, Montreal (CAN) 19-24 settembre 2010.

Lotti T., Cordola M., Kleerebezem R., Caffaz S., Lubello C., van Loosdrecht M.C.M. (2011) Inhibition effect of swine wastewater heavy metals and antibiotics on Anammox activity. Proceedings The 8th International IWA Symposium on Waste Management Problems in Agro-Industries. Çeşme (Turkey) 22-24 June 2011.

SUBMITTED OR YET TO BE SUBMITTED

Lotti T., Van der Star W.R.L., Kleerebezem R., Lubello C., van Loosdrecht M.C.M. The effect of nitrite inhibition on the anammox process.

Lotti T., Kleerebezem R., Lubello C., van Loosdrecht M.C.M. High purity anammox suspension in MBR: physiological and kinetic characterization.

Lotti T., Cordola M., Kleerebezem R., Caffaz S., Lubello C., van Loosdrecht M.C.M. Inhibition effect of swine wastewater heavy metals and antibiotics on Anammox activity.