Doctoral School in Environmental Engineering

Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process for treating ammonium-rich wastewaters

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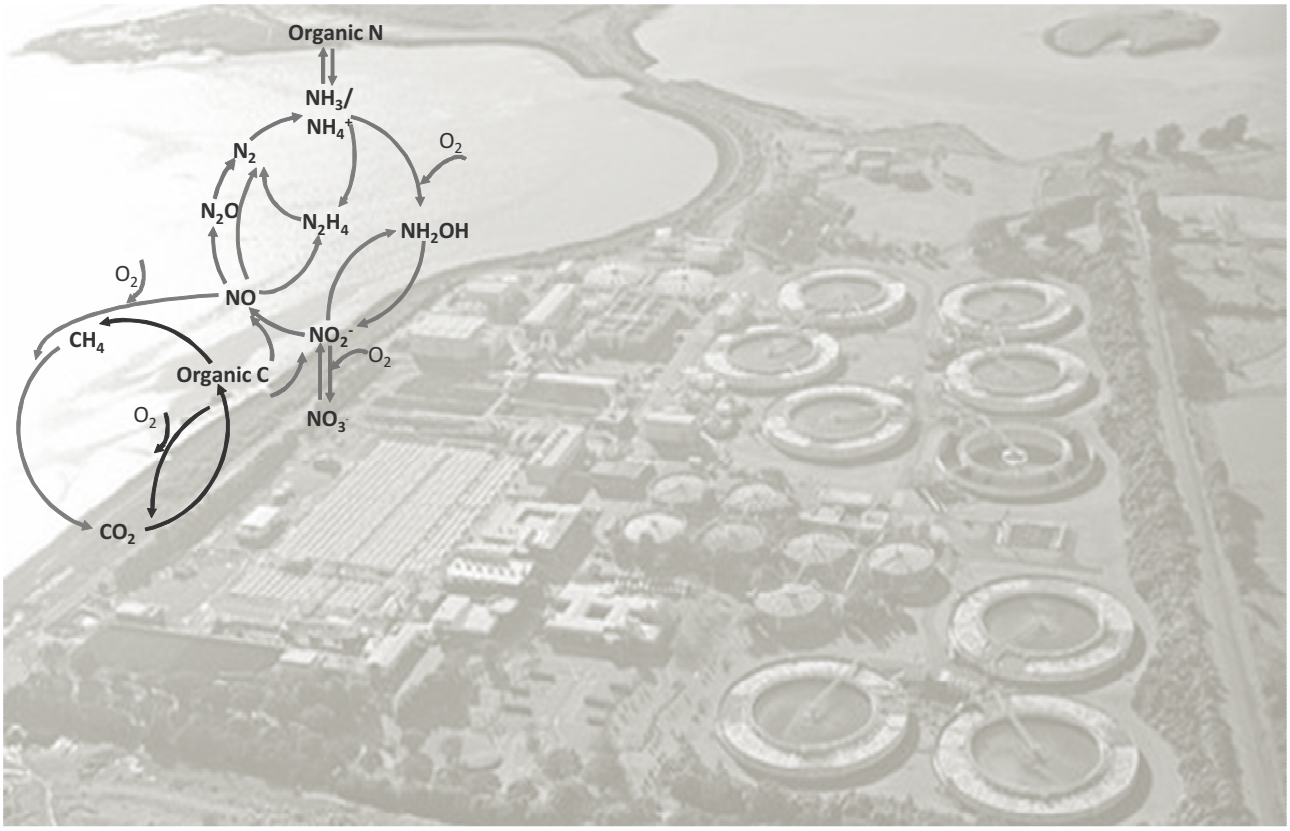

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to my family

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Nowadays, in a rapidly growing world, there is an increasing need for innovative solutions to deal with water challenges. Water problems are increasingly globalised and it is important to focus policy, planning and scientific investigation on water reducing, water treatment, water recycling and reuse.

Processes and technologies have to be implemented to reduce the environmental concentrations of pollutants released into the environment and to reduce the energetic requirements and the generation of secondary products. A sustainable water policy through facilitating innovation has been placed central in the Europe 2020 strategy.

The present work focus on nitrogen removal from high strength ammonium wastewaters. Biological nitrogen removal from strong nitrogenous wastewaters received a great deal of attention during the last years, due to the stricter limits set by the EU Standards for nutrient content in wastewater effluents. Currently, there is a wide range of European legislation covering many different aspects of water management. Among them, the Directive 91/271/EEC of the European Union, as amended by the Directive 98/15/EC, concerns the collection, treatment and discharge of urban wastewater and the treatment and discharge of wastewater from certain industrial sectors. Its aim is to protect the environment from any adverse effects due to discharge of such waters and evidences the concern of Europeans for sensitive areas which are subject to eutrophication. The Directive 91/676/EEC (Nitrates Directive) concerns the protection of waters against pollution caused by nitrates from agricultural sources. The Water Framework Directive (Directive 2000/60/EC) establishs a framework for the protection of inland surface waters (including rivers and lakes), transitional waters (estuaries), coastal waters and groundwater.

Some high strength ammonium wastewaters, such as anaerobic digester effluents and old landfill leachates, are further characterized by low biodedradable organic content (COD_{bio}). Those wastewaters are usually treated *in – situ* by conventional biological processes (nitrification – denitrification) or collected and recirculated to the conventional activated sludge (CAS) tanks in the wastewater treatment plants (WWTP), inducing operational and economic problems, as they cause nitrogen overloads and inhibition effects. The amount of COD_{bio} contained in these effluents is usually not enough to carry out the removal of nitrogen by the nitrification-denitrification process and an external carbon source must be added. Apart the high costs related to the external carbon source furniture, it must be taken into account the high energy consumption related to the oxygen supply for the oxidation of the ammonium (nitrification).

The aim of the research presented in this thesis is to characterize high strength ammonium wastewaters and to develop a more energetically efficient technology for nitrogen removal. Since the discovery of anammox bacteria, the traditional theory of biological nitrogen removal has been extended to new nitrogen metabolic pathways. The present work is focused on investigating the feasibility and sustainability of the Simultaneous partial nitritation, Anammox and Denitrification (SNAD) process in “1 reactor system” for treating anaerobic digested effluents and old landfill leachates.

SNAD process is a complex biological process where different populations with opposed environmental requirements coexist. Thus, the microbial community composition in the reactor has been investigated by analysis of Polymerase Chain Reaction (PCR) combined with sequential DNA sequencing and phylogenetic tree and fluorescence in situ hybridization (FISH). Special attention has been paid to wastewaters characterization, reactor configuration design, operative conditions and control process strategies in order to optimize the nitrogen

and carbon removal efficiencies. The main factors that influence the performance of the SNAD process have been studied, and a real time strategy, based on indirect on-line parameters, has been implemented.

This thesis addresses the microbiology, strategies and wastewater implications in the co-existence of nitritation, anammox and denitrification processes.

The research described in this thesis was conducted at the Department of Civil and Environmental Engineering of the University of Trento, Italy, and a three month – period, from 30 June 2011 to 30 September 2011, was spent at the Department of Microbiology of the Radbound University Nijmegen, Netherland. The experiments at the University of Trento focused on the develop of a sustainable nitrogen removal system to treat ammonium rich wastewaters using Simultaneous partial nitritation, Anammox and denitrification (SNAD) process in a "1 reactor system". A Sequencing Batch Reactor (SBR) has been equipped. A collaboration with the "Servizio Elettronico & Progettazione" of Physic Department of the University of Trento allowed to implement an acquisition control software and hardware, useful to manage the SNAD process in the SBR. The research at the Radbound University Nijmegen focused on the characterization of bacteria involved in SNAD process using microbiology techniques and microbial activity tests in order to perform phylogenetic analyses and to detect the potential microbial activity of each microorganism. A new PCR primer set to detect anammox bacteria was developed working together with the Servizio Opere Igienico-Sanitarie, Provincia Autonoma di Trento, Italy. Finally, due to reciprocal interests on modeling, plans for a future collaboration associated with the University of Naples, Italy, have been initiated, in order to define a mathematical model based on the ASMs and calibrate and validate the model using experimental data obtained by SNAD process developed in the "1-reactor system" in Trento (Italy).

The **first chapter** of this thesis starts with the description of the origin and effects of nitrogen pollution. Further it gives a general overview of nitrogen removal process from wastewater looking at new nitrogen metabolic pathways which have extended the microbial nitrogen cycle. The current knowledge on the stoichiometry, phylogeny, bioinformatics, kinetics and factors involved in kinetics of the nitrogen removal biological processes are reviewed. The advantages of the microbial co-existence are discussed and finally an overview of mathematical models based on ASMn family is given.

Chapter two describes the microorganisms community composition of the SNAD sludge sampled at the Wastewater Treatment Plant (WWTP) of Zürich, Switzerland, and used as inoculums to start a lab scale reactor. The community structure and phylogeny of the whole biomass was analyzed using the PCR technique combined with sequential DNA sequencing and phylogenetic tree construction. FISH analysis were performed in order to determinate the microbial spatial distribution.

The **third chapter** opens with a brief overview of landfill leachate composition. This is followed by a leachate characterization through chemical and respirometric analysis.

The **fourth chapter** deals landfill leachate treatments, focusing on biological process to treat old landfill leachates. Batch tests, performed simulating a cycle of a Sequential Bacth Reactor (SBR) at T=30°C, confirmed the sustainability of the SNAD process for the old landfill leachate treatment.

The **chapter fifth** shows the construction, start-up and continuous operation of an SBR reactor in view of performing the SNAD process for treating anaerobic digested effluents at moderate temperature. In this chapter, first the effect of temperature on the SNAD process are investigated, treating a synthetic wasteawater in a suspended/growth – granular SBR both at 30°C and 25°C. Then, the applicability to the anaerobic digester effluents is confirmed. Special emphasis was placed on the reactor configuration and operative conditions.

In the **chapter sixth** a correlation between the on-line ORP, pH, conductivity and DO profiles with the biological nitrogen and carbon removal was studied. Then, a real time control strategy, mainly based on the conductivity and ORP signals, was designed and successfully applied.

Finally the main results of the aforementioned chapters are summarized and discussed in the **summary and outlook section.**

I. Chapter

General introduction: Sustainable biological nitrogen removal processes from ammonium rich wastewater

General introduction: Sustainable biological nitrogen removal processes from ammonium rich wastewater

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ABSTRACT

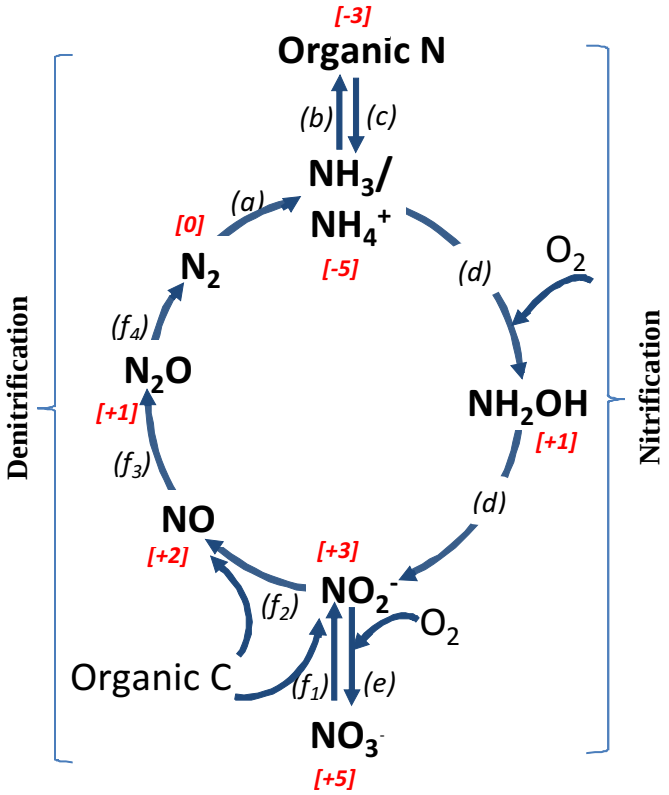
Biological nitrogen removal from strong nitrogenous wastewaters received a great deal of attention during the last years. Nitrogen removal performed by biological treatments is economically preferred over nitrogen recovery techniques for wastewaters containing less than 5 g N L^{-1}. Therefore, due to their simplicity and high cost-effectiveness, biological treatments are widely applied for nitrogen and carbon removal. Conventional biological treatments are carried out by means of the combination of autotrophic nitrification and heterotrophic denitrification. Conventional biological processes are generally performed to treat wastewaters with low nitrogen concentrations, as could be municipal wastewaters. Nevertheless, the high oxygen demand and the addition of external carbon source do not allow the application of the conventional biological processes for treating wastewaters with a high ammonium load and a low biodegradable organic matter content, such as anaerobic digester effluents and old landfill leachates. Since the discovery of anammox bacteria, the traditional theory of the biological nitrogen removal has been extended. Further, new nitrogen metabolic pathways have been discovered expanding the nitrogen cycle, such as the nitrite-dependent methane oxidation (n-damo), aerobic ammonia oxidation by archaea, dissimilatory nitrate reduction to ammonia. In particular, after the discovery of anammox reaction, several nitrogen removal processes have been developed in the wastewater treatments field, among them ANaerobic AMMonium OXidation (anammox); Nitritation and Partial Nitritation, such as Single reactor High activity Ammonia Removal Over Nitrite (SHARON), denitrification via nitrite, and innovative combinations of those processes. Several strategies have been adopted by using 2 separate reactors in series (*2-reactor system*), such as the shortcut nitritation and denitrification as well as SHARON/anammox, and accomplishing two or more processes in the same reactor (*1-reactor system*): Simultaneous Nitrification and Denitrification via nitrite (SND via nitrite), Completely Autotrophic Nitrogen removal Over Nitrite process (CANON), Oxygen-Limited Autotrophic Nitrification-Denitrification (OLAND), Single-stage Nitrogen removal using anammox and partial Nitritation (SNAP) and Simultaneous partial Nitrification, Anammox and Denitrification (SNAD) process. A description of the current knowledge of the innovative biological nitrogen removal processes from ammonium rich wastewaters is presented. Special importance is given to the influence of the wastewaters composition, to the environmental factors, to the reactor configurations on the nitrogen removal efficiency, to the microbial community and the co-existence of different biological processes. The present status, regarding practical applications of these processes, modeling and future developments, is provided looking at the sustainability of nitrogen removal technologies.

Keywords: aerobic ammonia oxidizing-bacteria, aerobic nitrite oxidizing-bacteria, anammox, archaea, autotrophic nitrogen removal, denitrifiers, CANON, co-existence, n-damo, nitrogen cycle; SNAD, wastewater treatment

1. Introduction

The most potentially hazardous pollutant of wastewaters is nitrogen, especially ammonium - nitrogen (NH_4^+-N) which has been identified as the main long-term pollutant as well as the main cause of acute and chronic toxicity in aquatic environments (Camargo and Alonso 2006; Kurniawan et al. 2006b). Nitrogen is an important element for life and is one of the essential nutrients for plants growth. Wastewater discharges containing nitrogen can lead to, amongst others, the oxygen depletion and the eutrophication in receiving waters. Nitrogen is a versatile element, existing in both inorganic and organic forms as well as in many different oxidation states from -3 (NH_4^+ or NH_3) to + 5 (NO_3^-) and chemical forms. The exchange of nitrogen between the atmosphere, biosphere, hydrosphere and geosphere in different forms is known as the “nitrogen cycle” and it is mostly governed by microbial activities (Francis et al. 2007). In particular, the nitrogen cycle is mainly driven by prokaryotic

organisms (both *Eubacteria* and *Archaebacteria*) that use nitrogen compounds for assimilatory, dissimilatory, or respiratory purpose. Until the last decade of the nineteenth century it was assumed that the microbial nitrogen cycle was essentially complete (Jetten 2008). Free-living or symbiotic N_2 gas-fixing bacteria, such as *Rhizobium*, reduced dinitrogen gas (N_2) to ammonium for assimilation (Beijerinck 1888; Fred et al. 1932). Biological nitrogen fixation is an important process, as it provides ammonia (NH_3) for plants and animals. Soil and aquatic microbes were responsible of the assimilation (uptake or immobilization) of inorganic nitrogen (in form of NH_3) (Yevdokimov and Blagodatsky 1993) and the production of ammonium (NH_4^+) from organic N compounds (ammonification /mineralization) (Stanford and Smith 1972). In soil, freshwater, and marine environments, two physiologically and phylogenetically distinct groups of chemolithotrophic nitrifying bacteria oxidized ammonia, via nitrite, to nitrate (Winogradsky 1890). Prokaryotes chemoorganotrophic microorganisms closed the cycle by returning the oxidized form of nitrogen back to N_2, a highly stable gas, in a four step denitrification process (Gayon and Dupetit 1886; Knowles 1982; Zumft 1997) (Fig. I.1). Further, heterotrophic bacteria and fungi were found to have the ability to oxidize a variety of nitrogenous compounds, nitrifying heterotrophs (Verstraete and Alexander 1972; Castignetti and Hollocher 1984) while chemolithotrophic ammonia oxidizers have been found to reduce nitrite to nitric oxide with nitrous oxide or dinitrogen gas as final products, denitrifying nitrifiers (Poth 1986).



(a) dinitrogen gas fixation by bacteria;
(b) ammonia assimilation/ immobilization.
(c) ammonification/mineralization;
(d) aerobic ammoniaoxidation by bacteria;
(e) aerobic nitrite oxidation by bacteria;
(f_1) denitrification – nitrate reduction;
(f_2) denitrification – nitrite reduction;
(f_3) denitrification – NO reduction;
(f_4) denitrification – N_2O respiration;

[*]oxidation states

Hydroxylamine (NH_2OH), nitric oxide (NO) are intermediary compounds do not released into the environment.

Fig. I.1 Classical Nitrogen-cycle.

Several discoveries of new nitrogen metabolic pathways have been made during the last 15 years, both expanding the nitrogen cycle and linking the carbon with nitrogen cycle (Jetten 2008; van de Leemput et al. 2011): anaerobic ammonium oxidation (anammox) (Strous et al. 1999b), aerobic ammonium oxidation by crenarchaea (AOA) (Könneke et al. 2005; Francis et al. 2007), nitrite-dependent anaerobic methane oxidation (n-damo) (Raghoebarsing et al. 2006), hyperthermophilic N_2-fixing methaneproducing archaea (Young 1992; Mehta and Baross 2006), dissimilatory nitrate reduction to ammonium (DNRA, also called nitrate ammonification)

(Woods 1938; Stanford et al. 1975), nitrate reduction to dinitrogen gas by unicellular eukaryotes foraminifera (Risgaard-Petersen et al. 2006), nitrite-oxidizing phototrophs (Griffin et al. 2007; Schott et al. 2010) (Fig. I.2). The overall biological reactions involved in the nitrogen and carbon cycles create a network of pathways. Fig. I.3, as adapted from Ettwig (2010), shows the nitrogen cycle coupled with carbon cycle.

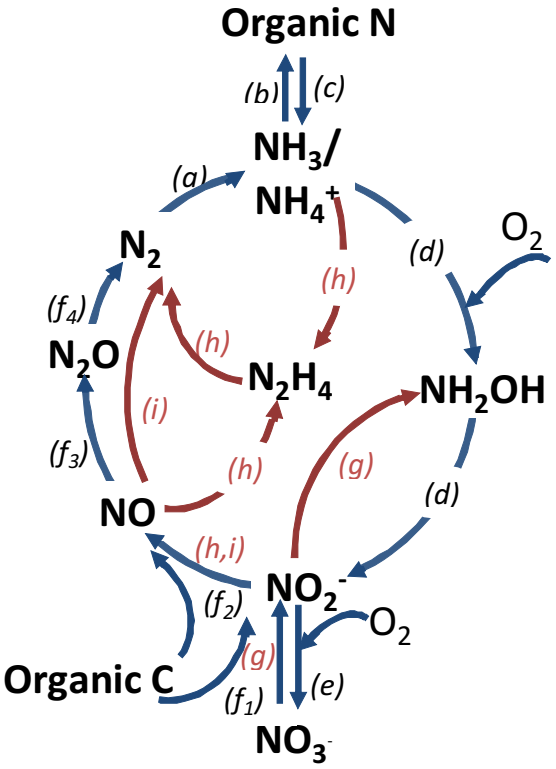


Fig. I.2 Overall Nitrogen cycle

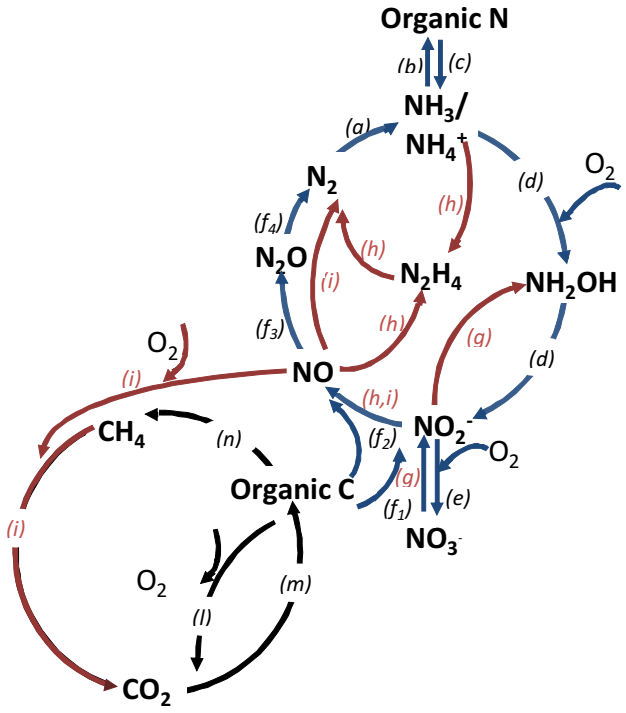


Fig. I.3 Nitrogen cycle coupled with Carbon cycle

(a) dinitrogen gas fixation by bacteria;
(b) ammonia assimilation/ immobilization.
(c) ammonification/mineralization;
(d) aerobic ammoniaoxidation by bacteria;
(e) aerobic nitrite oxidation by bacteria;
(f_1) denitrification – nitrate reduction;
(f_2) denitrification – nitrite reduction;
(f_3) denitrification – NO reduction;
(f_4) denitrification – N_2O respiration;
(g) *dissimilatory nitrate and nitrite reduction to ammonium (DNRA)*[*];
(h) *anaerobic ammonium oxidation* [*];
(i) *nitrite-dependent anaerobic methane oxidation by bacteria (proposed pathway)*[*].

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(h) *anaerobic ammonium oxidation*[*];
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(l) carbon oxidation
(m) carbon fixation
(n) methanogenesis.

Hydrazine (N2H4), hydroxylamine (NH2OH), nitric oxide(NO) are intermediary compounds do not release into the environment. * New pathway

The discovering biological pathways have opened up new possibilities in wastewater treatment plants (WWTP). Consequently, several nitrogen removal processes have been developed. These processes include the ANaerobic AMMonium OXidation (anammox) (Van Der Star et al. 2007); the nitritation and partial nitritation, achieved using either high temperature, i.e. Single reactor High activity Ammonia Removal Over Nitrite (SHARON) process (Mulder et al. 2001; van Hulle et al. 2007) or other suitable strategies (i.e oxygen limited conditions, high free ammonia levels); denitrification via nitrite (Glass and Silverstein 1998; Adav et al. 2010); Simultaneous Nitrification and Denitrification (SND) via nitrate and via nitrite (Münch et al. 1996; Yoo et al. 1999; Ruiz et al. 2006; Wu et al. 2007; Blackburne et al. 2008a). In particular, the anammox process has to be

preceded by a (partial) nitritation process. Most researches on autotrophic biological treatments for nitrogen removal has been directed towards understanding the integration of partial nitritation and anammox processes using both 2 separate reactors in series (*2-reactor systems*) such as SHARON/anammox (Van Dongen et al. 2001a) and accomplishing two or more processes in the same reactor (*1-reactor systems*): Completely Autotrophic Nitrogen removal Over Nitrite process (CANON) (Dijkman and Strous 1999; Sliekers et al. 2002), Oxygen-Limited Autotrophic Nitrification-Denitrification (OLAND) (Kuai and Verstraete 1998), Single-stage Nitrogen removal using anammox and partial Nitritation processes (SNAP) (Lieu et al. 2005; Furukawa et al. 2006).

Other researchers have studied the anammox process looking at the interactions between nitrogen and carbon cycle (Ahn et al., 2004, Kumar and Lin, 2010). Actually, this represents a common problem in the anammox system because most of the wastewaters contain carbon compounds as well as nitrogen. The combined system for nitrogen and carbon removal based on the partial nitritation, anammox and denitrification processes in *1-reactor systems* is known as the Simultaneous partial Nitrification, Anammox and Denitrification (SNAD) process (Chen et al. 2009; Wang et al. 2010; Lan et al. 2011; Daverey et al. 2012; Winkler et al. 2012b; Langone et al. 2013b). The SNAD process, under oxygen limitation, in one single reactor, has various advantages compared to the 2-stage systems and to the conventional biological treatments, such as lower capital costs, no need for external carbon additions, negligible sludge productions and lower energy and oxygen requirements.

The SNAD process comprises three main processes: one *micro-aerobic*, partial nitritation process (Eq. I-1), where about 50% of ammonia is oxidized to nitrite; one *anoxic*, anammox process (Eq. I-2), where ammonia and nitrite are converted to nitrogen gas producing a small amount of nitrate; and an *anoxic* one, denitrification process (Eq. I-3 - Eq. I-4), where organic carbon as electron donor could deoxidize nitrate and nitrite to dinitrogen gas.

$$NH_4^+ + 0,75O_2 + HCO_3^- \xrightarrow[\text{autotrophic bacteria}]{AOB} 0,5NO_2^- + 0,5NH_4^+ + 1,5H_2O + CO_2 \qquad \text{Eq. I-1}$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \xrightarrow[\text{autotrophic bacteria}]{anammox} 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{0.5}N_{0.15} \qquad \text{Eq. I-2}$$

$$5C + 4NO_3^- + 2H_2O \xrightarrow[\text{bacteria}]{\text{heterotrophic}} CO_2 + 4HCO_3^- + 2N_2 \qquad \text{Eq. I-3}$$

$$5.5C + 6NO_2^- + 1H_2O \xrightarrow[\text{bacteria}]{\text{heterotrophic}} 3.5CO_2 + 2HCO_3^- + 3N_2 \qquad \text{Eq. I-4}$$

The SNAD process has a high potential for the complete conversion of ammonium and organic carbon to dinitrogen gas and carbon dioxide from ammonium rich wastewaters characterized by a low biodegradability and a low biodegradable carbon (CODbio) – nitrogen (N) ratio, such as urban anaerobic digester effluents (Joss et al. 2009), swine digester liquor (Zhang et al. 2012) and old landfill leachates (Xu et al. 2010). Recently, it was also applied to treat some industrial wastewaters, such as opto-electronic wastewaters (Daverey et al. 2012). The main advantage of the SNAD process is the complete nitrogen and carbon removal, requiring a low energy demand. From an overview of literature, more studies are necessary to investigate how to improve the performance, the cost-effectiveness, the nitrous oxide emissions and the robust control of reactors. A key factor for the development of the SNAD process is a better understanding of both the biological processes involved in the nitrogen removal and the numerous microbial interactions using microbiological and modeling tools. In this contribution a general overview of nitrogen removal processes is presented looking at the nitrogen metabolic pathways which have extended the microbial nitrogen cycle, with a main focus on the partial nitritation,

anammox and denitrification processes and the co-existence of them in one single reactor. The reactor configurations applied up to date for nitrogen removal from ammonium rich wastewater are also presented.

2. Sustainable metabolic pathways for nitrogen removal from ammonium-rich wastewater

For each biological process involved in N removal, the stoichiometry, microbial and kinetic aspects are reviewed: the well known 'aerobic' ammonia and nitrite oxidizers bacteria, anaerobic ammonia oxidizers bacteria and denitrifiers. Also other nitrogen biological pathways are briefly analyzed, such as the aerobic ammonia oxidation by archaea, heterotrophic nitrification, nitrifiers denitrification, dissimilatory nitrate reduction to ammonium (DNRA) and nitrite-dependent anaerobic methane oxidation (n-damo).

2.1. Nitrification - Ammonia and nitrite oxidation by bacteria

Nitrification, the biological oxidation of ammonia to nitrate via nitrite by bacteria, occupies an essential position within the nitrogen cycle and has various direct and indirect implications for natural and man-made systems (Fiencke et al. 2005). Nitrification has traditionally been considered as a single process in wastewater treatment plants whereby ammonia/ammonium is oxidized to nitrate by specific chemical autotrophic microorganisms under strict aerobic conditions. Some nitrifying bacteria are known to have limited heterotrophic capability, as the consumption and the assimilation of simple organic compounds (Verstraete and Alexander 1972; Arp and Bottomley 2006). From a biological point of view, nitrification is composed of two steps: the oxidation of ammonia/ammonium (oxidation state -3/-5) to nitrite (oxidation state +3) by Ammonia-Oxidizing Bacteria (AOBs) and the oxidation of nitrite to nitrate (oxidation state +5) by Nitrite-Oxidizing Bacteria (NOBs). The first step is termed *nitritation* while the second step is called *nitratation*. After the anammox process discovery, the term "*partial nitritation*" was introduced. The term "*partial nitritation*" stands for the partial oxidation of ammonia to nitrite, with a residual fraction of ammonium not converted into nitrite. A stable partial nitritation is an essential step for the following anammox step (Fux et al., 2002). Nitritation (ammonia oxidation to nitrite) provides significant costs savings compared to the complete nitrification (ammonia oxidation to nitrate) because the stoichiometry indicates that it demands less oxygen and produces less sludge resulting in energy and volume requirement savings. Nitritation process can be coupled with anammox or denitrification "via nitrite" in order to achieve a more sustainable nitrogen removal solution than the conventional nitrification and denitrification "via nitrate".

2.1.1 Stoichiometry of nitrification

In the nitrification process, oxygen acts as the terminal electron acceptor both for ammonia- and for nitrite-oxidizing bacteria: electrons are removed from ammonia and nitrite and finally transferred to oxygen. Nitrifiers bacteria use mainly ammonia and nitrite as an energy source (catabolism) and only a negligible fraction is used for biosynthesis of new molecules, cell maintenance, and growth (anabolism), while carbon dioxide is used as a carbon source.

In the literature it is generally accepted that although ammonia (NH_3), and not ammonium (NH_4^+), is the actual substrate, the oxidation reaction of nitritation can be written using ammonium. Simplified oxidation

reactions for nitritation and nitratation are shown in Eq. I-5 and Eq. I-6, respectively, while the overall oxidation reaction for nitrification is shown in Eq. I-7.

$$NH_4^+ + 1.5O_2 \xrightarrow{AOBs} NO_2^- + 2H^+ + H_2O \qquad \Delta G°' = -275\ kJ\ mol^{-1} \qquad \text{Eq. I-5}$$

$$NO_2^- + 0.5O_2 \xrightarrow{NOBs} NO_3^- \qquad \Delta G°' = -74\ kJ\ mol^{-1} \qquad \text{Eq. I-6}$$

$$NH_4^+ + 2O_2 \longrightarrow NO_3^- + 2H^+ + H_2O \qquad \Delta G°' \cong -360\ kJ\ mol^{-1} \qquad \text{Eq. I-7}$$

The energy yields from ammonium and nitrite oxidation are modest, leading to small biomass yields and low maximum specific growth rates. As the energy generating reaction for the aerobic oxidation of ammonia is higher than the energy for the aerobic oxidation of nitrite, it is reasonable that AOBs grow faster than NOBs. Bock (1978) confirmed a minimum doubling times of 7–8 hours for AOBs and 10–13 hours for NOBs. The combined synthetis-oxidation reactions for nitritation and nitratation are reported in Eq. I-8 and Eq. I-9.

$$55NH_4^+ + 76O_2 + 109HCO_3^- \xrightarrow{AOBs} 54NO_2^- + C_5H_7O_2N + 57H_2O + 104H_2CO_3 \qquad \text{Eq. I-8}$$

$$400NO_2^- + NH_4^+ + 4H_2CO_3 + 195O_2 + HCO_3^- \xrightarrow{NOBs} 400NO_3^- + C_5H_7O_2N + 3H_2O \qquad \text{Eq. I-9}$$

Using the Eq. I-8 and Eq. I-9, the overall synthesis and oxidation reaction in the complete nitrification can be represented as follows (Eq. I-10):

$$NH_4^+ + 1.83O_2 + 1.98HCO_3^- \xrightarrow{AOBs} 0.98NO_3^- + 0.021C_5H_7O_2N + 1.041H_2O + 1.88H_2CO_3 \qquad \text{Eq. I-10}$$

$C_5H_7O_2N$ is the empirical cell formula usually utilized for nitrifying bacteria (Brown&Caldwell 1975). Nitritation is an acidifying process. Approximately 2 mol of bicarbonate (HCO^-_3) are consumed for every mole of ammonium oxidized producing carbon acid (H_2CO_3), resulting in 7.07 mg of alkalinity as $CaCO_3$ for every mg of nitrogen ammonium. In these equations, the oxygen requirements are 3.16 mg O_2 per mg NH_4-N oxidized and 1.11 mgO_2 per mg NO_2-N oxidized, respectively. Hence, the stoichiometric conversion of ammonia to nitrate requires 4.27 mgO_2 mgN^{-1}, complessively. Expressed as volatile suspended solids (VSS), growth yields for AOBs and NOBs are 0.15 mg cell/mg NH_4-N oxidized and 0.02 mg cell/mg NO_2-N oxidized, respectively. Expressed as chemical oxygen demand (COD), growth yields are 0.21 mg COD/mg NH_4-N oxidized for AOBs and 0.028 mg COD/mg NO_2-N oxidized for NOBs.

2.1.2 Mechanisms of nitrification

Nitritation. For the nitritation step, Suzuki et al., 1974 formulated the hypothesis that ammonia (NH_3), rather than ammonium (NH_4^+), is the actual substrate for AOBs. Anthonisen et al. (1976) observed that at higher concentrations, ammonia becomes inhibiting for both AOBs and NOBs. Van Hulle et al. (2007) using an experimental study verified both those hypothesis. Further, genetic studies demonstrated the enzyme involved in ammonia oxidation uses NH_3, not NH_4^+. The aerobic ammonia oxidation to nitrite proceeds through two consecutive intermediate oxidation reactions, and hydroxylamine (NH_2OH) is an intermediate (Kluyver and Donker 1926). First, ammonia is oxidized to hydroxylamine using O_2 as a substrate (electron acceptor) (Eq. I-11). One oxygen atom is added to ammonia, while the second oxygen is reduced to water. This reaction is catalyzed by a membrane-bound enzyme called ammonia monooxygenase (AMO). The second step of aerobic ammonia oxidation is the oxidation of hydroxylamine to nitrite during which four electrons are released (Eq. I-12). This second reaction is catalyzed by hydroxylamine oxidoreductase (HAO), a key enzyme in the chemoautotrophic respiratory chain, which is a soluble trimer of three identical octaheme subunits that could

serve to direct electrons along two separate pathways (Prince and George 1997). This allows that in every cycle of oxidation of ammonia, two hydroxylamine-derived electrons return to the ammonia monooxygenase enzyme (AMO) to regenerate hydroxylamine (Hooper et al. 1997; Whittaker et al. 2000).

$$NH_3 + O_2 + 2H^+ + 2e^- \xrightarrow{AMO} NH_2OH + H_2O \qquad \Delta G°' = -120\ kJ\ mol^{-1} \qquad \text{Eq. I-11}$$

$$NH_2OH + H_2O \xrightarrow{HAO} NO_2^- + 5H^+ + 4e^- \qquad \Delta G°' = -289\ kJ\ mol^{-1} \qquad \text{Eq. I-12}$$

Under anoxic conditions, *Nitrosomonas* spp. has also been shown to be a denitrifying organism that use nitrite reductase and nitrous oxide reductase enzymes to form nitrous oxide (N_2O) and dinitrogen (N_2) respectively (Poth and Focht 1985a; Poth 1986). *Nitrosomonas eutropha* can grow with nitrite (NO_2) as oxygen source (electron acceptor) instead of O_2 for ammonia oxidation to hydroxylamine and NO is formed (Schmidt and Bock 1997a, 1998). This process is known as nitrifier denitrification and its contribution has been observed at oxygen-limiting conditions or elevated nitrite concentrations (Colliver and Stephenson 2000).

According to Kampschreur et al. (2009), nitritation process is responsible for N_2O emission. In particular, N_2O is produced via hydroxylamine oxidation at 2 - 3 $mgO_2\ L^{-1}$, and it is favored at high ammonia and low nitrite concentrations (Wunderlin et al. 2012). The N_2O production in hydroxylamine oxidation could be related to an imbalanced metabolic activity of AOBs (Yu et al. 2010), or to a chemical decomposition of hydroxylamine as well as to a chemical oxidation with NO_2 as an electron acceptor (Ritchie and Nicholas 1972). In addition, N_2O is also produced by nitrifier denitrification by AOBs.

Free ammonia can be used directly as substrate or can made available by hydrolyzation of urea (Koops et al. 1991). Free ammonia (NH_3) is in chemical equilibrium with its ionic forms (NH_4^+) (Eq. I-13). In nitrification process, the ammonia oxidation releases protons which cause the acidification of the environment and thereby shift the NH_3/NH_4^+ equilibrium and the oxidation rate of nitritation.

$$NH_3 + H^+ \xleftrightarrow{Equilibrium} NH_4^+ \qquad \text{Eq. I-13}$$

Nitratation. Regarding the nitrite oxidizing bacteria, the use of nitrite ion (NO_2^-) or free nitrous acid (FNA or HNO_2) as substrate is not unequivocal. Hellinga et al., (1999) and Magrí et al., (2007) defined FNA as the direct substrate. The growth rate of NOB, thus, depended on the free nitrous acid concentration. Boon and Laudelout, (1962) and Park and Bae, (2009) suggested to consider nitrite (NO_2^-) as the substrate and free nitrous acid (FNA) as the inhibitor. Others considered the growth rate of NOBs depended on total nitrite concentration (TNN = NO_2^-N + HNO_2-N) according to the Monod Equation (Pambrun et al. 2006; Jiménez et al. 2011). Clear experimental results showed that the inhibitor is FNA (HNO_2) rather than nitrite (NO_2^-) (Vadivelu et al. 2006). However, the mechanism of NO_2^- oxidation remains uncertain. An empirical mechanism of NO_2^- oxidation has been proposed, where the oxidation of NO_2^- and the conversion of O_2 occurred simultaneously preventing the formation of H^+ gradient (Eq. I-14 - Eq. I-15).

$$2NO_2^- + 2H_2O \rightarrow 2NO_3^- + 4H^+ + 4e^- \qquad \Delta G°' = +83\ kJ\ mol^{-1} \qquad \text{Eq. I-14}$$

$$4H^+ + 4e^- + O_2 \rightarrow 2H_2O \qquad \Delta G°' = -157\ kJ\ mol^{-1} \qquad \text{Eq. I-15}$$

Nitratation is carried out by the enzyme nitrite oxidoreductase in *Nitrobacter* spp.. In the genera *Nitrococcus, Nitrospina,* and *Nitrospira* the key enzyme is called the nitrite-oxidizing system.

2.1.3 Phylogeny

Nitrification is performed by two specific genera of autotrophic bacteria, the Ammonium-Oxidizing Bacteria (AOBs) and the Nitrite-Oxidizing Bacteria (NOBs). Recent molecular techniques have shown that there are several genera of nitrifying organisms.

Aerobic AOB. The terrestrial AOBs are restricted to the beta- subdivision of the phylum proteobacteria (Fig. I.4), while the marine AOBs are found both to the beta- and gamma- subdivision of the phylum proteobacteria (Fig. I.4 and Fig. I.5). Each cluster identifies typical Ammonia-Oxidizing Bacteria of specific environments, including sewage, wastewater, marine and terrestrial environments. Soils are often dominated by *Nitrosospira* spp., while marine and freshwater often have mixtures of the genera of AOBs. Focussing on terrestrial AOBs, *Nitrosomonas* and *Nitrosospira* are the currently genera comprising the beta-proteobacteria. Controversial aspects are linked to (*i*) *Nitrosococcus mobilis* proposed to be reclassified to the genus *Nitrosomonas* spp. (cluster 7), (*ii*) *Nitrosolobus* and *Nirosovibrio* superseded by *Nitrosospira* and (*iii*) *Nitrosomonas cryotolerans and Nitrosomonas* Strain Nm143 placement (Norton 2011). In wastewater treatment plants are usually found beta- proteobacteria of the cluster 7 (*Nitrosomonas europea, Nitrosomonas mobilis* strains and *Nitrosomonas eutropha*) (Wagner et al. 1996; Juretschko et al. 1998; Kelly et al. 2005) and the cluster 8 (*Nitrosomonas nitrosa)*. They are capable of tolerating high-ammonia concentration. *Nitrosospira* have also been observed under cool temperature and high dissolved oxygen concentration (Park et al. 2002). Often, *Nitrosomonas* cluster 6A, related to *N. oligotropha*, have been found in pool with low ammonia/ammonium concentration (Park et al. 2002). In constructed wetland for wastewater treatment are often present both *Nitrosospira* and *Nitrosomonas* spp. (Gorra et al. 2007; Ruiz-Rueda et al. 2009).

Molecular techniques targeting the signature regions of the 16S ribosomal DNA (rDNA) and 16S rRNA of AOB have been successfully applied in both environmental and engineered systems in order to: investigate the phylogenetic tree and the affinities of members (Stephen et al. 1996; Kowalchuk et al. 1997); identify and direct microscopic enumerate through fluorescent in situ hybridization techniques (FISH) (Mobarry et al. 1996; Schramm et al. 1996, 1999; Juretschko et al. 1998; Okabe et al. 1999); quantify by real-time PCR (Polymerase Chain Reaction) (Hermansson and Lindgren 2001; Dionisi et al. 2002). On the other hand, the gene encoding AMO can be a function-specific target for detecting AOB in wastewater treatment processes. The *amoA*-DNA-based method enables the detection, quantification and phylogenetic analysis of AOB (Sinigalliano et al. 1995; Rotthauwe et al. 1997; Juretschko et al. 1998; Purkhold et al. 2000; Hoshino et al. 2001). DNA- or rRNA-based analysis gives little information on the specific activity under any different environmental conditions while Aoi et al., 2004 analyzed *amoA*- messenger RNA (mRNA) as an indicator of the ammonia oxidation activity because the turn-over of mRNA is rapid in living bacterial cells, with most mRNA having a half-life of only a few minutes (Alifano et al. 1994).

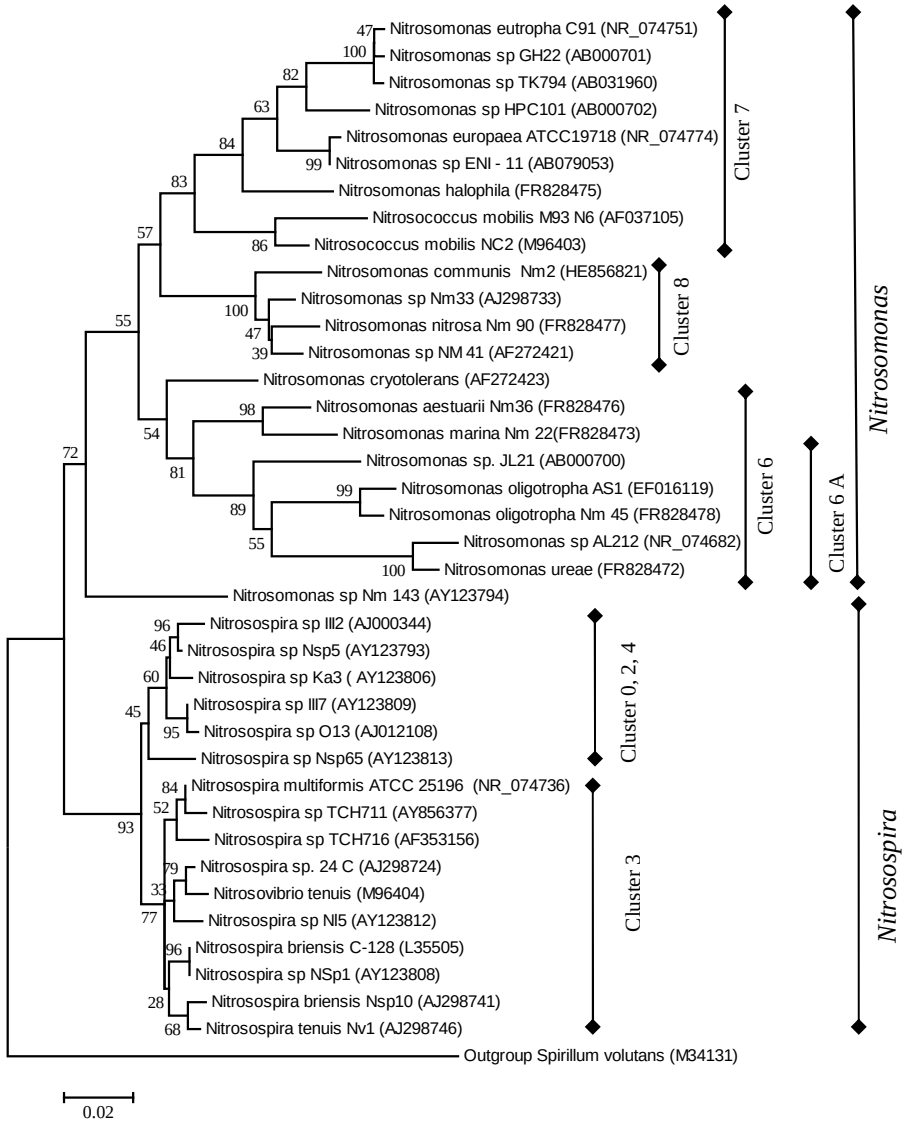

Fig. I.4 16S ribosomal RNA phylogenetic tree for the cluster of beta- proteobacterial AOB, based on high quality sequences (>1200 bp) from isolates
The scale bar indicate 0.02. Adapted from Norton (2011)

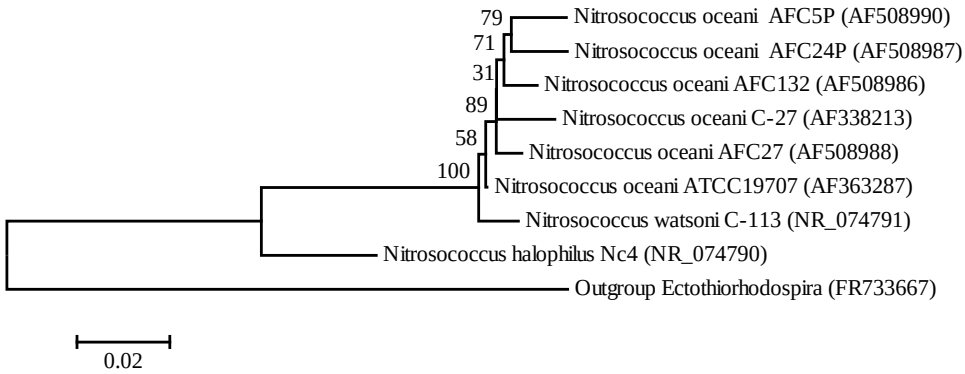

Fig. I.5 16S ribosomal RNA phylogenetic tree for the cluster of Gamma- proteobacterial AOB, based on high quality sequences (>1200 bp) from isolates.
The scale bar indicate 0.02. Adapted from Norton (2011)

Aerobic NOB. NOBs proliferate in a broad range of environmental conditions (terrestrial, marine, acidic) and have diverse lifestyles (lithoautotrophic, mixotrophic, and heterotrophic) (Daims et al. 2011). NOBs are comprised of five genera, which have been found in several lineages of Bacteria (Fig. I.6). *Nitrobacter* spp. are located in the alpha-proteobacteria lineage whereas *Nitrococcus* spp. in the gamma-proteobacteria. *Nitrospina* spp. are temporarily placed in delta-proteobacteria but could belongs to a separate bacterial phylum while *Nitrospira* spp. occupy its own branching lineage (Starkenburg et al. 2011). A new candidate genus have been found in Siberian Arctic, the "*Candidatus* Nitrotoga", located in the beta-proteobacteria (Alawi et al. 2007). *Nitrospina* and *Nitrococcus* have been recovered from marine environments and are characterized as obligate halophilic (Starkenburg et al. 2011) while *Nitrobacter* spp. and *Nitrospira* spp. assumed to be the major players in nitrite oxidation in freshwater environments and wastewater treatment plants. For decades, *Nitrobacter* spp. were mainly regarded to be responsible for the nitrite oxidation in wastewater treatment plant and was the most famous nitrite oxidizer genus. Recently, independent molecular techniques (FISH with rRNA – targeted oligonucleotide probes, PCR) and metagenomics-based analysis showed that *Nitrobacter* spp. cannot be relevant for nitrite oxidation in most wastewater treatment plants while *Nitrospira* spp. are much more abundant in sewage treatment systems (Daims et al. 2011). Further, Schramm et al. (1999) demonstrated that *Nitrospira* spp. have much higher affinity for nitrite that *Nitrobacter* spp.. *Nitrospira* spp. are more likely to dominate nitrite oxidation under low ammonium and low nitrite concentrations (Blackburne et al. 2007). *Nitrobacter* spp. can be found in systems treating wastewater with high nitrogen loads (Mobarry et al. 1996; Gieseke et al. 2003).

Blackburne et al. (2007) have found that the yield of *Nitrospira* spp. (gVSS gN^{-1}) is higher than that of *Nitrobacter* spp. and is likely similar to that of the ammonia oxidizers.

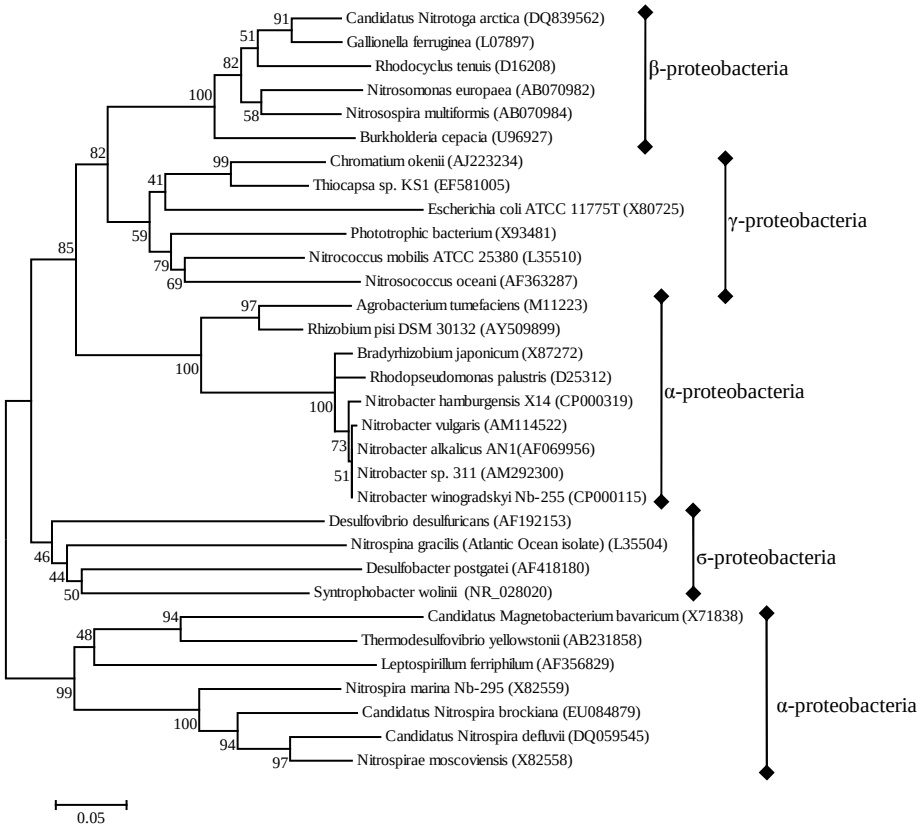

Fig. I.6: Phylogenetic tree, based on protein sequences showing the phylogenetiv affiliation of the currently know NOB. The scale bar indicate 0.05. Adapted from Daims et al. (2011).

Molecular techniques targeting the signature regions of the 16S ribosomal RNA (rRNA) have been applied to detect NOBs. In particular, *Nitrobacter* spp. show high 16 rRNA similarities to their closest non-nitrifying bacteria suggesting that the 16 rRNA is too conserved to explicitly resolve lineage within the genus Nitrobacter (Daims et al. 2011). A better approach to identify *Nitrobacter* spp. uses genes encoding subunits of the key enzyme of nitrite oxidation, nitrite oxidoreductase (*Nxr*), as functional and phylogenetic markers. Poly et al., 2008 used PCR primers targeting the gene of the *Nxr* alpha subunit of *Nitrobacter* spp., *nxrA* genes. Also other two genes from the *Nxr* cluster are used as molecular markers, *nxrB* and *nxrC* (Vanparys et al. 2007)

2.1.4 Bioinformatics

Aerobic AOB. More than a century has passed since the discovery of the nitrification process and, today is finally known genomes from AOB representative of several ecotype in beta-proteobacteria and gamma-proteobacteria. AOBs have a small genome (~ 3 megabases) and it contains genes necessary for the ammonia catabolism, energy and reductant generation, biosynthesis, CO_2 and NH_3 assimilation and iron acquisition. Genes encoding transporters for inorganic ions were plentiful. The gene profiles reveal limited genes for catabolism and transport of complex organic compounds, whereas genes for sucrose synthesis/degradation have been identified (Chain et al. 2003; Arp et al. 2007)).

Aerobic NOB. Until now, five genomes of NOBs have been sequenced (*Nitrobacter* spp., *Nitrococcus* spp. *and Nitrospira* spp.). On average, *Nitrobacter* genomes is 4.1 megabases and encode approximately 4000 genes. It is relatively small compared to its closest non-nitrifying bacteria. It contains genes for nitrite oxidoreductase (*Nxr*). The functional gene *nxrA*, encoding the catalytic subunit of the nitrite oxidoreductase in nitrite-oxidizing bacteria (NOB) has been detected. Cytochromes associated with a dissimilatory nitrite reductase (*NirK*), PII-like regulators, and polysaccharide formation were identified (Starkenburg et al. 2008). The high diversity of genes identified may reflect the high diversity of NOBs and their flexibility. It is interesting note that *Nxr* can behave as a dissimilatory nitrate reductase during anaerobic growth, reducing nitrate to nitrite under anaerobic conditions at pH 6 to 7 (Tanaka et al. 1893). This properties agrees with the fact that *nxrA* and *nxrB* are homologos of *narGH* that comprise subunits of dissimilatory nitrate reductse, found in some denitrifying bacteria (Kirstein and Bock 1993).

2.1.5 Kinetics

AOBs and NOBs are characterized by different general kinetics (Eq. I-16, Eq. I-17). Biochemical experiments performed on different nitrifying cultures indicated that nitritation and nitratation kinetics are strongly influenced by the nature of nitrifying cultures and by many physico-chemical environmental factors such as substrate concentration, intermediate and final products concentration, temperature, dissolved oxygen, pH, and various inhibitors such as salts and so on. The difference in sensitivity of AOBs and NOBs towards these influences determines the dominance of one group over the other. The specific autotrophic growth rate (μ_{Aut}) can be expressed by the interactive Monod model for growth which is used to describe the effect of limiting substrate on microbial growth.

Monod – Based growth Kinetics:

$$\mu_{AOB} = \mu_{AOB}^{MAX} \frac{S_{NH4}}{K_{S,NH4}^{AOB} + S_{NH4}} \cdot \frac{S_O}{K_O^{AOB} + S_O} \qquad \text{Eq. I-16}$$

$$\mu_{NOB} = \mu_{NOB}^{MAX} \frac{S_{NO2}}{K_{S,NO2}^{NOB} + S_{NO2}} \cdot \frac{S_O}{K_O^{NOB} + S_O} \qquad \text{Eq. I-17}$$

where μ_{AOB} and μ_{NOB} are the actual specific growth rate of AOB and NOB, respectively; μ_{AOB}^{MAX} and μ_{NOB}^{MAX} are the maximum specific growth rate of AOB and NOB; S_{NH4} and S_{NO2} are the NH_4^+-N and NO_2^--N concentrations in the bulk liquid and $K_{S,NH4}^{AOB}$ and $K_{S,NO2}^{NOB}$ are the NH_4^+-N and NO_2^--N half saturation constants for AOB and NOB, respectively. Finally, S_O is the O_2 concentration in the bulk liquid, while K_O^{AOB} and K_O^{NOB} are the oxygen half saturation constants for AOB and NOB, respectively.

Several models describing nitrite build-up during nitrification ($NH_4^+/NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$) have been developed as reviewed by Sin et al., (2008) (Hellinga et al. 1999; Hao et al. 2002; Wett and Rauch 2003; Carrera et al. 2004; Wyffels et al. 2004; Pambrun et al. 2006; Kaelin et al. 2009; Park and Bae 2009).

Carbon dioxide, the carbon source for autotrophic growth, is present in abundance, so that it is not taken into account in the kinetic expressions. The nitritation and nitratation kinetics could be different both in processes and in models considered. In particular, some differences are in the form of the substrate considerated (dissociated/undissociate) to model nitritation and nitratation. In addition, some substrates can be also inhibitors. Among models and theories, Jubany et al., 2008 expressed the AOBs inhibition/limitation by TAN and NOB inhibition/limitation by TNN with a Haldane model while AOB inhibition by TNN and NOB inhibition by TAN with a non-competitive model.

2.1.6 Factors involved in nitrification kinetics

As autotrophic microorganisms are very sensitive to change in environmental conditions, a number of factor are found to affect the kinetics of nitrification.

Free ammonia and ammonium concentration

Free ammonia (FA or NH_3-N) and ammonium (NH_4^+-N) concentrations have a great influence on AOBs and NOBs kinetics. In particular, free ammonia (NH_3-N) acts both as substrate (Suzuki et al., 1974) and inhibitor for AOBs and as inhibitor for NOBs (Anthonisen et al. 1976). Free ammonia (NH_3-N) is in chemical equilibrium with its ionic form (NH_4^+-N) and it is calculated as a function of pH, temperature and total ammonium nitrogen (TAN= NH_3-N+ NH_4^+-N) (Wiesmann 1994) (Eq. I-18 - Eq. I-20). The formation of FA is favorable at a basic pH and at high temperature (Fig. I.7).

$$FA(mgNL^{-1}) = \frac{TAN}{1+\frac{10^{-pH}}{K_e^{NH}}} = \frac{TAN}{1+\left(\frac{10^{-pH}}{e^{-6344/(273+T(°C))}}\right)} = \frac{TAN}{1+e^{6344/(273+T(°C))}\cdot 10^{-pH}} \quad \text{Eq. I-18}$$

$$TAN = NH_3 - N + NH_4^+ - N \quad \text{Eq. I-19}$$

$$K_e^{NH} = \frac{NH_3 - N \cdot H^+}{NH_4^+} = e^{-6344/(273+T(°C))} \quad \text{Eq. I-20}$$

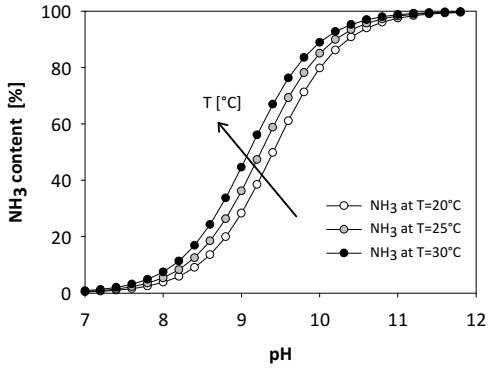

Fig. I.7 NH_3/NH_4^+ equilibrium (calculated from Wiesmann et al., 1994)

Anthonisen et al. (1976) stated that aerobic ammonia oxidizers are inhibited at NH_3-N concentrations between 10 – 150 mg NH_3-N L^{-1} while inhibition of nitrite oxidation is observed at NH_3-N concentration of 0.08–0.82 mg NH_3-N L^{-1} (Anthonisen et al. 1976; Philips et al. 2002a). As nitrite oxidizers are more sensitive than the ammonia oxidizers to free ammonia, working at high pH (7.5 – 8) and, thus, at high free ammonia concentrations could be a way to outcompete NOBs. A summary of main literature findings on the FA initiated inhibition thresholds on AOBs and NOBs is given in Table I-1.

Table I-1 FA inhibition thresholds. The corresponding nitrite concentrations at pH 7.5 are also shown

Culture	FA (mg NH_3-N L^{-1})	Total nitrogen ammonium at pH 7.5 at 35°C (mg TAN L^{-1})	Reference
AOB	10 - 150	290 - 4330	(Anthonisen et al. 1976)
	73	2100	(Hellinga et al. 1999)
	>>16	>>460	(Vadivelu et al., 2006)
	300	8660	(van Hulle et al. 2007)
NOB	0.08-0.82	2-24	(Anthonisen et al. 1976)
	0.04–0.08 *Nitrospira* spp.	1 -2	(Kim et al. 2008)
	30 ~ 50 *Nitrobacter* spp.	860 -1440	(Kim et al. 2008)
	1 -9 *Nitrobacter* sp. anabolism inibition 100%	29 - 260	(Vadivelu et al., 2007)
	1 -9 *Nitrobacter* sp. catabolism inibition 12 – 25%	29 - 260	(Vadivelu et al., 2007)

Vadivelu et al. (2006) showed that FA up to 16.0 mg NH_3-N L^{-1} at pH of 7.1 and 30°C (1574 mg NH_4-N L^{-1}) did not have any inhibitory effect on either the catabolic or anabolic process of a *Nitrosomonas* culture.

Literature on the NH_3–N inhibition of NOBs shows disagreement about the threshold NH_3-N concentration and its inhibition degree. Kim et al. (2008a) reported different threshold for *Nitrobacter* spp. and *Nitrospira* spp.. In particular, *Nitrobacter* spp. were inhibited at 30~50 mg NH_3-N L^{-1} and *Nitrospira* spp. were inhibited at 0.04~0.08 mg NH_3-N L^{-1}. The authors also showed that free ammonia inhibition of nitrite oxidizing bacteria was reversible. Turk and Mavinic (1989) showed an adaptation of the nitrite oxidizing bacteria to FA, confirmed also by other studies (Villaverde 2000). The higher resistance of *Nitrobacter* spp. was also confirmed by Vadivelu et al., (2007), who stated that the respiration (catabolism) of *Nitrobacter* spp. was inhibited by FA even at concentrations in the range of 1 – 9 mgNH_3-N L^{-1} at pH of 7.3 and 22°C (100 - 1000 mg NH_4-N/L). However, this inhibitory effect was limited (inhibited by 12 - 25%). Further, they found the biosynthesis (anabolism) of NOBs ceased completely at a FA concentration of 7.5 mgNH_3-N L^{-1}, although at this point the oxygen consumption rate was only reduced by 25%.

Nitrite and free nitrous acid (FNA)

Nitrite, under certain environmental conditions, can be accumulate in WWTPs. The protonated form of nitrite, free nitrous acid (HNO_2-N or FNA), has been found to cause a severe inhibition to numerous biological processes. In particular, Anthonisen et al. (1976) had first reported that both the inhibition of AOBs and NOBs was related to concentrations of FNA rather than the nitrite itself (NO_2^-), as the undissociated form is easily transported over the cell way by a passive transport. The nitrite produced by nitritation process will exist in equilibrium with the un-ionized form (HNO_2), depending on the pH and the temperature. The formation of FNA

is favorable at an acidic pH (Fig. I.8). FNA can be determined through the total nitrite (TNN=HNO_2-N+NO_2^--N) concentrations by the following formula (Eq. I-21- Eq. I-23):

$$FNA(mgNL^{-1}) = \frac{TNN}{1+\frac{K_e^{NO}}{10^{-pH}}} = \frac{TNN}{1+\left(\frac{e^{-2300/(273+T(°C))}}{10^{-pH}}\right)} = \frac{TNN}{1+e^{-2300/(273+T(°C))}\cdot 10^{pH}} \quad \text{Eq. I-21}$$

$$TNN = HNO_2 - N + NO_2^- - N \quad \text{Eq. I-22}$$

$$K_e^{NO} = \frac{NO_2 - N \cdot H^+}{HNO_2 - N} = e^{-2300/(273+T(°C))} \quad \text{Eq. I-23}$$

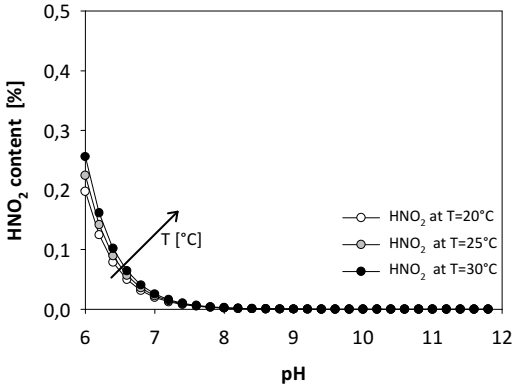

Fig. I.8 HNO_2/NO_2^- equilibrium (calculated from Wiesmann et al., 1994)

In terms of nitrite, Silva et al. (2011) in their study reported that, using an initial pH of 7.7 and T=30°C, at 25 mg NO_2-N L^{-1} (0.001 HNO_2-N L^{-1}), complete nitrification well proceeded while at 100 and 200 mg NO_2-N L^{-1} (mg 0.004–0.008 HNO_2-N L^{-1}) inhibition effects on the nitrification respiratory process was evidenced. The authors also demonstrated that the nitrite oxidizing process was more sensitive to the presence of nitrite than the ammonium oxidizing process. Anthonisen et al. (1976) reported that the inhibition on AOBs and NOBs will be initiated at a FNA concentration of 0.22 e 2.8 mg HNO_2-N L^{-1}. Using the Eq. I-21 and varying the pH, Anthonisen and coauthors defined a boundary zone of FNA inhibition to the nitrification process. Later studies have found variable inhibitory FNA threshold levels. A range of 0.2-1.72 mg HNO_2-N L^{-1} has resulted in a 50% reduction in AOB activity (Hellinga et al. 1999; Vadivelu et al. 2006; Torà et al. 2010). Inhibition on NOB was observed even at lower HNO_2^--N concentrations.

Table I-2 reported some FNA and nitrite concentrations at pH 7 that have been reported to inhibit both the ammonia- and nitrite- oxidizing bacteria by 50% and 100%, respectively (Zhou et al. 2011).

Table I-2 FNA inhibition thresholds. The corresponding nitrite concentrations at pH 7 and T=30°C are also shown

Culture	100% inhibition		50% inhibition		Reference
	FNA (mg HNO_2-N L^{-1})	Total Nitrite at pH 7 (mg TNN L^{-1})	FNA (mg HNO_2-N L^{-1})	Nitrite at pH 7 (mg TNN L^{-1})	
AOB	-	-	0.2	1010	(Hellinga et al. 1999)
NOB	0.023	116	0.0175	88	(Vadivelu et al. 2006)

The range of inhibition thresholds reported above shows that NOBs are more sensitive to FNA than AOBs by approximately one order of magnitude. FNA inhibited both the growth (0.10–0.40 mg HNO_2-N L^{-1}) and the energy production abilities (0.50– 0.63 mgHNO_2-N L^{-1}) of *Nitrosomonas* culture (Vadivelu et al. 2006). Yang et al. (2003) found that FNA inhibition on nitritation can be reversible and AOBs recovered their activity after a short period of 12 days. On the contrary, FNA concentrations start to affect NOBs activity in the range of 0.011

- 0.07 mg HNO_2-N L^{-1}, while a complete inhibition was observed at 0.023 - 0.22 mg HNO_2-N L^{-1} (Anthonisen et al. 1976; Vadivelu et al. 2006; Zhang et al. 2010a).

The wide range of inhibition thresholds values, both for AOB and NOB, could be due to the diversity of microbial community and to contrasting FNA inhibition effects. For istance, *Nitrosomonas europaea* has some different metabolic properties as compared to *Nitrosomonas eutropha*, such as the ability to use nitrite as an electron acceptor anaerobically (Zhou et al. 2011). *Nitrospira* spp. have been reported to be more sensitive to FNA than *Nitrobacter* spp. (Blackburne et al. 2007). Different species and strains within a genus could possess distinct tolerances towards FNA. Jiménez et al., (2011) demonstrated that the nitrite oxidation rate (NOR) depended33 on the TNN concentrations independently of the free nitrous acid (FNA) concentration, thus FNA cannot be considered as the real substrate for NOB.

pH

The pH of wastewaters has a strong influence on nitrification as it plays an important role on nitrifying bacteria activity. The pH impacts are both direct and associated with nutritional effect (indirect) and it is difficult to distinguish them. The cellular environment affects enzymatic activity, so at extremes values of pH (as well as temperature) the native structure of the enzyme could be directly compromised and the molecule will become inactive in a reversible way (Gaudy and Gaudy 1980; Quinlan 1984; Paredes et al. 2007). Using batch experiments based on respirometric techniques, the direct effect of pH on the nitritation (Jiménez et al. 2012) and nitratation process (Jiménez et al. 2011) has been determined and pH inhibition constants have been proposed for both ammonia- and nitrite- oxidation. Jiménez et al., (2011) showed that pH values below 6.5 caused the complete inhibition of the NOB activity, but high pH values did not affect it, and similar NOB activities were measured at pH values ranging from 7.5 to 9.95.

In addition, pH plays an important role on TAN (total ammonium nitrogen), total nitrite nitrogen (TNO_2) and (total inorganic carbon TIC) speciation, affecting the substrate/inhibitors chemical equilibria, substrates availability and inhibition phenomena and thus the biological processes performances. (Magrí et al. 2007; Ganigué et al. 2012).

Considering the availability of CO_2 and NH_3 and the potential adverse impact of NH_3 and HNO_2, pH around 7.5 would be the most favorable for the complete nitrification (i.e. NH_3 and HNO_2 concentration are higher at higher and lower pH, respectively). Alleman (1984) observed an optimum range of 7.9 – 8.2 for ammonia oxidizers (*Nitrosomonas* spp.) and of 7.2 – 7.6 for nitrite oxidizers (*Nitrobacter* spp.). In natural environments, nitrification has been also reported to occur at pH values as low as 4 (De Boer et al. 1991, 1995).

However, the most probable mechanism that influence how pH impacts on AOBs and NOBs is related to the speciation of ammonia and nitrite. In general, the maximum specific autotrophic growth rate (μ_{Aut}) is extremely sensitive to the pH of the liquid bulk. It is well accepted, in WWTPs, that the optimum pH for both ammonium oxidizers and nitrite oxidizers lies between 7 and 8.5 (Ekama and Wentzel 2008). The specific growth rate (μ_{Aut}) is maximum for the range 7.2 <pH< 8.0 but decrease as the pH decreases below 7.2 and as the pH increases up 9 – 9.5. Below pH 7, nitrification rate could decreases since carbon limitation, due to CO_2 stripping, will occur (Wett and Rauch 2003) and since pH has a direct effect on the specific growth rate (Van Hulle et al. 2007). Studies have shown that maximum μ_{Aut}^{max} can be expressed as a percentage of the highest value at the optimum pH and different model have been proposed (Quinlan 1984; Siegrist and Gujer 1987; Magrí et al. 2007; Park et al. 2007; van Hulle et al. 2007).

Temperature

The impact of the temperature on nitrifying activity is normally much more pronounced than the effect of pH. Temperature is a mean parameter in the nitrification process and, as for the pH influence, the precise effect on the biological process is difficult to determine due to its interaction with chemical equilibria, mass transfer and growth rate. Increasing temperature multiple effects occur: a decrease of the solubility of ammonia gas and a shift of the NH_4^+/NH_3 equilibrium in favor of NH_3 gas, an increase of mass transfers of O_2, CO_2 and an increase of the activity of the organisms according to the Arrhenius principle until a certain critical temperature above which biological activity decreases again.

The range of the temperatures favorable to nitrification is quite wide. The lower limit would be 8-10°C (Jones and Hood 1980; Choi et al. 1998; Park et al. 2008) whereas the higher limit would be between 35°C and 45°C (Willers et al. 1998). The thermal death point of pure cultures of *Nitrosomonas* spp. lies between 54 – 58°C (Barrit 1933). Correlation of the maximum growth rate of nitrifying bacteria and temperature is described in the Arrhenius equation at temperature of 5° - 40°C (Eq. I-24), that for small temperature differences can be simplified (Eq. I-25):

$$\mu_{Aut,T}^{max} = \mu_{Aut,20°C}^{max} \exp\left[-\frac{E_a(20-T)}{293 \cdot R(273+T)}\right] \qquad \text{Eq. I-24}$$

$$\mu(T) = \mu(T_r) e^{\vartheta(T-T_r)} \qquad \text{Eq. I-25}$$

where $(\mu_{Aut}^{max})_T$ is the maximum specific growth rate at the actual temperature T, Tr is the reference temperature (often taken as 20°C), E_a is the activation energy and θ is the Arrhenius constant, θ =1,08 - 1,12 (Painter and Loveless 1983; Henze et al. 2000).

As reported in Table I-3 the activation energy of the ammonia oxidation process is higher than that of the nitrite oxidation process. As consequence of this, an increase of temperature implies an increase on the ammonia oxidation rate larger than that by the nitrite oxidation rate. This means that temperature has distinct effects on the competition between AOB and NOB and could be used as a key operation parameter to achieve a stable nitritation, as AOB would out-compete NOB at the relatively higher temperatures. As reported in Fig. I.9, the maximum specific growth rate of the AOBs will be higher than that of NOBs at temperatures above 20-25°C. This principle is the basis of the SHARON process, which consists of a chemostat reactor operated at a hydraulic retention time (HRT) of 1 d and 30°C to promote the growth of the AOB and to washout the NOB (Mulder et al. 2001; van Hulle et al. 2007).

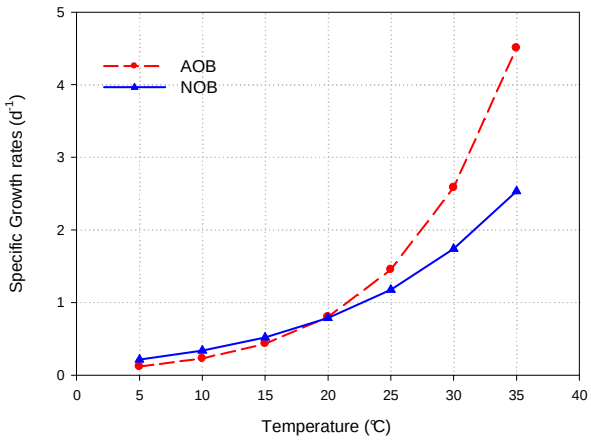

Fig. I.9 Maximum Growth rate of AOBs and NOBs at different temperature

Table I-3 Activation energy of ammonia- nitrite oxidation		
Ea *Nitrosomonas*	86,4*	KJ mol^{-1}
Ea *Nitrobacter*	58,4*	KJ mol^{-1}
growth rate *Nitrosomonas*	0,801	d^{-1} at 20°C
growth rate *Nitrobacter*	0,788	d^{-1} at 20°C

* at 2 – 30°C *Nitrosomonas europaea* (Wijffels, 1995)

Recently, Kim et al. (2008b) calculated the energy of activation of ammonia- and nitrite oxidation on the basis of Arrhenius equation. The activation energies of ammonia oxidation were 87.1 and 38.6 kJ mol^{-1} in the temperature ranges 10–20 °C and 20–30 °C, respectively, while for the nitrite oxidation, the activation energy was obtained as a constant value of 34.2 kJ mol^{-1} in the temperature range of 10–30 °C. The authors explained that different activation energies in the lower and higher temperatures of ammonia oxidation might be attributed to the shift of rate-limiting step between the two enzyme-catalysis steps (AMO and HAO). Similar results were achieved by Guo et al. (2010). Further, Guo et al. (2010), studying long–term effects of temperature on nitritation, achieved a stable nitritation at low temperature by decreasing gradually the temperature from 25°C to 15°C and controlling aeration duration. This experimental evidence might extend shortcut nitrogen removal via the nitrite applicability, particularly on treating wastewater in the cold regions.

Dissolved Oxygen

In aerobic nitrification bioprocesses, the dissolved oxygen (DO) concentration is of high importance for both AOBs and NOBs bacteria. According to Monod kinetic, as dissolved oxygen decreases it becomes the growth limiting substrate. The reported values range for the level of oxygen half saturation constant (*Ko*) is wide both for AOBs and NOBs. The *Ko* values of AOB and NOB cultures with negligible oxygen mass transfer resistances (i.e. flocs) were found to be considerably lower compared to floccular aggregates, granular biomasses and biofilms. This could be explained considering that both oxygen mass transfer from the gas to the liquid phase and oxygen consumption by microorganism has a critical importance (Blackburne et al. 2007, 2008b). Due to the oxygen mass transfer resistance in the liquid bulk, the DO concentration would not be uniform for bacteria inside aggregates, granules and biofilm. Consequently, different specific oxygen uptake rates would be achieved for different bacteria, depending on biomass density, flocs, granules and biofilm size and shape. The oxygen mass transfer kinetics will affect the true bacterial *Ko* and a high "apparent *Ko*" value will be detected for systems with high oxygen mass transfer resistances (Blackburne et al. 2008b).

However, note that both working at high and low DO, it is confirmed the difference in oxygen affinity of AOBs compared to NOBs. In other words, the activity of nitrite oxidizers is more significantly influenced by oxygen limitation than that of ammonium oxidizers (Philips et al. 2002a). Imposing oxygen-limiting conditions can be considered a sustainable way to outcompete nitrite oxidizers and favor nitrite accumulation. AOBs dominate NOBs at low DO concentration, but this depends on temperature too. In fact, given free nitrous acid (HNO_2) and free ammonia (NH_3) concentrations that don't inhibit the process, at temperature higher than 15°C AOBs always dominate NOBs while at T lower than 15°C AOBs dominate NOBs only for DO < 2mg L^{-1} (Fig. I.10).

It is also suggested that a possible mechanism for the inhibition of nitrite oxidation by a low oxygen concentration is based on the accumulation of free hydroxylamine, intermediate product of the ammonia oxidation (Yang and Alleman 1992), rather than a lower NOB oxygen affinity constants. Kindaichi et al. (2004) and Harper et al. (2009), in their study on nitrifying biomass were unable to identify nitrite-oxidizing bacteria (NOB) in the NH_2OH-treated biofilms, which suggested that NH_2OH may also inhibits NOBs (as a side-effect).

Moreover, aeration patterns have been proposed to be an alternative parameter to wash out NOBs and prevent the nitrite oxidation to nitrate. Frequent aeration switching on/off can increase the stress on nitrite oxidizers (Yoo et al. 1999; Hidaka et al. 2002). Several authors confirmed that nitrite build-up could be achieved working

at low oxygen concentration and fast alternations of the aeration conditions in the system (Wett 2007; Vázquez-Padín et al. 2009c).

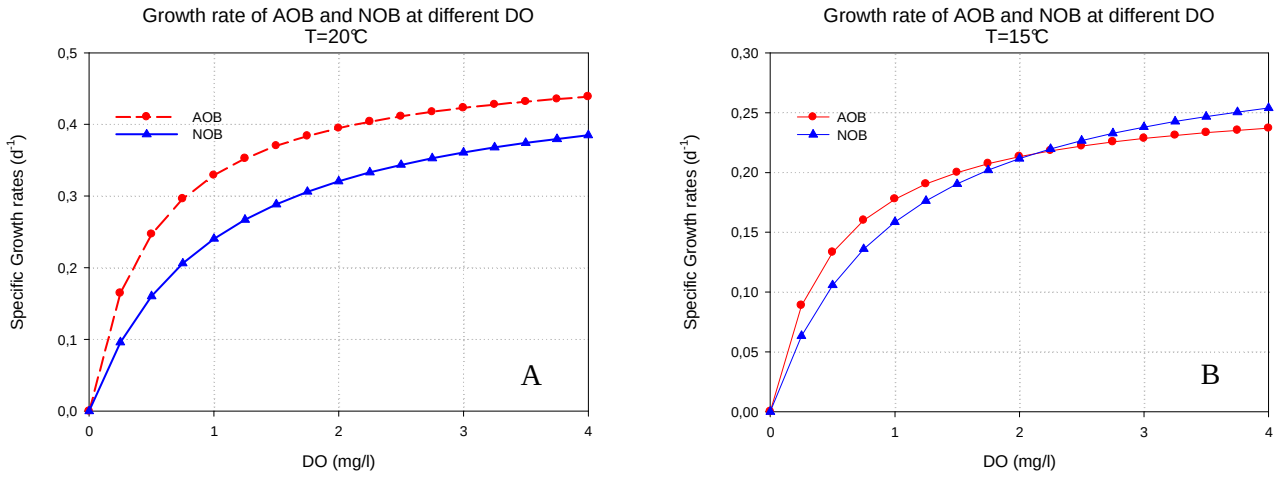

Fig. I.10 Maximum growth rate of AOBs and NOBs at different temperatures.
(A) T= 20°C, (B) T=15°C

Sludge age

Ammonium oxidizers and nitrite oxidizers can be selectively accumulated by appropriately adjusting the sludge retention time (SRT) in a suspended-growth system (Hellinga et al. 1998). Due to their different growth rates, AOB need a longer retention time than NOB at temperature below 15°C while the trend is reversed at temperature above 20-25°C. At high temperature (T=30°C), the selection of the AOB on the basis of different growth rates has been used in the SHARON process, which operates at an HRT (equal to SRT) of 1 day under high temperature (T=30°C) and at high oxygen concentrations to favour the growth of ammonia oxidizers and to washout the nitrite oxidizing bacteria (Hellinga et al. 1998; van Hulle et al. 2007). However, as recently reviewed by Van Hulle et al., (2010) successful nitritation were reported under longer sludge age, working on other selection pressures, such as oxygen limitation (Pollice et al. 2002; Peng and Zhu 2006)

Alkanility

The oxidation of 1 mole of ammonia to nitrite consumes approximately 2 moles of bicarbonate, which correspond to 7.07 g of alkalinity (as $CaCO_3$) consumed per gram of N oxidized, while the oxidation of 1 mole of nitrite to nitrate does not consume a significant alkalinity content. Hence, an alkalinity consumption to ammonia consumption ratio of 7 or more is considered as an indicator of a complete nitritation process. Depending on the alkalinity of the wastewater, the nitritation process could be complete or partial, since the whole load of ammonium or only a fraction would be converted into nitrite. In order to accomplish the nitritation with the anammox process a molar ratio 1:1 between consumed ammonium and bicarbonate is appropriate, determining a conversion of approx. 50% of ammonium to nitrite while the rest remains as ammonium (Van Hulle et al. 2010). Recently, Li & Irvin, (2007) used a multiple way control mechanism to inhibit NOB, working at high temperature and low oxygen concentration. Further they controlled the alkalinity to ammonia ratio in order to achieve a partial nitritation process. If the denitrification process, through the nitrite or nitrate route, is coupled with the nitritation/nitratation process, alkalinity will be produced, equilibrating the system. Also anammox process partially equilibrates the system, as it both consumes alkalinity and hydrogen ions. The alkalinity difference between influent and effluent had been used as indicators for nitrification and denitrification

in a Sequencing Batch Reactor (SBR), and could be utilize in the new biological nitrogen removal configurations, which include the integration of partial nitritation, anammox (Bagchi et al. 2010).

Salinity

The presence of salts in wastewaters may negatively affect bacteria and inhibit their activity. Salinity influences the life strategies of bacteria. Bacteria can tolerate salinity, being able to survive in environments with high salinity by maintaining their cytoplasm at the same osmotic level as that of the surrounding environment (Bollmann and Laanbroek 2002). However, the effect of salinity on nitrogen removal, is not clear at all, mainly because different experimental conditions were used in the literature studies (Paredes et al. 2007). Nevertheless, different degrees of inhibition on nitrification process have been reported in literature, mainly depending on the adaptation of biomass to the saline environments (Table I-4).

It has been confirmed that the nitrite oxidizers were more sensitive to salt concentration than the ammonium oxidizers (Vredenbregt et al. 1997; Campos et al. 2002; Mosquera-Corral et al. 2005), even if some researcher concluded that, compared to nitrite oxidizers, ammonia oxidizers were more sensitive to short- and long-term salt stress (Moussa et al. 2006; Sudarno et al. 2011). In saline environments, the adaptation of a microbial community to the high salinity concentration has a significant effect on their species diversity. *Nitrosomonas europaea* was detected at higher levels in saline environments compared to the levels in freshwater systems (Tal et al. 2003). In addition, Ye and Zhang (2011) demonstrated that the ammonia-oxidizing bacteria dominates over ammonia-oxidizing archaea in a saline nitrification reactor under low DO and high nitrogen loading.

Table I-4 Effects of salinity on the nitrification processes.

	Salinity	Inhibition	System	Reference
non adapted biomass	5.8 g NaCl L^{-1}	0%	SHARON	(Mosquera-Corral et al. 2005)
	30 g NaCl L^{-1}	20%	nitrification reactor	(Dincer and Kargi 2001)
	30 g NaCl L^{-1}	55%	activated sludge	(Panswad and Anan 1999)
	66 gNaCl L^{-1}	95%	SBR	(Moussa et al. 2006)
adapted biomass	8.3 g Na_2SO_4 L^{-1}	higher inhibition on NOB rather than AOB	activated sludge	(Campos et al. 2002)
	>13.7 g NaCl L^{-1}	inhibition	activated sludge	(Campos et al. 2002)
	19.9 g $NaNO_3$ L^{-1}	inhibition	activated sludge	(Campos et al. 2002)
	25 g NaCl L^{-1}	0%	SHARON	(Mosquera-Corral et al. 2005)
	30 g NaCl L^{-1}	30%	activated sludge	(Panswad and Anan 1999)
	40 g NaCl L^{-1}	60%	PNBR.	(Aslan and Simsek 2012)
	78 g NaCl L^{-1}	0%	SBR	(Dahl et al. 1997)

Other sources of inhibition

Nitrification may also be influenced by several inhibitory compounds. Among them, the influence of heavy metals on the nitrification has been thoroughly studied. You et al. (2009b) showed that the inhibition degrees for the nitrification activity was nickel (Ni) > cadmium (Cd) > lead (Pb). No significant inhibition in the nitrification reaction of the activated sludge was observed even when as much as 40 ppm Pb were used. In addition, no synergistic effect was found when different heavy metals were simultaneously added in different concentrations, and the overall inhibition effect depended on the heavy metal with the highest toxicity. Cecen et al. (2010) showed that copper (Cu) proved to be the most inhibitory metal on nitrification, while nickel (Ni) was in second place, Cobalt (Co) appeared to be the least inhibitory metal and the inhibitory effect evolved only very slowly. The author suggested to pay attention to factors such as the exposure time besides the metal concentration and speciation (labile and free metal). However inhibitor-resistant nitrifying cultures can be obtained from long-term batch incubations of decaying activated sludge incubated with high levels of added inhibitor (Yeung et al. 2013). Chromium, nickel, copper, zinc, lead and cadmium inhibit both steps of nitrification reaction but the bacteria are not inhibited according to the same pattern (Grunditz et al. 1998). Carucci et al. (2006) reported significant inhibition of nitrification by lincomycin, a lincosamide antibiotic. Free cyanide cause a lag phase on nitrification in activated sludge. However, the continuous exposure to the toxicity of free cyanide progressively increase its toxicity over time (Kim et al. 2011). Also benzene, toluene and xylene affect nitrification activity, mainly the ammonia oxidizing bacteria (Zepeda et al. 2006). In literature, opposite effects of volatile fatty acid on nitrite oxidation have been reported (Eilersen et al. 1994; Oguz et al. 2007). A selective inhibition of nitrite oxidation can be achieved using chlorate (Xu et al. 2011). High chloride concentration were used to select ammonia oxidizing bacteria over nitrite oxidizing bacteria (Chen et al. 2003). Allylthiourea ATU (86µM) is an inhibitor of the aerobic ammonia oxidation while sodium azide (50µM) is an inhibitor of aerobic nitrite oxidation, both used in research studies to inhibit AOB and NOB, respectively (Ginestet et al. 1998).

2.2. Ammonium oxidation by archaea

The aerobic oxidation of ammonia is a central bioprocess in the nitrogen cycle coupling ammonia production from mineralization of organic matter with denitrification process. Over a century from their discovery, bacterial nitrifiers were assumed to be the only microorganisms capable of the autotrophic nitrification. Recently, it was demonstrated that also Archaea are involved in the aerobic nitrification as well as bacteria. For years, microbiologists characterized the Archaea as obligate extremophiles, that are able to live in environments too harsh for other organisms, i.e. sulphur- metabolizing thermophiles. The first observation of nitrification in the Archaea was reported by Könneke et al. (2005), who isolated a marine Crenarchaeote, *Nitrosopumilus maritimus*, that grows chemolithoautotrophically by aerobically oxidizing ammonia to nitrite, suggesting that nitrifying marine Crenarchaeota may be important to the global carbon and nitrogen cycles. 16S rRNA phylogeny places this organism firmly in the Group 1.1a Crenarchaeota. Ammonia Oxidizing Archaea (AOAs) contain putative ammonia mono-oxygenase genes *amoA, amoB and amoC* (Francis et al. 2007). Nevertheless, in the analysis of genome, hydroxylamine oxidoreductase (HAO) was not identified, indicating that other different mechanism than known for AOBs occurred (Hallam et al. 2006). AOAs are predominantly autotrophic but facultative and like heterotrophs Crenarchaeote are able to use solved organic carbon, such as amino acids, as carbon source (Herndl et al. 2005).

Other two Archeaea species were detected able to perform the aerobic oxidation of ammonia: "*Candidatus* Nitrososphaera gargensis" (usually detected in soils), and "*Candidatus* Nitrosocaldus yellowstonii". The *amoA* genes had been used to detect aerobic ammonia oxidizing Archaea (AOA) in natural samples. However, the *amoA* gene of AOB and AOA are distantly related, and different PCR primer sets are used for the AOB and AOA detection. AOAs are found to thrive in various habitats including hot/thermal springs, marine and fresh waters, marine sponge, agriculture soils, estuarine sediments and wastewater treatment systems (Francis et al. 2007; You et al. 2009a). Phylogenetic analysis of several hundred archaeal *amoA* sequences revealed diverse and distinct AOA communities which can be associated with different habitats and sampling sites, with little overlap between water columns and sediments. In the natural environments, AOBs and AOAs co-occurr and AOAs appear to be much more abundant than AOBs in the ocean and in soils (Leininger et al. 2006; Mincer et al. 2007). However, the mechanisms of the competition are not completely understood, yet. In particular ammonia concentrations could be the major selection pressure of one species over the others. For example, AOBs grow in agricultural soil, with high concentrations of ammonium, which are typical of mineral fertilization strategies (100 – 1000 mg L^{-1}) (Nicol et al. 2011). On the contrary, AOAs were observed in soil with particular low ammonium concentration (Offre et al. 2009). While AOBs typically grown in media in the range of 20 – 350 mg TAN L^{-1} and concentration lower than 1 mg TAN L^{-1} are limiting, AOAs grow in media containing 7 - 20 mg TAN L^{-1} (Koops et al. 2003). Ammonium concentrations higher than 40 mg TAN L^{-1} cause the inhibition of the AOAs (Hatzenpichler et al. 2008). Other studies, suggest that AOAs are well adapted to grow in the nutrient limiting (oligotrophic) environment (Martens-Habbena et al. 2009). Nevertheless, AOA could also adapt to higher ammonia concentrations.

Archaeal ammonium-oxidizer *amoA* sequences have been found in some wastewater treatment plant (Park et al. 2006; Zhang et al. 2009). Cao et al. (2011) and Park et al. (2006) used PCR primers targeting archaeal *amoA* genes to retrieve and compare 75 sequences from the activated sludge of WWTPs across the US. 50 of 75 sequences were archaeal *amoA* sequences The presence of AOAs appeared in WWTPs operating at low DO concentrations for simultaneous nitrification and denitrification and high Sludge Retention Time (SRT) (Cao et

al. 2011). AOAs were also detected in wetland systems (You et al. 2009a; Wang et al. 2011a; Sims et al. 2012). In particular, Sims et al. (2012) showed a strong correlation between the abundance of AOAs and AOBs and nitrification activity in wetland soils. The results suggested that AOAs are more persistent and more abundant than AOB in the nutrient-depleted (oligotrophic) wetlands. The authors investigated the seasonal and spatial distributions of AOB and AOA. AOAs were detected both in the top soil and in the water. AOB were more sensitive to low temperature than AOA. Finally, the results confirmed that ammonium concentrations is one of the major factor influencing AOA and AOB population in wetlands, although other factors such as temperature, dissolved oxygen, and soil organic matter are involved. Recently, the cooperation between marine anammox bacteria and ammonia- oxidizing archaea in a laboratory-scale model system under oxygen limitation was succesflul demostrated (Yan et al. 2012).

2.3. Denitrification

The denitrification activity was observed in the late nineteenth century, when nitrogen losses in agriculture soil were noticed. In 1875, Meusel associated microorganisms with nitrogen losses (Meusel 1875). Gayon and Dupetit (1882) introduced the term of denitrification and the modern era of studies of the nitrate and nitrite reduction. Denitrification involves the reduction of the oxidised forms of nitrogen –nitrate (NO_3^-), nitrite (NO_2^-), nitric oxide (NO) and nitrous oxide (N_2O)– into dinitrogen gas (N_2). This process is used as an alternative to the oxygen respiration, under low oxygen or anoxic conditions (Sørensen 1978). Heterotrophic denitrification has been considered for a long time to be the only anoxic process contributing significantly to the loss of inorganic nitrogen, playing a fundamental role both in oceanography and wastewater treatment.

The sequential production of intermediates via a linear pathway of four reductive steps was first proposed by Payne (1973) and has been widely accepted (Eq. I-26):

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2 \qquad \text{Eq. I-26}$$

2.3.1 Stoichiometry of denitrification

The main pathways of denitrification can be summarized by the following nitrate and nitrite reduction reactions (Eq. I-27 - Eq. I-28).

$$0.2NO_3^- + 1.2H^+ + e^- \xrightarrow{heterotrophic} +0.1N_2 + 0.6H_2O \qquad \text{Eq. I-27}$$

$$0.34NO_2^- + 1.36H^+ + e^- \xrightarrow{heterotrophic} +0.17N_2 + 0.68H_2O \qquad \text{Eq. I-28}$$

Denitrification process and, in particular, the reduction of nitrate and nitrite could be accomplished with a variety of electron donors and carbon sources, such as: methanol, acetate, glucose, ethanol (Constantin and Fick 1997; Adav et al. 2010). Methane was recently used by Modin et al. (2007), while Elefsiniotis et al. (2004) investigated the use of volatil fatty acid (acetic, butyric and propionic acid) from an acid-phase digester effluent. A widespread use of methanol (CH_3OH) had been gained, as it was a relatively inexpensive external carbon source. The combined dissimilation and synthesis equations for denitrification via nitrate and via nitrire, using methanol as an electron donor, are following reported (Eq. I-29 - Eq. I-30) (Ahn 2006):

$$NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7O_2N + 0.47N_2 + 1.68H_2O + HCO_3^- \qquad \text{Eq. I-29}$$

$$NO_2^- + 0.53CH_3OH + 0.67H_2CO_3 \rightarrow 0.04C_5H_7O_2N + 0.48N_2 + 1.23H_2O + HCO_3^- \qquad \text{Eq. I-30}$$

In these equations, the theoretical methanol requirement for nitrate reduction to dinitrogen gas, considering the synthesis requirement, is 2.46 mg CH_3OH per mg NO_3-N, corresponding to 1.7 mg COD mg NO_3-N^{-1}. The methanol requirement for nitrate reduction to dinitrogen gas is 1.21 mg CH_3OH per mg NO_2-N. Considering the acetate (CH_3OOH) instead of methanol as carbone source for the whole denitrification process, the requirement of acetate is 2.67 mg CH_3OOH per mg NO_3-N, corresponding to 2.86 mg COD mg NO_3-N^{-1}.

Expressed as volatile suspended solids (SSV), the growth yield for heterotrophic denitrifiers is 0.45 mg cell per mg NO_3-N reduced, using methanol and 0.67 mg cell per mg NO_3-N reduced using acetate. Finally, in the denitrification process, alkalinity is produced. Approximately 1 mol of bicarbonate (HCO_3^-) is produced for

every mole of nitrate or nitrite reduced, resulting in 3.57 mg of alkalinity as $CaCO_3$ for every mg of nitrate or nitrite reduced.

2.3.2 Mechanisms of denitrification

A number of thorough reviews have been published during the last years discussing the biology and molecular basis of the denitrification (Knowles 1982; Zumft 1997; Philippot 2002; Kraft et al. 2011).

Denitrification can be seen as the assemblage of nitrate (NO_3^-) respiration, nitrite (NO_2^-) respiration combined with nitric oxide (NO) reduction and nitrous oxide (N_2O) respiration. The three respiratory complexes maintain a certain degree of independence, thus, denitrification is best described as a modular organization (Zumft 1997). In each reaction of the denitrification process, the oxidised nitrogen substrates serve as an electron acceptor in the subsequent respiration usually coupled to the oxidation of organic compounds, which are used as electron donors for energy generation. Denitrification pathways are now also well- characterized on the enzymatic level (Berks et al. 1995; Zumft 1997). Each step of denitrification is carried out by specialized enzymes: nitrate reductase (*nar - narG, narH, narI or nap- napA, napB*), nitrite reductase (the copper-containing *nirK* and the cytochrome c-type *nirS*), nitric oxide reductase (*nor -norB, norC, norZ*) and nitrous oxide reductase (*nosZ*).

In denitrification, the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) is catalyzed by the membrane bound complex nitrate reductase (*nar*), which could be also present in several non-denitrifying bacteria (Zumft 1997; Kraft et al. 2011). In addition, in some denitrifying bacteria, the nitrate reduction step in the denitrification process is not dependent on the presence of *nar* gene but on a periplasmic nitrate reductase gene (*nap*) (Bedmar et al. 2005). The reduction of nitrite (NO_2^-) to nitric oxide (NO) is catalyzed by two isofunctional periplasmic enzymes, which are evolutionary unrelated: a copper-containing enzyme, *nirK*, and a cytochrome c-type *nirS* (Zumft 1997; Kraft et al. 2011). The product of *nirS* and *nirK* is nitric oxide (NO), that is a radical which reacts with many other molecules. It is now well accepted that the reduction of nitric oxide (NO) to nitrous oxide (N_2O) is catalyzed by the membrane bound nitric oxide reductase (*nor*) (Zumft 2005; Kraft et al. 2011). Finally, the last of the four enzymes required for the complete denitrification is the multicopper enzyme nitrous oxide reductase, *nos*, that reduce nitrous oxide (N_2O) to dinitrogen gas (N_2). *Nos* gene could be either a periplasmic or a membrane bound enzyme depended on the bacteria (Kraft et al. 2011). However, also other non-denitrifying microorganisms use nitrous oxide as electron acceptor and reduce NO to dinitrogen via *nos* (Zumft,1997).

Dinitrogen gas is the main end product of denitrification while the nitrogenous gases (nitric oxide and nitrous oxide) are obligatory and free intermediates. Nevertheless, these gases may be released as end products when denitrification is not complete. Thus, denitrification could be also a source of environmental trouble, as it may produce N_2O emissions, that is a powerful greenhouse gas.

Factors influencing the N_2O production during denitrification have been identified in the literature, related to an imbalanced activity of nitrogen-reducing enzymes. Wunderlin et al. (2012) confirmed that in anoxic conditions, N_2O emission is low under optimal growth conditions for heterotrophic denitrifiers (e.g. no oxygen input and no limitation of readily biodegradable organic carbon). Limited carbon substrate may lead to an incomplete denitrification and a N_2O production (Itokawa et al. 2001). Further, N_2O can also be released in high quantities under low oxygen conditions (Noda et al. 2003). Indeed, denitrification also occurs in the presence of oxygen (Bell et al. 1990). Dissolved oxygen acts as an inhibitor of N_2O reductase, *nos* (Bonin 1996; Baumann et al. 1997; Wunderlin et al. 2012). N_2O reductase is more sensitive to oxygen than the other enzymes. Therefore,

aerobic denitrification is often incomplete. An increase in N_2O formation has been observed when conditions switch from anaerobic to aerobic (Frette et al. 1997). In addition, several authors have shown that high nitrite concentrations lead to a lower denitrification rate and N_2O emissions (Von Schulthess et al. 1994; Wunderlin et al. 2012). This may be due to the inhibition of N_2O reductase by NO or NO_2.

Over denitrification, also other processes could be involved in N_2O emission under low oxigen concentration. Tallec et al. (2008) reported that below 0.3 mg O_2 L^{-1}, heterotrophic denitrification appeared to be the major process responsible for the N_2O emission, while from 0.4 to 1.1 mg O_2 L^{-1}, N_2O emissions were due to two processes: (i) heterotrophic denitrification (40%) and autotrophic nitrifier denitrification (60%).

2.3.3 Phylogeny

Denitrification occurs in natural aqueous habitats (Trevors and Starodub 1987; Gao et al. 2009), in engineered systems (Wiesmann 1994) and in soil (Trevors 1985). Denitrification is carried out by a wide diversity of bacteria as well as by archaea and fungi (Focht and Verstraete 1977; Kobayashi et al. 1996; Philippot 2002). The denitrification pathway in archaea (Philippot 2002; Cabello et al. 2004) and some eukaryotes (Risgaard-Petersen et al. 2006) is much less understood than in bacteria.

However, denitrifiers are somewhat more frequent within the phylum proteobacteria (alpha, beta, gamma, and epsilon divisions), although there is no an identifiable pattern of their distribution (Zumft 1997; Kraft et al. 2011). Among the gram-negative proteobacteria, there are the H_2 oxidizing *Pseudomonas* (γ- proteobacterium), the aerobic and H_2 oxidizing *Paracoccus* (α- proteobacterium) and the lithotrophic sulfur oxidizing *Thiobacillus* (β- proteobacterium) which can degrade complex or toxic natural and synthetic organic compounds. Among the N-fixation bacteria, denitrification was detected in *Alcaligenes (*aerobic β-proteobacterium), in *Azospirillum, Magnetospirillum, Bradyrhizobium* and *Rhizobia (*α-proteobacteria) and *Azoarcus* (β-proteobacterium). *Azoarcus tolulyticus* is a N-fixation bacteria which degrades toluene and exhibits the complete denitrification (β-proteobacterium). On the contrary, *Azoarcus evansii* degrades a variety of monoaromatic compounds under denitrifying conditions but is unable to use toluene. However, denitrification and N-fixation can proceed concomitantly. Among the denitrifiers *Pseudomonads*, N-fixation strains have been reported for P. *fluorescens*, some P. *stutzeri* and P. *pseudoflava.* Among the photosynthetic denitrifying bacterium, *Rhodobacter* sp *(*α-proteobacterium) were identified.

Anoxic-microaerophilic denitrification. Most denitrifiers are heterotrophic organisms which use an organic carbon source to reduce nitrate and nitrite to dinitrogen gas. In heterotrophs, a wide range of organic substrates is known to support denitrification: glycerol, acetic acid, lactic acid (Akunna et al. 1993) methanol (Akunna et al. 1993; Kalyuhznaya et al. 2009), sugars and alcohols, diverse complex organic molecules (Burford and Bremner 1975), wastewater organic carbon (Gayon and Dupetit 1886; Matějů et al. 1992), aromatic hydrocarbons (Rabus and Widdel 1995, 1996), to name a few. Generally denitrification is nearly exclusively a facultative anaerobic or microaerophilic process. However, metagenomic studies on uncultivated bacteria suggested that nitrate respiration could be also performed by strictlpy anaerobic lifestyle bacteria (Walsh et al. 2009).

Aerobic denitrification. The complete aerobic denitrification at high dissolved oxygen (DO) concentrations may also occurr and it is well reviewed (Zumft 1997; Ahn 2006). Most aerobic denitrifiers are heterotrophic organisms, such as the *Paracoccus* species (Ludwig et al. 1993). *Magnetospirillum magnetotacticum* is also a bacterium that denitrifies only under microaerophilic but not under anaerobic conditions, consuming oxygen

(Bazylinski and Blakemore 1983). Also *Pseudomonas* stutzeri reduced nitrate to nitrogen gas without nitrite accumulation under aerobic conditions (Su et al. 2001).

A variety of molecular methods have been applied to investigations of denitrifying genes in natural environments as reviewed by Wallenstein et al. (2006). Due to the enormous diversity, abundance and widespread distribution of denitrifying microorganisms, the detection of denitrifiers requires the use of functional genes rather than 16S ribosomal RNA (rRNA) genes (Kraft et al. 2011). The expression of *narG*, *napA*, *nirS*, *nirK* and *nosZ* in different environments had been investigated (Philippot et al. 2001; Nogales et al. 2002; Throbäck et al. 2004) using different microbial techniques, i.e PCR and FISH. The nitrite reductase genes *nirS* and *nirK* have frequently been used as marker genes for denitrifying bacteria in environments samples (Braker et al. 1998; Pratscher et al. 2009), such as estuarine sediments (Bulow et al. 2008), sea (Ward et al. 2009), and activated sludge (Pratscher et al. 2009; Zafiriadis et al. 2011). Nevertheless, Philippot (2002) showed that the phylogeny of *nir* and *nor* genes, coding for the key enzymes nitrite reductase and NO reductase in the denitrification pathway, does not always agree with the phylogeny of the 16S rRNA gene. In their study on denitrification, Cuhel et al. (2010) suggested that counting functional genes is not sufficient for a comprehensive understanding of how denitrification is developing. Today, the identification of relevant environmental denitrifiers in situ remains a challenge (Kraft et al. 2011).

2.3.4 Bioinformatics

Genomes size among denitrifying genera varies within a wide range. Among the highest, there is the genome of *Bradyrhizobium japonicum* (8.7 Mb) (Kündig et al. 1993), while, among the lowest, there is the genome of *Neisseria gonorrhoeae* (2.2 Mb) (Dempsey and Cannon 1994). Different data were obtained for *Pseudomonas*, resulting in 5.9 Mb for P. *aeruginosa* PAO (Holloway et al. 1994), 6.5 Mb for P. *aeruginosa* C (Schmidt et al. 1996), 6.63 Mb for P. *fluorescens* SBW25 (Rainey and Bailey 1996) and 4.3 Mb for P. *stutzeri*. Among the other, genes related to the NO_3^-, NO_2^-, NO and N_2O reduction were identified (Zumft 1997).

2.3.5 Kinetics

Denitrification is a four-step process ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Usually, it is modeled as one or two step model ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$.) as the production of NO and N_2O does not contribute significantly to the total mass –flow of nitrogen in the biological system. Recently, N_2O modeling has received attention as N_2O is a powerful greenhouse gas. Most models only consider N_2O production by denitrifying microorganisms. A three-step denitrification model including the reduction of nitrate, nitrite and nitrous oxide has been formulated by von Schulthess et al., (1994). Samie et al., (2011) considered as a four-step denitrification process, based on ASM1, to simulate soluble N_2O production by heterotrophic denitrifying biomass and estimate the gaseous emissions from the reactors.

Further, different denitrification rate can be considered, depending on the organic source type. Mainly, two organic sources come from the wastewater, i.e. readily biodegradable organic (RBCOD) and slowly biodegradable organics (SBCOD). A third source is the slowly biodegradable organic generated by the biomass itself through death and lysis of organism mass (the endogenous mass loss/respiration) that is utilized in the same way as the wastewater SBCOD, but recognized separately. The RBCOD can be used at a high rate, using

the Monod equation. The SBCOD must be first broken down (hydrolysed) and then utilized. The SBCOD hydrolysis rate is slow and forms the limiting rate in the utilization of SBCOD (Ekama and Wentzel 2008).

2.3.6 Factors involved in denitrification kinetics

Scientists have investigated the factors controlling denitrification in an attempt to better understand the process. Many studies have been focalized on the roles of NO_3^- availability, O_2, and pH in controlling denitrification process both in aquatic and in soil systems. There is a large diversity of bacteria, archaea, and fungi capable of denitrification, and their community composition depends on the long-term environmental drivers (Wallenstein et al. 2006). Thus, the denitrifying community may differ in physiology, and environmental tolerances to pH, Temperature (T) and oxygen (O_2). Growth rate, and enzyme kinetics could vary.

The rate of denitrification processes generally increases exponentially with increasing temperature. Most of the studies of the effect of temperature on denitrification have been carried out with soil in which there appears to be a marked temperature dependence. This is in contrast to aquatic sediments where lower temperature variation can be measured and controlled (Heinen 2006). Low temperatures, caused by seasonal changes, affect denitrification by reducing the microbial growth rate and decreasing the rate of biologically-mediated reactions (Madigan and Martinko 2006). Mckenney et al. (1984) successful applied the Arrenhius type equation to study the kinetic of several denitrification steps in the temperature range of 5 to 25°C in soils. However above 60 - 75°C, a rapid decline of denitrification activity was observed both in soil and in aquatic systems (Knowles 1982). Several authors investigated denitrification at temperature lower than 20°C (Welander and Mattiasson 2003; Elefsiniotis et al. 2004). Further, low temperatures exacerbate the effects of inhibitory compounds (Hajaya et al. 2011).

Experiments on the effect of pH on denitrifiers activity in soils have clearly showed that pH has a remarkable effect on denitrification (Cuhel et al. 2010). In general, denitrification rate increases with increasing pH values up to the optimum pH, while the N_2O emmission decreases. A clear optimum in denitrification rate exists around pH=7 to 7.5, and denitrification almost ceases for pH values of 4 or 10 (Heinen 2006). At low pH values, the nitrous oxide reductase, which reduces N_2O, is progressively inhibited such that the overall rate of denitrification decreases and the mole fraction of N_2O produced increases. Glass and Silverstein (1998) showed that denitrification activity on an activeted sludge was significantly inhibited at pH of 6.5- 7. At higher pH (pH 7.5 – 9), denitrification was achieved with a high nitrite accumulation. The pH also regulate the chemical compounds equilibria. Abeling and Seyfried (1992) concluded that FNA inhibited denitrifiers. The toxicity threshold in their study was reported to be 0.04 mgHNO_2-N L^{-1} on activated sludge. Ma et al. (2010) revealed that FNA exhibited a similar inhibitory effect on nitrate and nitrite reducers. The nitrate and nitrite reduction activities were reduced to 40% in the FNA concentration range of 0.01-0.025 mgHNO_2-N L^{-1} and completely stopped at the FNA concentration of 0.2 mgHNO_2-N L^{-1}.

However, some denitrifiers lack key enzymes to obtain a complete denitrification. The lack of some enzymes can determine the production and the accumulation of free intermediates. Accumulation of nitrite has been often observed in denitrification systems, both in soil and aquatic environments (Betlach and Tiedje 1981). Nitrite accumulation may result from nitrate inhibition of nitric oxide reductase (Payne 1973). On the contrary, Betlach and Tiedje (1981) reported that nitrate did not inhibit nitrite and nitrous oxide reduction. The accumulation of nitrite seemed to depend on the relative rates of nitrate and nitrite reduction. Bilanovic (1999) obtained denitrification under high nitrate concentration (500-750 mg NO_3^--N L^{-1}). Nitrite accumulation was only

observed initially but its concentration decreased with time, reaching zero when acetate or anaerobic digester effluent was used as carbon source. However nitrite accumulation limits the expression of *nar* (nitrate-), *nir*, (nitrite) and *nos* (nitrous oxide) reductase (Glass et al. 1997). Adav et al. (2010) studied the capacity of supplementary carbon sources (acetate, methanol, and ethano) on the *nir* enzyme reactivation.

Both the synthesis and activity of nitrate reductase (*nar*) are inhibited by oxygen. Nitrous oxide reduction, carried out by nitrous oxide reductase (*nos*), is more sensitive to oxygen inhibition than the other steps in denitrification (Krul and Veeningen 1977). The nitrous oxide reductase is inhibited by DO concentrations less than 0.2 mg/L (Kumar and Lin 2010).

The availability of electrons in organic carbon compounds is one of the most important factors controlling the activity of the heterotrophs. As can be observed from denitrification stoichiometry, for a complete denitrification an influent COD/NO_3^--N ratio over 2.86 is required. Experience suggests a COD/N ratio over 7 would be appropriate. Moreover, the influence of the type of carbon source on the denitrification rate have been widely studied. Constantin and Fick (1997) showed that the specific denitrification rate obtained using acetic acid was higher than that measured using ethanol, as acetic acid is a directly assimilable carbon source while the ethanol must be first changed into acetate. Further, Akunna et al. (1993) showed that using acetic and lactic acids media the nitrate/nitrite reduction was essentially denitrification activity, while ammonium production by DNRA occurred using glucose and glycerol media.

Production of N_2O can be obtained by heterotrophic denitrifiers, when an imbalanced activity of nitrogen-reducing enzymes occur (Wunderlin et al. 2012), e.g. due to oxygen inhibition (Baumann et al. 1997; Lu and Chandran 2010), nitrite accumulation (Von Schulthess et al. 1994), as well as a limited availability of biodegradable organic compounds (Itokawa et al. 2001).

Brunet and Garcia-Gill (1996) showed that at extremely low concentrations of metal sulfides, nitrate was reduced via denitrification whereas at higher metal sulfides concentrations, reduction to ammonia (DNRA) and incomplete denitrification took placed. Finally, the reductase enzymes involved in denitrification are susceptible to inhibition by a variety of compounds, such as pesticide, azide, cyanide, acetylene, nitrapyrin (nitrification inhibitor) and sulfur compound (Knowles 1982). Chloramphenicol is an antibiotic, used as inhibitor of denitrification process in research studies (300 mg L^{-1}). It acts inhibiting the formation of the peptidic bonds for the proteins synthesis (Brooks et al. 1992).

2.4. Heterotrophic nitrification and Nitrifiers denitrification

Among autotrophic nitrifiers and heterotrophic denitrifiers which perform nitrification and denitrification respectively, other organisms participate to the nitrogen cycle (Stein 2011).

Heterotrophic nitrification. Aside the autotrophic nitrifying bacteria, several heterotrophic bacteria are capable to oxidize ammonia (Van Niel et al. 1993), hydroxylamine (Ralt et al. 1981), organic nitrogen compounds (Castignetti and Hollocher 1984) to nitrite and nitrate: This process is termed, in literature, as heterotrophic nitrification while the bacteria responsible are called heterotrophic nitrifiers. Many heterotrophic nitrifiers are also able of aerobic denitrification, such as *Thiosphaera pantotropha* (Robertson and Kuenen 1988). Thus, the nitrite and nitrate produced during the heterotrophic nitrification are immediately reduced to N-oxides or dinitrogen gas via denitrifies enzymes. This is the so-called simultaneous nitrification-denitrification process.

Autotrophic denitrification. On the contrary, many autotrophic bacteria are capable to utilize inorganic compounds as electron donors, such as sulfur compounds (Beijerinck 1920), hydrogen, ferrous iron (Straub et al., 1996), arsenite (Sun et al., 2008), or nitrite (De Bruijn et al. 1995) to produce N_2, both in anoxic and micro-aerobic conditions. T. *pantotropha* is a facultatively anaerobic, autotrophic sulfur bacterium (Robertson and Kuenen 1984). *Thiobacillus* denitrificans is a widely distributed and well-characterized obligate chemolithoautotrophic bacterium, which is able to couple the oxidation of inorganic sulfur compounds (such as hydrogen sulfide and thiosulfate) to denitrification. The oxidation of Fe(II) is also coupled to a complete denitrification (Straub et al. 1996; Blöthe and Roden 2009).

Among the other, the denitrifying nitrifiers can play an important role. Initially, the nitrifying bacteria seemed to be excluded from being considered denitrifiers, as they are involved in the aerobic branch of the N cycle. Using ^{15}N as a tracer, evidence of denitrifying activity by *Nitrosomonas* species have been reported, producing N_2O from nitrite. Indeed, hydroxylamine is an intermediate in the oxidation of ammonia to nitrite. In the absence of oxygen, hydroxylamine can be oxidized with nitrite as electron acceptor, while nitrous oxide is produced (De Bruijn et al. 1995). *Nitrosomonas eutropha* is an obligate lithoautotrophic nitrifying, but under anaerobic conditions could be stimulated by the presence of nitrogen oxide and acts as a denitrifying organism, using hydrogen as the electron donor and nitrite as the electron acceptor (Schmidt and Bock 1997b). *N. europaea* also produce N_2O in the denitrification process (Poth and Focht 1985b). The denitrifying nitrifiers microorganisms are mainly detected in bioreactors which have a combination of aerobic and anaerobic ammonia oxidation, as denitrifying nitrifiers operate under aerobic conditions during ammonia oxidation but they are enhanced under micro-aerobic condition, while anaerobic conditions are required for the growth of some of denitrifying nitrifiers (Stein 2011). Denitrifying nitrifiers were also detected at elevated nitrite concentration (Colliver and Stephenson 2000).

2.5. Anammox - Anaerobic Oxidation of Ammonium

Ammonium oxidation was long assumed to be a strictly aerobic process. However, based on thermodynamic calculations, in 1977, Broda proposed the existence in nature of lithotrophic organisms that could derive their energy for growth from the oxidation of ammonium using nitrite as electron acceptor and producing dinitrogen gas (Broda 1977). In the early 1990s, anaerobic ammonium oxidizing (anammox) bacteria were discovered in a denitrifying bioreactor and patented (Mulder 1992). Later, experiments with labeled $^{15}NH_4^+$ and unlabeled NO_2^- confirmed that N_2 was generated by anammox reaction (Mulder et al. 1995). As illustrated by Kuenen (2008), this discovery led to the realization that a substantial part of the nitrogen losses in the marine environment were due to the activity of these bacteria, as well as the denitrification process. Considering both the reduction of costs of ammonium removal from wastewaters and the reduction of CO_2 emission, the anammox process was further proposed as an innovative and sustainable wastewater treatment for nitrogen removal (Jetten et al. 1997). Until now, many research groups studied and are studying various microbial and industrial aspects of the anammox process.

2.5.1 Stoichiometry of Anammox

Several basic studies investigated the stoichiometry of the anammox reaction based on mass balance over anammox enrichment cultures. Under steady state condition, ammonium, nitrite and bicarbonate are converted according the following catabolic and anabolic reactions (Eq. I-31 - Eq. I-32), respectively. The overall reaction is expressed in the Eq. I-33 (Strous et al. 1998):

$$NH_4^+ + NO_2^- \xrightarrow{Anammox} N_2 + 2H_2O \qquad \Delta G^{\circ\prime} = -358\ kJ\ mol^{-1} \qquad \text{Eq. I-31}$$

$$0.066CO_2 + 0.033H_2O + 0.26NO_2^- \rightarrow 0.066HCO_3^- + 0.26NO_2^- \xrightarrow{Anammox} 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} \qquad \text{Eq. I-32}$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \xrightarrow{Anammox} 1.02N_2 + 0.066CH_2O_{0.5}N_{0.15} + 0.26NO_3^- + 2.03H_2O \qquad \text{Eq. I-33}$$

The anammox reaction is associated with a considerable release of Gibbs free energy and is mediated by a group of microorganism associated with the phylum of *Planctomycetes*. The main product of the anammox process is dinitrogen gas, but about 10% of the nitrogen feed is converted to nitrate. The overall nitrogen balance gave a ratio of ammonium to nitrite conversion of 1:1.32 and a ratio of nitrite conversion to nitrate production of 1:0.26. Nitrate production is a fundamental part of anammox reaction and has be used to measure the growth of anammox bacteria, at it is only involved in the anabolic reaction (Eq. I-32). NO_2^-N/NH_4- N and NO_3^-N/NH_4- N ratios which differ from the stoichiometric values reported above, have been found in some systems, indicating the co-occurrence of other biological processes, such as denitrification or DNRA under anoxic conditions (Strous et al. 1999b; Ahn et al. 2004) and nitritation under micro-aerobic conditions (Third et al. 2001).

Bicarbonate is utilized as the sole carbon source for the synthesis of biomass ($CH_2O_{0.5}N_{0.15}$), as this microorganisms are autotrophs. Nitrite can act as electron acceptor in the energy generating reaction (Eq. I-31) as well as electron donor for the CO_2 reduction to biomass, producing nitrate (Eq. I-32). Approximately 1 mol of bicarbonate (HCO_3^-) is consumed to oxidize 15 mol of ammonium, resulting in 0.23 mg of alkalinity as $CaCO_3$ for every mg of nitrogen ammonium. The alkalinity consumption is significantly lower than the requirements of nitritation process. However, in anammox reaction hydrogen ions are consumed and a pH decrease of the system

can be detected. In anammox stoichiometric equations, are required about 0.066 mol of fixed carbon per mol of ammonium and 0.05 mol of fixed carbon per mol of nitrite. Although the energy yield from anammox catabolic reaction is similar to the energy of AOB, the maximum specific growth rate of anammox bacteria is lower, with value between 0.054-0.2 d^{-1} (Strous et al. 1999b; Van Der Star et al. 2008). Expressed as volatile suspended solids (VSS), growth yield for anammox bacteria is 0.11 mg cell per mg NH_4-N oxidized. Expressed as chemical oxygen demand (COD), growth yield is 0.08 mg COD per mg NH_4-N oxidized. The decay rate of anammox bacteria is also low. It is estimated one order of magnitude lower that the maximum growth rate, in the range of 0.001 – 0.008 (Koch et al. 2000a; Dapena-Mora et al. 2004; Scaglione et al. 2009).

2.5.2 Mechanisms of anaerobic ammonia oxidation

The biochemistry of the anammox bacteria is very complex and only recently almost resolved. Initially it was conceived that anammox process involved the turnover of hydroxylamine (NH_2OH) and hydrazine (N_2H_4) as intermediates (Van de Graaf et al. 1997). In the original model, it was proposed that hydroxylamine was the true intermediate that would combine directly with ammonium to form hydrazine. In this case, hydroxylamine would have to be formed directly from nitrite (NO_2^-) or nitric oxide (NO). The conversion of hydrazine to dinitrogen gas was postulated as the reaction generating the electron equivalents for the reduction of nitrite to hydroxylamine (Jetten et al. 1999). The model consisted in three subsequent steps: (i) the four-electron reduction of nitrite to hydroxylamine; (ii) the condensation of the hydroxylamine with ammonium to hydrazine; and (iii) the oxidation of hydrazine to dinitrogen gas (Van de Graaf et al. 1997; Jetten et al. 2001) (Fig. I.11).

An analysis of the genome revealed the apparent lack of a nitrite-hydroxylamine oxidoreductase and the presence of a nitrite-nitric oxide oxidoreductase, *nirS*, in the genome of K. *stuttgartiensis*. In addition, anammox bacteria are characterized by a separated membrane–bound compartment, termed "anammoxosome", which contains large quantities of a hydroxylamine oxidoreductase (HAO)-like enzyme (Schalk et al. 2000). HAO-lime enzyme was postulated to be responsible for the oxidation of hydrazine to N_2 gas. HAO can oxidize both hydroxylamine and hydrazine. However, a recent work has revealed that the anammox organisms not only expresses the HAO enzyme in high concentrations, but also encodes a true hydrazine-oxidizing enzyme (HZO) (Shimamura et al. 2007), which has a significantly higher affinity for hydrazine than HAO and is strongly inhibited by hydroxylamine. Thus, the postulated pathway for energy metabolism and hydrazine formation was modified. A plausible alternative three steps cyclic pathway was suggested (Kartal et al. 2011): (i) the one-electron reduction of nitrite to nitric oxide (NO) by *nirS*; (ii) the condensation of one molecule of NO with one molecule of the ammonium with the concomitant input of three electrons generating hydrazine; and (iii) the oxidation of hydrazine, catalyzed by HZO, yielding four electrons and dinitrogen gas as the end product. Kartal and coauthors have recently proposed the biochemical pathway and enzymatic machinery of "Ca. K. stuttgartiensis", resolving the genes and proteins central to anammox metabolism (Fig. I.12). A set of three redox reactions (Eq. I-34 - Eq. I-36) involving N_2H_4 and nitric oxide (NO) was proposed to explain the overall anammox stoichiometry (Eq. I-31):

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \qquad \text{Eq. I-34}$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \qquad \text{Eq. I-35}$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \qquad \text{Eq. I-36}$$

Several studies showed that anammox bacteria are not strictly lithotrophic but their metabolism is versatile, as they are able to use a range of organic electron donors and inorganic electron acceptors (Güven et al. 2005; Strous et al. 2006; Kartal et al. 2007b). Short chain fatty acids, (i.e acetate, formate, propionate) can be metabolized by anammox bacteria producing ammonium and nitrite from nitrate, completely changing the stoichiometry described above. This characteristic in future could lead to apply the anammox process for the treatment of nitrate rich wastewaters using limited amounts of organic material (Kartal et al. 2007b).

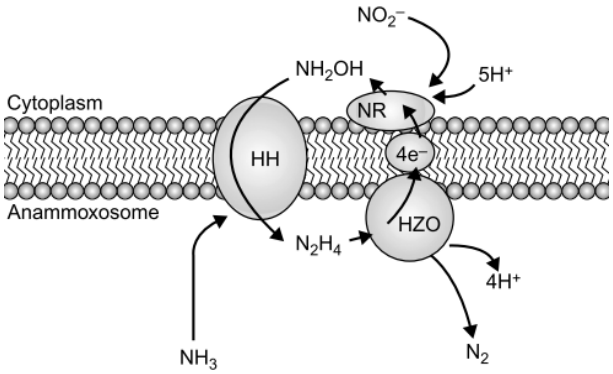

Fig. I.11 Past postulated mechanism of anaerobic ammonium oxidation.
NR is a nitrite-reducing enzyme and NH_2OH is the assumed product; HH (hydrazine hydrolase) condenses hydrazine out of ammonia and hydroxylamine; HZO is a hydrazine-oxidising enzyme (which might be equivalent to hydroxylamine oxidoreductase, HAO). Picture from Jetten et al. (2001).

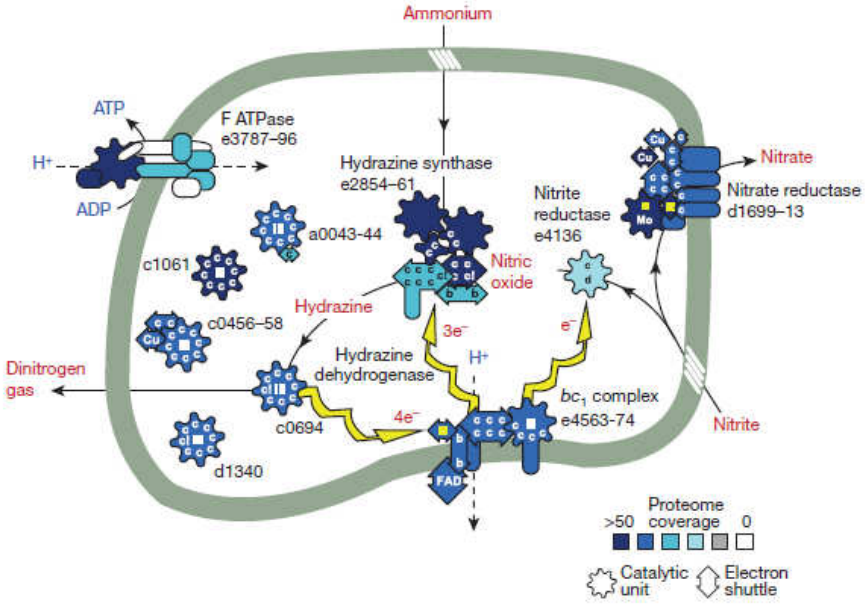

Fig. I.12 Current biochemical pathway and enzymatic machinery of *K. stuttgartiensis*.
The anammoxosome, an intracytoplasmic compartment bounded by a membrane (grey line), is the locus of anammox catabolism. Identifiers of open reading frames and the degree to which the encoded respiratory protein complexes were detected in the proteome are indicated. Hydrazine synthase depicted in the centre of the figure is also loosely membrane associated. Yellow arrows, electron flow; yellow square, iron–sulphur clusters. Picture from Kartal et al. (2011)

2.5.3 Phylogeny

Strous et al. (1999a), were able, using an isolation strategy based on density gradient centrifugation, to isolate, from an enrichment culture, bacterial species with a high anammox activity and capable of CO_2 fixation (Strous et al. 1999b). The authors characterized the 16S ribosomal RNA (rRNA) gene of the isolated bacteria, which was given the status of "*Candidatus* Brocadia anammoxidans". 16S rRNA gene phylogeny confirmed that the organism was a member of Planctomycetes (Strous et al. 1999a). Using the previously developed 16S rRNA sequences and FISH probes, research efforts resulted in other seven anammox species: "*Candidatus* Brocadia fulgida", "*Candidatus* Jettenia asiatica", "*Candidatus A*nammoxoglobus propionicus", "*Candidatus* Kuenenia Stuttgartiensis", "*Candidatus* Scalindua wagneri", "*Candidatus* Scalindua sorokinii", "*Candidatus* Scalindua brodae" (Strous et al. 1999a; Schmid et al. 2000, 2003; Kuypers et al. 2003; Kartal et al. 2007b; Quan et al. 2008). As can be seen in Fig. I.13, the identified anammox families and species are characterized by different evolutionary distances, probably due to their habitats and ecological niches rather than difference in their physiologies, metabolisms and ultrastructures (Kuenen 2008). Recently, a new divergent Brocadia-like anammox phylotype has been found in a WWTP, for which the name "*Candidatus* Brocadia sinica" has been proposed (Hu et al. 2010; Oshiki et al. 2011).

Anammox bacteria have been found in different environments. As they need the simultaneous presence of ammonium and nitrite, they can be found near to the aerobic–anaerobic interface of sediments and water bodies, in marine and fresh aquatic environments and in wastewater treatment plants. As recently reviewed by Trimmer and Engstrom (2011), anammox bacteria were found in marine or estuarine sediments, deep marine sediments, shallower coastal sediments (temperate and artic), and oceanic Oxygen Minimum Zones (OMZs), in which the oxygen saturation is at its lowest level (at at depths of about 200 to 1,000 metres). Anammox process plays an important role in the global nitrogen turnover.

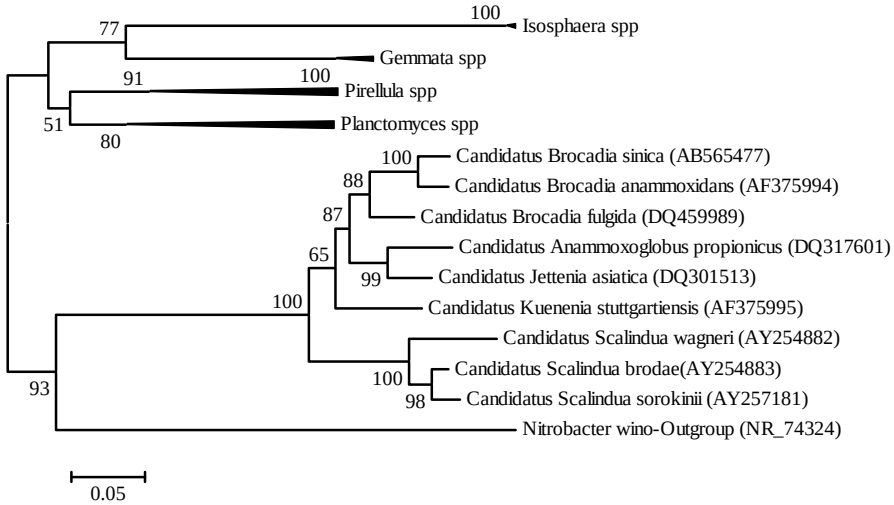

Fig. I.13. A 16s ribosomal RNA-gene-based phylogenetic tree of anammox bacteria,
The tree illustrates the relationships of the different families of anaerobic ammonium oxidation bacteria among the Planctomycetes (genera Pirellula, Planctomyces, Gemmata and Isophaera). The triangles indicate phylogenetic groups. The scale bar represents 5% sequence divergence. Adapted from Kuenen (2008)

The "*Candidatus* Scalindua" family has been found principally, but not exclusively, in marine environments, such as the Black Sea, the coasts of Namibia, Chile, Peru and, more recently, in a fresh-water lake in western Tanzania (Kuypers 2005; Schubert 2006; Hamersley 2007). "*Candidatus* Brocadia" and "*Candidatus* Kuenenia" species are the most commonly found organisms in the enrichments from wastewater-treatment plants (Innerebner et al. 2007; Hu et al. 2010). In a lab scale competition experiments between "*Candidatus* A.

propionicus" and "*Candidatus* B. anammoxidans", the higher capacity of "*Candidatus* A. propionicus" for propionate metabolism have been demonstrated, pointing out a defined ecological niche for this organism (Kartal et al. 2007b).

Up to day several groups of methods are used in detection of anammox bacteria based on their physiological and biochemical characteristics (Li and Gu 2011). The most commonly used phylogenetic biomarker for studying anammox communities is the 16S rRNA gene, using different microbial techniques (PCR, FISH). Usually, amplification of 16S rRNA genes of either planctomycetes or anammox bacteria is usually the first step for the identification of anammox bacteria in the environment (Kuenen 2008; Jetten et al. 2009). The next step, over the amplification of 16S rRNA, is the utilize of functional gene targeting anammox bacteria. The *hao/hzo* gene has been used as an additional genetic marker for anammox bacteria (Schmid et al. 2008; Hirsch et al. 2011), even if some phylogenetic diversity were found for *hao/hzo* gene compared to 16S rRNA gene (Li et al. 2010). Another functional genetic marker for anammox bacteria is the anammox cd1 nitrite reductase *nirS* gene, which is very different from the *nirS* sequences of denitrifiers. Currently, PCR primer sets designed for the amplification of *nirS* gene of "*Candidatus* Scalindua spp." are available (Lam et al. 2009; Li et al. 2011a). However, further analysis of environmental clones, metagenomes study and genome sequencing of the other anammox bacteria will be necessary to better identify environmental anammox bacteria diversity (Kraft et al. 2011).

2.5.4 Bioinformatics

The first metagenome of an anammox bacterium came from an enrichment culture of "*Candidatus* Kuenenia stuttgartiensis" in 2006 (Strous et al. 2006). The genome stuttgartiensis has been assembled by a metagenomics approach from a laboratory reactor fed with synthetic wastewater. The genome of "*Candidatus* Kuenenia Stuttgartiensis" was almost complete (98.5%) and it is 4.2 megabases, encoding 4663 genes involved in nitrogen metabolism and substrate uptake reactions, cell carbon synthesis and intermediary anabolism, other catabolic and anabolic pathways (Strous et al. 2006). The presence of cytochromes makes this bacteria brightly red.

In 2012, the genome of "*Candidatus* Scalindua profunda" has been assembled by a metagenomics approach from a marine anammox enrichment culture (Van de Vossenberg et al. 2012). Scalindua bacteria are most likely a very specialized group that are well adapted to marine environmental conditions. The genome of "*Candidatus* Scalindua profunda" encoded 4756 genes, but only about a half of them showed the highest identity to the "*Candidatus* Kuenenia stuttgartiensis", mainly due to the adaptations of Scalindua to the substrate limitation of the ocean, to salinity and other environmental factors (Van de Vossenberg et al. 2012). The genomes of other species are underway.

2.5.5 Kinetics

The specific anammox growth rate (μ_{AN}) can be expressed by the interactive Monod model for growth which is used to describe the effect of limiting substrate on microbial growth (Eq. I-37):

Monod – Based growth Kinetic

$$\mu_{AN} = \mu_{AN}^{MAX} \frac{S_{NH4}}{K_{S,NH4}^{AN} + S_{NH4}} \cdot \frac{S_{NO2}}{K_{S,NO2}^{AN} + S_{NO2}} \qquad \text{Eq. I-37}$$

where μ_{AN} is the actual specific growth rate of anammox; $\mu_{AN}{}^{MAX}$ is the maximum specific growth rate; S_{NH4} and S_{NO2} are the NH_4^+-N and NO_2^--N concentrations in the bulk while $K_{S,NH4}{}^{AN}$ and $K_{S,NO2}{}^{AN}$ are the NH_4^+-N and NO_2^--N half saturation constants for anammox, respectively. Carbon dioxide, the carbon source for anammox growth, is present in abundance, so that is not taken into account in the kinetic expression. In addition, some substrates can also inhibit the anammox process, such as nitrite and they could be also included in the kinetic law. The anammox submodel has been implemented in the two-step nitrification ASM1 described by Wyffels et al., 2004 (Van Hulle et al. 2003; Dapena-Mora et al. 2004).

2.5.6 Factors involved in nitrification kinetics

Anammon is a promising biological nitrogen removal process with attractive application prospects. Nevertheless, some studies reported many factors influencing the anammox process. However, these works were sometimes carried out under very different operational conditions, such as pH and temperature and using different anammox-type bacteria. Further, both short- and long- term effect have been studied using batch and continuous tests, entailing that the results of inhibition tests are often difficult to compare and to use in order to develop an anammox reactor operational strategy. As recently reviewed by Jin et al. (2012), anammox process is inhibited by a lot of factors which are commonly present in the practical applications: substrates (ammonia and nitrite), organic matter (nontoxic organic matter and toxic organic matter), salts, heavy metals, phosphate and sulfide, oxygen. The authors conducted a detailed review of previous researches on the inhibition of the anammox processes, but more recent works have been published, due to the interest in anammox process. For the application of avammox process in wastewater treatment plants, the prevention of inhibitions is fundamental, especially as anammox bacteria are characterized by very growth rates and high doubling time (10 -24 day) (Strous and Jetten 2004).

Free ammonia and ammonium

Total nitrogen ammonium (TAN) up to concentrations of 1 gTAN L^{-1} in continuous operation SBR during 1 week do not inhibit anammox process (Strous et al. 1999b). This result was partially confirmed by Dapena-Mora et al. (2007) in batch tests, who found a 50% inhibition of anammox process at concentration of 770 gTAN L^{-1}. Further, Jung et al. (2007) observed free ammonia (NH_3-N or FA), rather than ammonium itself (NH_4^+-N) was the true inhibitor of the anammox process, even at levels as low as 1.7 mgFA L^{-1}. However more recent literature does not consider FA to be an important parameter at FA concentrations below of 13–38 mg FA L^{-1} (Waki et al. 2007; Fernández et al. 2012).

Both short- and long-term effects of ammonia on the anammox process have been investigated by Fernández et al. (2012). In short-term tests, a decrease of 50% in the anammox activity was observed at FA concentrations of 38 mgFA L^{-1}. Long-term tests conducted in a Sequencing Batch Reactors, enriched with anammox biofilm biomass, showed performance instability when the FA concentrations reached values between 35-40 mgFA L^{-1}. In an anammox biofilm reactor, Waki et al. (2007) also reported FA as one of the inhibitory factors of the

anammox reaction with FA levels of 13–90 mgFA L^{-1}, while Tang et al. (2010a) suggested that FA concentrations of 57–187 mgFA L^{-1} may be toxic to anammox microorganisms. Aktan et al. (2012), in their study, investigated the inhibitory effect of FA on anammox bacteria in a continuous lab-scale upflow fixed-bed reactor with Kaldnes biofilm carriers (T=34°C and pH=8). Results showed that anammox process inhibition was not observed when the FA concentration in the reactor gradually increased up to 150 mgFA L^{-1}. However, anammox activity suddenly dropped to 10 % when the free ammonia concentration reached to 190 mgFA L^{-1}. In the reactor, the main anammox organisms were phylogenetically related to "*Candidatus* Kuenenia stuttgartiensis", "*Candidatus* Jettenia asiatica" and "*Candidatus* Brocadia anammoxidans".

Some discrepancies between the different threshold can be attributed at different operative conditions, microbial populations and physical structures of the sludge (flocculent sludge, biofilm or granular sludge). Granular and biofilm may provide a suitable circumstance for anammox bacteria to tolerate FA inhibition compared to single cells.

Nitrite and free nitrous acid

Nitrite, as ammonium, is a substrate of the anammox process. However, high-concentrations of nitrite severely inhibited anammox bacteria. Unlike AOBs and NOBs, which are inhibited by the free nitrous acid (FNA or HNO_2-N), the inhibition of anammox bacteria is caused by the ion itself (NO_2^--N). Recently, Lotti et al. (2012b) performed nitrite inhibition tests, using a manometric approach. The authors obtained a similar decrease of the anammox activity under different pH, confirming that nitrite rather than nitrous acid is the actual inhibiting compound. The authors also showed that the nitrite inhibition was reversible and depended on the time of exposure and on the concentration of NO_2^-, as total nitrite nitrogen (TNN). Any toxic effects (irreversible inhibitions) were detected, thus the authors concluded that nitrite inhibitions on anammox process is reversible. Reversibility by nitrite inhibition was also confirmed by other studies (Bettazzi et al. 2010; Kimura et al. 2010). In literature, a wide range of nitrite concentrations at which the inhibition occurs have been reported, under different experimental conditions and operating modes. Different thresholds are reported for short and a long term effects of nitrite on anammox process. Usually when only short-term effects are evaluated, high values of nitrite threshold had been found: 50% reduction at 400 mg NO_2-N L^{-1} (Strous et al. 1999b; Lotti et al. 2012b); 50% reduction at 350 mg NO_2-NL $^{-1}$ (Dapena-Mora et al. 2007); 25% reduction at 60 mg NO_2-NL^{-1} (Bettazzi et al. 2010); 37 % reduction at 430 mg NO_2-NL^{-1} (Kimura et al. 2010). Scaglione et al. (2012) reported for anammox granules, mainly Brocadia species, a reduction of anammox activity of 40% at 500 mg NO_2-N L^{-1} for an exposure time of 3–4 h. The activity loss was substantial after prolonged exposure (24 h). However the inhibition was reversible after washing. Lotti et al. (2012b) reported a 50% reduction at 4000 mg NO_2-N L^{-1}, which is a significantly high nitrite concentration compared to other studies.

During long period, the inhibition had been observed at much lower but contradictory concentrations, as it depends on the operative reactor conditions. Nitrite concentrations as low as 5 mg NO_2-N L $^{-1}$ has been reported as inhibitive in an intermittently aerated one reactor nitritation-anammox process (Wett 2007). In contrast to the inhibition at this low levels, Strous et al. (1999a) found that dispersed anammox aggregates completely lost their activity in a nitrite solution of 100 mg NO_2-N L^{-1} in an SBR reactor conducted for 1 week. Significant long-term inhibitions in the presence of 80 mg NO_2-N L $^{-1}$ has been reported (Fux et al. 2004; Fernández et al. 2012). Further, a full scale anammox reactor worked at typical concentration of 40 – 80 mg NO_2-N L $^{-1}$ (Van Der Star et al. 2007). Repeated additions of nitrite higher than 30 mg NO_2-N L $^{-1}$ caused losses of activity, and the inhibition

was found to increase after each spike (Bettazzi et al. 2010). However, inhibition appears to be more severe in case of suspended and flocculent biomass, suggesting that the outer layer of the biofilm or granules can protect the anammox bacteria in the inner part, mainly due to diffusion (Cho et al. 2010; Fernández et al. 2012). Lotti et al. (2012b) in their experiments showed that the inhibition increased as increasing exposure times to high nitrite concentrations.

Presence of ammonium (2 g TAN L^{-1}) during nitrite exposure resulted in a stronger loss of activity after the nitrite exposure (50% and 30% in presence and absence of ammonium respectively) (Lotti et al. 2012b). Those results were also found by Jaroszynski et al. (2011). The authors observed at low FA levels nitrite concentrations up to 250 mg NO_2-N L^{-1} did not cause inactivation of anammox consortia over a 2-days exposure time. Moreover, Lotti et al. (2012b) showed that the presence of oxygen during nitrite incubation led to a maximum activity reduction of 32%. However, at 2g N L^{-1} of ammonium and nitrite, the presence of 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).

Organic matter

Anammox bacteria are chemoautotrophic microorganisms that require inorganic chemicals as their energy source, like bicarbonate. The concentration of bicarbonate in the influent of the anammox process is therefore important but the stoichiometry requirement is significantly lower compared to nitrification stoichiometry. However, an appropriate addition of inorganic carbon in the influent can promote the growth or enhance the activity of anammox bacteria until an influent bicarbonate and nitrogen ratio of 10. Higher ratios led to an anammox process inhibition (Liao et al. 2008).

Some kinds of nitrogen-rich wastewaters, such as landfill leachate and wastewater from digested animal waste, contain both biodegradable and refractory organic matter. High concentrations of biodegradable organic carbon in the influent wastewater may inhibit anammox bacteria. Biodegradable organic carbon may cause a heterotrophic denitrifying bacteria competition with anammox bacteria for nitrite (Molinuevo et al. 2009). This effect is concentration dependent and it has been usually observed when influents with a C/N ratio higher than 2 are treated (Kumar and Lin 2010). Because the heterotrophic bacteria grow faster than the autotrophic anammox bacteria under high concentrations of biodegradable organic matter, the heterotrophic bacteria may eliminate the anammox bacteria and thereby reduce the anammox nitrogen removal capability (Chamchoi 2008; Ruscalleda et al. 2008; Kumar and Lin 2010). As reviewed by Jin et al. (2012), this type of inhibition could be considered as a phenomenon of ‘‘out-competition’’.

Further, the presence of biodegradable organic carbon may activate different anammox metabolic pathways, which could inhibit the main anammox bacteria activity, reducing nitrogen removal efficiency, i.e., using organic matter rather than ammonium and nitrite as a substrate (Güven et al. 2005; Kartal et al. 2007b). As proposed by Jin et al. (2012), this kind of inhibition could be considered as a phenomenon of ‘‘metabolic pathway conversion inhibition’’. Anammox microorganisms have the capacity to couple the oxidation of various organic acids to the reduction of nitrate. Güven et al. (2005) demonstrated that anammox bacteria have a versatile metabolism because propionate and acetate were shown to be potential substrates for anammox bacteria. Kartal et al. (2007b) reported that “*Candidatus* Anammoxoglobus propionicus’ outcompeted other anammox bacteria and heterotrophic denitrifiers for the oxidation of propionate in the presence of ammonium, nitrite and nitrate, still using CO_2 as the only carbon source. Further, Kartal et al. (2008) showed that anammox bacteria can also be

enriched to high densities in the presence of acetate. The enriched biomass "*Candidatus* Brocadia fulgida" can oxidize other organic compounds such as formate, propionate, monomethylamine and dimethylamine (Kartal et al. 2008).

Anammox bacteria may also be inhibited by some toxic organic compounds, such as methanol (CH_4O). Jensen et al. (2007) reported that 96-128 mg L^{-1} methanol could almost completely inhibit the activity of anammox bacteria in marine sediment. In artificial systems, different thresholds for methanol have been reported, mainly due to the different anammox bacteria investigated (Jin et al. 2012).

Oshiki et al. (2011), using an enrichment of "*Candidatus* Brocadia sinica", proved that the anammox activity lost 86% at 32 mg L^{-1} methanol, but Isaka et al. (2008), using mainly "*Candidatus* Kuenenia stuttgartiensis", observed that 160 mg L^{-1} methanol could only reduce anammox activity by 71% in batch experiments. Güven et al. (2005) showed that 16 mg L^{-1} methanol deactivated anammox process irreversibly. Tang et al. (2010b), successfully cultured a Brocadia-like anammox enrichment from methanogenesis sludge, which contained high concentrations of both organic matter (acetate) and methanol. Recently, the mechanism of methanol inhibition of anammox process was found to be related to the formaldehyde inhibition. As the toxic action only occur in actively metabolizing enrichments, it has been suggested that formaldehyde, which is produced from methanol, rather than methanol itself, is the actual inhibitor (Isaka et al. 2008). Methanol may be converted to formaldehyde intracellularly, then formaldehyde may destroy enzyme and protein activity by irreversibly cross-linking the peptide chains, thus causing the irreversible inhibition of anammox process (Isaka et al. 2008). Alcohol, aldehydes, ethanol, phenol and antibiotics, are common types of toxic organic matter that are able to inhibit anammox bacteria by microbial poisoning or enzyme inactivation and the inhibition is often not reversible (Jin et al. 2012).

Temperature and pH

Several authors found that the optimum temperature for the anammox process was around 30–40°C (Strous et al. 1999b; Egli et al. 2001). Thus, the application of the anammox process has been usually focused on the treatment of wastewaters with temperatures around 30°C in order to operate under optimum conditions. However, the applicability of the anammox process at lower temperature is challenging. Dosta et al. (2008) investigated the short-and long- term effects of temperature on the anammox biomass, using batch tests. They found that the maximum activity of non-adapted anammox biomass ranged between 35 and 40°C, while a temperature of 45°C caused an irreversible loss of the activity due to the biomass lysis. The long term effects were studied using an SBR at different temperatures, and the system was successfully operated at 18°C. Lower temperature led to a significant instability, with nitrite accumulation. As reviewed by Van Hulle et al. (2010), small differences in the optimal temperature were found for "*Ca.* K. stuttgartiensis" (T = 40°C (Strous et al. 1997)) and "*Ca.* B. anammoxidans" (T = 37°C (Egli et al. 2001)). However, anammox process have been successfully operated at a low temperature of 20°C (Cema et al. 2007; Isaka et al. 2007). Anammox bacteria could adapt to low temperature conditions by acclimatization (Dosta et al. 2008).

In natural environments, anammox bacteria have a wider temperature range, between −1.3°C to 85°C (Gao and Tao 2011). Rysgaard et al. (2004) found anammox activity in Arctic sediments between −1.3 and 30°C, the optimum temperature being 12°C, while Byrne et al. (2009) found presence and activity of anaerobic ammonium oxidizing bacteria at deep-sea hydrothermal vents.

The optimal pH interval for anammox process is 6.7–8.3 with an optimum of 8. (Strous et al. 1997). Further, both temperature and pH affects FA and FNA concentrations, therefore, the temperature and pH control are important parameter, that can be used to monitor and control the anammox process.

Salinity

The increase of the osmotic pressure due to salts may impact negatively anammox activity. Nevertheless, anammox bacteria can grow both in freshwater and marine environments, where the salt concentrations are high. Hence, anammox reaction is a promising process for the treatment of wastewater with high salts concentrations.

The salt tolerance depends on the kind of the anammox biomass and on the biomass acclimatation. In natural saline ecosystems only anammox species belonging to "Scalindua" genus have been detected (Schmid et al. 2003). The other genera are known to inhabit freshwater ecosystems. However, Kartal et al. (2006) reported that the freshwater anammox species "*Candidatus* Kuenenia stuttgartiensis" successfully adapted to salts (90% NaCl and 10% KCl) concentrations as high as 30 g L^{-1}. Higher salt concentrations reversibly inhibited anammox bacteria, but the inhibition was reversible.

Yang et al. (2011) reported a stable anammox process in an up-flow column reactor at 30 g NaCl L^{-1}, after a gradual adaption process. Different results have been obtained using adapted and non-adapted biomass. Jin et al. (2011) observed that a saline shock loading of 30 g NaCl L^{-1} caused a 67.5% decrease in the specific anammox activity compared to the reference biomass, while the acclimatized anammox biomass displayed an activity just 45.1% lower than that of the reference biomass. Dapena-Mora et al. (2010) showed that when the anammox biomass is continuously subjected to high NaCl concentrations its resistance to salt clearly increases. Further, the authors observed a stimulatory effect of salt on the anammox activity at concentrations up to 6 and 15 g NaCl L^{-1}, while higher salt concentrations caused a decrease in the activity. Windey et al. (2005), studying the performance of the OLAND process for the treatment of high salinity wastewater. The authors found that the activity of the salt-adapted anammox biomass decreased 59% at 30 g NaCl L^{-1}. Zhang et al. (2010b), conducting a CANON process in a sequencing batch biofilm reactor (SBBR), achieved the maximal total nitrogen (TN) removal rate when the salinity was maintained at a constant value of 10.0 g NaCl L^{-1}. Oshiki et al. (2011), studying the "*Candidatus* Brocadia sinica", found that anammox activities were 70% of baseline in the presence of 30 g L^{-1} salinity. Thus, it seems clear that the anammox biomass is highly resistant to the presence of salt concentration (NaCl), even if the salt adaption ability of other anammox species should be studied in future. However, the salinity inhibition depends also on the types of salts. Dapena-Mora et al. (2007), conducting batch tests on anammox biomass, showed that NaCl concentrations below 6 g L^{-1} did not affect the anammox activity while KCl and Na_2SO_4 had adverse effect at concentrations higher than 7.5 g $L^{-1}$and 7.1 g L^{-1}, respectively . It was reported that salinity in certain concentrations (3–15 g NaCl L^{-1}) promoted the formation of anammox granular sludge and increased the retention of bacteria in the reactor (Fernández et al. 2008; Dapena-Mora et al. 2010).

Oxygen

Anammox bacteria are anaerobic and are inhibited by dissolved oxygen (Strous et al. 1997; van Dongen et al. 2001b). Nevertheless, inhibition caused by low concentration of oxygen (0.25–2%) was demonstrated to be reversible (Strous et al. 1997; Egli et al. 2001), but probably irreversibly at high levels (>18% air saturation) (Egli et al. 2001). Lotti et al. (2012b) recently reported the short- and long- effect of oxygen exposure on anammox process. After 1 h exposure in the presence of 5 mg O_2 L^{-1}, any inhibition effect on anammox process was detected for concentrations up to 2000 mg N L^{-1} of ammonium and nitrite. Further, after an exposure for 24 h to 2000 mg N L^{-1} ammonium and nitrite (actively metabolizing cells), the authors reported that 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).

Other sources of inhibition

There are few studies on the heavy-metal and antibiotic inhibitions of anammox bacteria, even if some kinds of nitrogen-rich wastewaters, such as landfill leachates or anaerobic digester effluents, often contain heavy metal ions and antibiotic traces. Recently, Lotti et al. (2012a) using a *Brocadia* enrichment, investigated the short effects of heavy metals and antibiotics on anammox bacteria. Anammox activity was inhibited by 50% after an exposition of 24 hours at 1.9, 3.9, 650 and 1,100 mg L^{-1} of copper, zinc, sulfathiazole and oxytetracycline, respectively. The authors investigated the effect of prolonged exposure (14 days) to the antibiotics oxytetracycline and sulfathiazole at concentration of 100 mg L^{-1}. The anammox activity decreased by 75% and 50% respectively. Both anabolism and catabolism reactions were active during the inhibition tests, as any discrepancy from the accepted anammox stoichiometry was found. Fernández et al. (2009) measured a 40% decrease in activity in the continuous operation of an anammox bioreactor after tetracycline hydrochloride was fed for 30 days at a concentration of 10 mg L^{-1}.

Dapena-Mora et al. (2007) reported inhibition effects on Anammox process due to different compounds. Concentrations up to 1 g L^{-1} of allylthiourea and chloramphenicol, used as nitrification and denitrification inhibitors, caused no significant decrease of the maximum anammox activity, implying that allylthiourea and chloramphenicol could be used as an useful method to distinguish anammox activity from the activity of other microorganisms. Further, polymerising flocculant positively charged compounds, usually used for a physical–chemical process-like flocculation process were tested. The complete inhibition of anammox activity was measured at concentration higher than the values adopted in the flocculation process.

Phosphate is commonly used as a pH buffer in batch experiments. A difference in tolerance for phosphate exists between different anammox species. The anammox species most common used for inhibition tests is "*Candidatus* Kuenenia stuttgartiensis". Using a culture of "*Candidatus* K. stuttgartiensis", Egli et al. (2001) did not see any inhibitory effect of phosphate up to 620 mgPO_4^{3-}-P L^{-1} tested. In batch tests using a biomass enriched in bacteria belonging to the specie "*Candidatus* K. stuttgartiensis", Dapena-Mora et al. (2007), observed an inhibition of 50% of anammox activity at concentration of 620 mgPO_4^{3-}-P L^{-1}. Performing batch tests with sludge from a highly loaded lab-scale rotating biological contactor containing "*Candidatus* K. stuttgartiensis", Pynaert et al. (2003) measured an anammox activity inhibition of 63% at 55.8 mgPO_4^{3-}-P L^{-1}, of 80% at 111 mgPO_4^{3-}-P L^{-1}. No further decrease was observed at higher concentrations. A lower tolerance to phosphate was measured by Van de Graaf et al. (1996), who observed a loss of activity of "*Candidatus* B. anammoxidans" when incubated the anammox reactor with more than 62 mgPO_4^{3-}-P L^{-1}.

Some kind of wastewaters contain sulphur compounds (sulphate or sulphide). Sulphate (SO_4^{2-}) is usually present in wastewaters such as seafood processing, leather tanning, oil refining and alcohol fermentation over carbon and nitrogen concentration. Recently, simultaneous removal of ammonium and sulphate by anammox bacterias have been reported by Yang et al. (2009). Sulphate (SO_4^{2-}) is often reduced to suphide (H_2S) in anaerobic conditions by sulphate reducing bacteria which use organic carbon as electron donor. Sulphide may act as inorganic inhibitors of anammox process. Dapena-Mora et al. (2007) showed an inhibition of 50% on anammox process at low sulphide concentration of 9.6 mgS L^{-1} while Van de Graaf et al. (1996) showed any inhibition effect of anammox process to at least 64 mgS L^{-1} in batch and continuous experiments. This large difference in sulphide inhibition could be explained either by the two different species of anammox bacteria utilized in the two experiments ("*Candidatus* K. stuttgartiensis" vs "*Candidatus* B. anammoxidans") or by the addition of nitrate as electron donor for the anammox biomass in Van de Graaf et al. (1996) since sulphide could reduce nitrate to nitrite, which is the preferable electron donor of the process (Van Hulle et al. 2010).

2.6. DNRA

Dissimilatory nitrate reduction to ammonium (DNRA), also called nitrate ammonification or autotrophic denitrification, is a potentially significant pathway of the nitrogen cycle (Tiedje 1988). For a long time it was generally assumed that most of the nitrate in nature is mainly reduced and transformed via denitrification to dinitrogen gas and any particularly high relevance was assigned to DNRA. While denitrification represents a nitrogen removal pathway producing N_2, DNRA is a nitrogen-conserving mechanism that transforms nitrate (NO_3^-) to ammonium (NH_4^+), a more bio-available inorganic nitrogen form, with nitrite (NO_2^-) as a free intermediate.

In DNRA process, nitrate and nitrite are reduced to ammonium. Information on the physiology, phylogeny and on enzymes involved in DNRA has been obtained by characterizing isolated DNRA-performing strains (Bonin 1996; Strohm et al. 2007). Recently, Kraft et al. (2011) reviewed the mechanisms of DNRA: (i) the reduction of nitrate to nitrite is assumed to mostly being catalyzed by the periplasmic nitrate reductase complex *NapAB*, however, same organisms also present a membrane-bound nitrate reductase, *NarGHI*; (ii) the reduction of nitrite to ammonium is catalyzed by *NrfA*, a pentaheme cytochrome c nitrite reductase, without the release of any intermediate. The authors reported that there are some indications that N_2O is also released as a byproduct of DNRA. NO and NH_2OH are also probable intermediates. The ability to carry out DNRA is phylogenetically widespread in nature and the functional gene *NrfA*, which reduce nitrite to ammonium, had been detected in diverse groups of bacteria as reviewed by Kraft et al. (2011): gamma-, delta- and epsilon-proteobacteria and in members of the bacteroides and sulfate reducing delta-proteobacteria.

Most organisms that carry out DNRA also can reduce nitrate to nitrite except some sulfate reducing bacteria which are only able to use nitrite (Dannenberg et al. 1992). DNRA has been shown to be mediated by obligate anaerobes, facultative anaerobes and some aerobes microorganisms (Tiedje 1988; Cole 1996). Although, the conditions promoting DNRA and heterotrophic denitrification are similar (absence of oxygen, and available nitrate and organic substrates) some mechanisms could drive the competition between those two biological processes. The conditions that favor the one or the other nitrate respiration pathway remain to be univocally determined. In contrast to denitrification, dissimilatory nitrate reduction to ammonium (DNRA) is assumed to occur when nitrate in comparison to organic carbon is limiting. This behavior was explained by the fact that DNRA, despite having a lower energy yield, consumes less nitrate per carbon than denitrification (Cole and

Brown 1980; Tiedje 1988). Further, DNRA is only predicted at strictly anoxic conditions, whereas denitrification is predicted in anoxic conditions and at both low and high oxygen concentrations. The ratio between the available electron donor compared to nitrate ($C:NO_3^-$), the kind of electron donor and the prevailing redox potential in the environment are hypothesized factors for the selection of the predominating nitrate reduction process (Tiedje 1988; Akunna et al. 1993). Van de Leemput et al. (2011), using simulation, based only on the stoichiometry and energy yield of the redox reactions involved in the nitrogen cycle, and assuming competition for resources between alternative pathways, showed that in hypothetical freshwater and marine sediment situations, at relatively low nitrate inflow levels, DNRA outcompeted denitrification at low oxygen inflow levels, while at high nitrate inflow levels, DNRA was outcompeted by denitrification at different oxygen concentration.

Recently, the role of DNRA has been established in different environments, showing the importance for the terrestrial nitrogen cycle (Rütting et al. 2011). In tropical forest soils, DNRA was shown to be at least three times higher than denitrification (Templer et al. 2008), accounting in some cases for nearly 99% of nitrate reduction (Huygens et al. 2007). In contrast, DNRA has been shown to account for only 5-15% of nitrate reduction in temperate freshwater environments, such as riparian fens, wetlands and paddy soils (Ambus et al. 1992; Yin et al. 2002; Matheson et al. 2003), where denitrification was responsible for the majority of nitrate removal through the production of N_2 gas. DNRA is measured under anoxic conditions and in deeper sediment layers (Jørgensen 1989). In natural systems, DNRA activity has been found coupled with sulfur cycling (Brunet and Garcia-Gill 1996). There has been recent indication that sulfate reduction is preferred over DNRA if both electron acceptors are present (Marietou et al. 2009). Evidence for sulfide-enhanced DNRA activity can be found in stratified aquatic microbial ecosystems (Brettar et al. 2006). In a mariculture system, Sher et al. (2008) showed that the addition of sulfide in the anaerobic reactor treating high nitrate and carbon stream, increased the nitrate reduction rate, pointing to the important role of DNRA in the anaerobic reactor. The importance of nitrate reduction to ammonium in fermenting conditions, performed by fermentative bacteria (e.g., Enterobacteriaceae, Aeromonas, Vibrio spp., Clostridium sp.) have been demonstrated (Cole and Brown 1980; Cole 1996). Akunna et al. (1994) detected nitrate reduction to ammonium in anaerobic sludge using glucose at various nitrate concentrations together with denitrification and methanogenic activities. Thus, DNRA needs to be considered also in WWTPs to optimize nitrogen removal.

Evidence for DNRA has been detected in the Benguela upwelling system (Kartal et al. 2007a), where anammox bacteria actively remove massive amounts of N (Kuypers 2005). This result showed that DNRA could be also performed by anammox bacteria, providing NH_4^+, that could be used consequently for the traditional anammox pathway producing N_2 gas. "*Ca.* K. stuttgartiensis" is capable of dissimilatory nitrate reduction to ammonium (DNRA), reducing NO_3^- even in the presence of high ammonium concentration (140 mg TAN L^{-1}) (Kartal et al. 2007a). The overall process could be seen effectively concealed as a denitrification process, as in anoxic systems, starting from NO_3^-, dinitrogen gas (N_2) is produced.

Coupled DNRA and denitrification and coupled DNRA–anammox processes are indistinguishable with most conventional activity assays and isotope tracer experiments activity assays, while the application of specific gene markers could help to assess the importance of the DNRA in the environment (Francis et al. 2007; Kartal et al. 2007a).

2.7. N-damo

For years, the aerobic methane oxidation was believed to be the only process to convert methane. The aerobic methane oxidation is performed by several specialized groups of bacteria that are widely distributed in the environment, which are able to oxidize methane under oxic condition, producing CO_2 (Hanson and Hanson 1996). Recently, microbes that couple the anaerobic oxidation of methane to the dinitrogen formation were observed. A novel group of microorganisms, affiliated to the bacterial phylum NC10, named "*Candidatus* Methylomirabilis oxyfera", performing the nitrite-dependent anaerobic methane oxidation, were discovered in two different freshwater systems in The Netherlands (Raghoebarsing et al. 2006; Ettwig et al. 2008). Later, enrichments cultures were obtained (Ettwig et al. 2009; Hu et al. 2009). Nitrite-dependent anaerobic methane oxidazing (n-damo) bacteria are capable of oxidizing methane under anoxic conditions, using oxygen originating from nitric oxide and, at the same time, produces dinitrogen gas, without a nitrous oxide reductase (Ettwig et al. 2010). Based on these observations, a new reaction pathway was proposed (Eq. I-38), which is characterized by a very high free Gibbs energy (Ettwig et al. 2010):

$$3CH_4 + 8NO_2^- + 8H^+ \xrightarrow{n-damo} 3CO_2 + 4N_2 + 10H_2O \qquad \Delta G^{\circ\prime} = -928\ kJ\ mol^{-1}\ CH_4 \qquad \text{Eq. I-38}$$

N-damo bacteria are mesophilic with regard to temperature and pH (enriched at 25– 30 °C and pH between values of 7–8) and are characterized by very low growth rate (doubling time 1–2 weeks) (Ettwig et al. 2010). The genome of 'M. oxyfera', was assembled from metagenomic data, resulting in a 2.7 megabases chromosome. The genome contained genes for the pathway of aerobic methane oxidation as well as an incomplete gene set for denitrification, missing a gene for nitrous oxide reductase (Ettwig et al. 2010). The lack of nitrous oxide reductase indicated that another denitrifying pathway had to be operative (Wu et al. 2011). Ettwig et al. (2010) explained that in the last step of this 'alternative' denitrification or methane pathway, a not yet known enzyme catalyzes both the formation of dinitrogen and oxygen from two molecules of nitric oxide. The oxygen produced in the last step is used by particulate methane monooxygenase (pMMO) in the first step for the 'conventional' methane oxidation pathway. Thus, n-damo bacteria grows anaerobically, but produces oxygen for the aerobic oxidation of methane (Ettwig et al. 2010).

To identify the presence of 'NC10' phylum bacteria, 16S rRNA primer set can be used (Ettwig et al. 2009). In addition, to identify *C.* M.oxyfera- like bacteria on a functional level, specific primer set targeting the *pmo*A can be used. In fact, the *pmo*A gene encodes one of the subunits of the pMMO complex that facilitates the aerobic conversion of methane to methanol and, since genome of M. oxyfera contained the complete pathway to oxidize methane aerobically, *pmo*A could serve as a functional marker. Recently, Luesken et al. (2011a) developed specific *pmoA* primers to monitor and identify "Ca. Methylomirabilis oxyfera"-like methanotrophs.

N-damo bacteria could be applied in wastewater treatment systems to remove trace of methane, usually present in anaerobic digester effluent. In 2009, from a mixed sample of freshwater sediment, anaerobic sludge, and return activated sludge, an enrich NC10 bacteria culture had been reached (Hu et al. 2009). Later, Luesken et al. (2011b) investigated the diversity and the enrichment of nitrite-dependent anaerobic methane oxidizing bacteria from activated sludge of WWTPs with relatively high sludge retention times (SRT = 8- 30 d) and a BOD/N ratio lower than 4.7. The authors, using molecular diagnostic methods based on the 16S rRNA gene analysis, detected the presence of M. oxyfera in nine out of ten WWTPs tested, Netherlands. Methane is a greenhouse gas in earth's which contributes to the greenhouse effect when released to the environment. Hence, it should be removed if present in wastewaters and possible used in an energy-efficient way. Nitrite-dependent

anaerobic methane oxidazing (n-damo) bacteria could play an important role in developing new sustainable wastewater treatment implementations (Luesken et al. 2011b).

3. Advantages and issues in the co-existence of microorganisms for wastewater treatment

Conserving energy and resources in WWTPs is nowadays a blessing rather than a duty or a burden. Therefore more attention is being paid to the selection of processes that conserve energy and resources. The discovery of anammox bacteria opened new ways in nitrogen removal from wastewaters. Also the discovery of the versatility of processes such as the heterotrophic nitrification, the autotrophic denitrification and other microbial pathways such as the aerobic ammonia oxidation by archaea, the dissimilative nitrate reduction to ammonia (DNRA) and the nitrite- dependent methane oxidation (n-damo) may lead to the development of new treatment processes.

Some researchers have reported that anammox bacteria can co-exist with other bacteria, such as the aerobic nitrifying and denitrifying bacteria, and this co-existence can play an important role in treating nitrogen- and carbon- containing wastewaters (Third et al. 2001; Ruscalleda et al. 2008; Chen et al. 2009; Kumar and Lin 2010).

3.1 Anammox– and Aerobic Ammonia Oxidizing- Bacteria

Ammonium removal with the anammox process always consists of the nitritation process followed by the anammox process. Processes like SHARON has been developed to carry on the nitritation process (Hellinga et al. 1998). First, "2-reactors systems" have been implemented, such as the SHARON/anammox (Van Dongen et al. 2001a; van Dongen et al. 2001b; Vázquez-Padín et al. 2009b), to treat wastewaters with high ammonia concentrations and no carbon content.

Later, a complete autotrophic nitrogen removal process (ammonia to N_2) in "1-reactor systems" had been implemented by a mixed population of aerobic ammonia oxidizers and anammox bacteria under oxygen limitation conditions (Third et al. 2001; Sliekers et al. 2002). In the Completely Autotrophic Nitrogen removal Over Nitrite (CANON) process, in one single reactor, the aerobic ammonia-oxidizers and the anaerobic ammonia oxidizers simultaneously oxidize ammonia to dinitrogen gas and a small amount of nitrate is produced. Oxygen limited conditions are necessary both to avoid inhibition of anammox bacteria by oxygen and to achieve appropriated conditions to obtain nitritation process. The growth of NOBs (and subsequent nitrate production) is prevented due to their lower affinity to oxygen compared to AOB ($K_{O\_AOB} < K_{O\_NOB}$) (Wiesmann 1994) and due to their lower affinity to nitrite compared to anammox bacteria ($K_{NO2\_anammox} < K_{NO2\_NOB}$) (Van Der Star et al. 2008). A combined *Nitrosomonas/Brocadia* cultures was obtained by Third et al. (2001), which converted ammonium into mainly dinitrogen gas and some nitrate according to the following reaction:

$$2.5NH_4^+ + 2.1O_2 \xrightarrow{AOB+Anammox} 1.15N_2 + 0.2NO_3^- + 2.8H^+ \qquad \text{Eq. I-39}$$

Theoretically, the potential advantages of the nitrogen removal via nitritation and anammox process are: (a) the decrease (theoretically ~ 60%) of oxygen consumption by limiting the ammonia oxidation to only 50% of nitrite (partial nitritation); (b) the reduction of organic carbon uptake as no denitrification is required; (c) the reduction of sludge production as only autotrophic bacteria are involved in the nitrogen removal process.

Nevertheless, in CANON systems the presence of nitrite oxidizing bacteria have been detected, mainly caused by NH_4^+-N limitation. Third el al. (2001) observed that under NH_4^+-N limitation only the 31% of NO_2^- generated by AOB reacted in anammox process, while the 69% of nitrite reacted in nitratation process. Possible

inhibitions of nitrite oxidizers by high free ammonium levels (Anthonisen et al. 1976) and high temperatures (Hellinga et al. 1998) have been also suggested. Recently, the possibility of using salt stress as a technical approach to inhibit the nitrite-oxidizing bacteria (NOB) activities during the start-up of a CANON process from activated sludge have been investigated (Zhang et al. 2010b). In addition, the salinity kept at a moderate level could also strengthen the biomass granulation (Kartal et al., 2006; Liu et al., 2008).

The main systems adopted to perform the CANON process, allowing the co-existence of these AOB and anammox bacteria species are biofilm systems (Rotating Biological Contactor (RBC), Moving-Bed Biofilm Reactor (MBBR)), or granular systems (Granular Sequencing Batch Reactor (GSBR), Upflow Anaerobic Sludge Blanket (UASB)) or reactors with an intermitted aeration (Sequencing Batch Reactor (SBR), RBC, Membrane BioReactor (MBR)) (Van Hulle et al. 2010). Usually systems with efficient retention of biomass in order to cultivate slowly growing bacteria are preferable.

As reviewed by Van Hulle et al. (2010), among the CANON configurations reported in literature, one of maximum nitrogen removal rate (NRR) was 1.5 kg N m^{-3} day^{-1}, achieved in a gas-lift reactor (Sliekers et al. 2003). This value is lower compared to the NRR of the 2-separate reactor configuration "SHARON-anammox", where a maximum value of 9 kg N m^{-3} day^{-1} has been reported using a gas-lift anammox reactor (Van Der Star et al. 2007). However, NRRs lower than 1.5 kg N m^{-3} day^{-1} have been also reported in "2-reactors systems", where anammox process had been carried out in SBR, MBR or UASB configurations.

Usually granular and biofilm systems are adopted to perform the CANON process. The distribution of AOBs and anammox bacteria in CANON systems, performed in a granular reactor, was investigated by Nielsen et al. (2005). Under oxygen limited conditions (< 0,1 mgO_2 L^{-1}), AOBs were restricted to the outer shell of the CANON aggregates, while anammox bacteria were found in the central anoxic parts. The larger type aggregate (>500 micron) accounted for about 68% of the anammox potential whereas 65% of the nitrification potential was found in the smaller aggregates (<500 micron). Fluorescence in situ hybridization (FISH) analysis of the CANON biomass showed that about 40% of the community consisted of AOBs, and 40% of anammox. No NOBs had ever been detected. The NOBs are only able to develop in CANON reactor after prolonged exposure (>1 month) to ammonium limitation (Third et al, 2001). The spatial distribution of AOB and anammox bacteria was also confirmed by Li et al. (2011b) in a lab-scale granular SBR, working at DO <0.8 mg L^{-1}. The authors found that the inner zone and the outer layer of the granules appeared to have an obvious difference because of the different situation of dissolved oxygen. In the outer layer of a granule, biomass was mostly micrococcus, presumably aerobic ammonia-oxidizing bacteria. On the other hand, in the inner layer of a granule, biomass was typical cauliflower-like aggregates, presumably anammox organisms. In addition, lots of cavities were present in the granules, which can enhance substrate transfer from the bulk to granules and intermediate or by-product transfer from inside granules to the bulk. The biofilm structure of a Rotating Biological Contactor performing the CANON process was analyzed by Pynaert et al. (2003), which found that the biofilm was largely a nitrifying community, with anammox bacteria spread throughout the biofilm. The AOBs were shown to be active at around the same depth as the anammox bacteria, possibly having two functions: either they consume O_2 (thus producing NO_2^-), protecting the anammox microorganism and supplying substrate, or they have some kind of anoxic metabolism.

The "1-reactor systems" for nitritation and anammox process have some advantages compared to the "2-reactor systems". Because only one reactor is required in the CANON systems, the capital and operational costs of the "1- single reactors" are lower compared to the "2- separate reactor" configurations. In addition, the "1-

reactor systems" may prevent inhibition effect, especially when biofilm or granular systems are used to perform the process. Biofilm and granular biomass structures create a mass transfer resistance which depends on the biofilm/granular thickness, protecting anammox bacteria by the inhibition of high concentrations of oxygen and nitrite in the bulk liquid. Usually, ammonium concentrations in the bulk are much higher than the oxygen or nitrite concentration, thus ammonium diffusion into the biofilm/granule will not limit the process rate. Then, the fluctuations of the ammonia and nitrite concentrations in the bulk and the ammonia and nitrite gradient concentration in the granule and biofilm may enable the co-existence of sublineage of *Nitrosomonas, Nitrobacter* or *Nitrospira* species (Maixner et al. 2006), getting more flexible the biological nitrogen process. Beyond CANON, different names have been used to describe the "1-syngle reactor" system, where the harmonious co-existence and cooperation of aerobic (AOB) and anaerobic ammonium-oxidizing (anammox) bacteria is achieved treating ammonium-rich wastewater, without organic carbon content. Among them, the OLAND-process (Oxygen Limited Autotrophic Nitrification and Denitrification) (Kuai and Verstraete 1998; Philips et al. 2002b), the aerobic/anoxic deammonification or DEMON process (Seyfried et al. 1998; Wett 2007) and the SNAP process (Single-stage Nitrogen removal using Anammox and Partial nitritation) (Lieu et al. 2005; Furukawa et al. 2006).

3.2 Anammox bacteria and Aerobic Ammonia Oxidizing Archaea

Mimicking the oxygen minimum zones, the cooperation between marine anammox bacteria, ammonia oxidizing bacteria (AOBs) and ammonia oxidizing archaea (AOAs) have been successful achieved in a laboratory-scale model system under oxygen limitation and low ammonium concentrations (Yan et al. 2012). The applicability of a biological process where anammox bacteria, AOB and AOA co-exist might be useful for the treatment of very dilute waste streams at ambient temperature. However, the presence of archaea has been investigated in a lab scale anaerobic up-flow granular bed anammox reactor (Cho et al. 2010), but any archaea have been detected. Further researches are necessary to study the applicability of anammox bacteria and ammonia oxidizing archaea co-existence in WWTPs.

3.3 Anammox– and Denitrifying- Bacteria

The co-existence of anammox and denitrifying bacteria have been reported in literature both in natural and artificial systems (Kumar and Lin 2010) even if denitrification is favorite over anammox process. In fact, the standard free energy ($\Delta G°$) of denitrification reaction is higher than the anammox reaction; therefore, the denitrification is thermodynamically more feasible. Further, denitrifiers have higher growth yield compared to autotrophic anammox bacteria.

In WWTPs, the co-existence of anammox and denitrification in one reactor would be useful to reduce the nitrate concentration in the reactor, produced by anammox process itself, improving the the total nitrogen removal via denitrifiers (Ruscalleda et al. 2008; Ni et al. 2012). Under anoxic conditions, nitrate can be reduced by denitrifiers to dinitrogen gas or to nitrite as intermediate which can be further utilized by anammox bacteria for the oxidation of ammonium (Kumar and Lin 2010; van Hulle et al. 2010). However, heterotrophic microorganisms could reduce also nitrite to gaseous nitrogen in anoxic denitrification, competing with anammox bacteria (Ahn et al. 2004). The coupling of anammox and denitrification processes will be successful only when the denitrification reaction is not competing with anammox for NO_2^-. Denitrification could be limited by the low

availability of easily biodegradable organic carbon; thus, denitrifiers are not able to dominate and cannot outcompete anammox bacteria in environments. The C/NH_4^+ ratio in the wastewater, and therefore, the C/NH_4^+ in the liquid bulk, is one of the most critical factor determining the co-existence of anammox and denitrification processes. Experimental studies reported that influent C/NH_4^+ ratios lower than 2 are necessary to achieve a stable co-existence between anammox and denitrifiers (Kumar and Lin 2010). Recently, it has been demonstrated that for granular systems when C/NH_4^+ ratio is over 3.1, anammox microorganisms could not compete with denitrifiers, which led to the reduction in the number of anammox bacteria and an increase of denitrifiers quantity (Ni et al. 2012). Further, at high C/NO_3^- ratio, nitrate will probably be reduced to ammonia due to dissimilative pathway (DNRA). The possibility of dissimilatory nitrate reduction to ammonium by anammox bacteria was also studied (Kartal et al. 2007a)

Nevertheless, the key role of the mechanism of the denitrification-anammox co-existence is not completely clear. Anammox bacteria could also use organic carbon. Unexpectedly, Sabumon (2007) found that in the presence of organic matter, ammonia was oxidized anoxically to nitrate by an unknown mechanism unlike anammox process. The authors hypothesized an enzymatic anoxic oxidation of NH_4^+ to NO_3^-, followed by denitrification via autotrophic and/or heterotrophic routes, i.e using hydrogen sulfide ions or organic carbon respectively. Further, it was showed that anammox bacteria have a more versatile metabolism than initially assumed. Güven et al. (2005) demonstrated that anammox biomass could oxidize propionate to CO_2 with nitrate and/or nitrite as the electron acceptor. Kartal et al. (2007b) reported that the anammox bacteria "*Ca.* Anammoxoglobus propionicus" was shown to outcompete both other anammox bacteria and denitrifiers for propionate. Further, "*Ca.* B. fulgida' was able to oxidize other organic compounds such as acetate, formate, monomethylamine, and dimethylmine with nitrate and/or nitrite as the electron acceptor (Kartal et al. 2008).

3.4 Anammox bacteria – DNRA

Anammox process can be coupled with dissimilatory nitrate reduction to ammonia (DNRA). The dissimilatory nitrate reduction to ammonium also proceeds in two steps: first nitrate is reduced to nitrite, then nitrite is reduced to ammonium. In artificial systems with NO_3^- limiting conditions and under anoxic conditions, NO_3^- can be reduced by bacteria which promote the dissimilatory reduction of NO_3^- to NH_4^+ (known as, DNRA) and release NO_2^- as a free intermediate. Subsequently, NO_2^- can be utilized by anammox bacteria for the anaerobic oxidation of NH_4^+ (Kumar and Lin 2010). A similar coupling between anammox and denitrification processes was observed in the marine environments (Dalsgaard et al. 2005)

Recently, Kartal et al. (2007a) verified that anammox bacteria were able to perform by itselfs the dissimilatory nitrate reduction to ammonia. The nitrogen gas production by anammox bacteria through nitrate reduction proceeds via two reactions: dissimilatory nitrate reduction to ammonium followed by anaerobic oxidation of ammonium.

3.5 Anammox- and n-damo- bacteria

The feasibility of a co-existence of anammox and n-damo bacteria has been successful tested by Luesken et al. (2011c) in a lab –scale reactor. Those results open the possibility to implement, in the near future, new biological configuration in WWTP to treat wastewaters that contain substantial amounts of both methane and ammonium.

3.6 Anammo- and–filamentous bacteria

Cho et al. (2010) reported *Chloroflexi*-like filamentous bacteria present mainly at the surface of the granules and around the anammox bacterial cluster. Several studies reported that *Chloroflexi*-like bacteria were present in a highly enriched anammox biomass (Li et al. 2009, 2011c; Xiao et al. 2009; Cho et al. 2011). The high detection frequency of *Chloroflexi*-like bacteria in an anammox reactor suggests that this group may be important for granulation. *Chloroflexi*-like bacteria could degrade and utilize macromolecules which derived from biomass decay and reinforce the structure of granules with a network of filamentous biomass (Miura et al. 2007; Miura and Okabe 2008; Li et al. 2009; Cho et al. 2010).

3.7 Aerobic Ammonia Oxidizing-, Anammox- and Denitrifying Bacteria

The co-existence of partial nitritation, anammox, and denitrification processes in one single reactor is a challenging process for treating wastewaters with high ammonium concentrations and a low carbon content, such as anaerobic digester effluents and old landfill leachate. The process is known as Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process. It consists in one single reactor where different biological processes take place. The potential advantages of the nitrogen removal via SNAD process are: (a) the decrease (theoretically about 60%) of oxygen consumption by limiting the ammonia oxidation to 50% nitrite (partial nitritation) (b) the reduction (theoretically 100%) of organic carbon uptake during anoxic respiration, (c) lower sludge production, as the main processes are carried out by autotrophic bacteria, (d) a high total nitrogen removal efficiency compared ti the CANON process. In addition, compared to the 2 – reactor systems, the SNAD process requires lower capital and operational costs.

The co-existence of nitritation, anammox and denitrification processes has been reported in literature in engineered systems both at lab-scale (Chen et al. 2009; Xu et al. 2010; Lan et al. 2011; Li et al. 2011b; Daverey et al. 2012; Winkler et al. 2012a; Zhang et al. 2012) and full scale (Wett 2007; Joss et al. 2009; Wang et al. 2010). Partial nitritation requires an aerobic condition for ammonium oxidation by chemolithoautotrophic AOBs with oxygen as electron acceptor. The residual ammonium and the nitrite produced can be removed by anammox process. In the anammox process, autotrophic bacteria utilize carbon dioxide as carbon source and nitrite as electron acceptor for ammonium oxidation under anoxic conditions. In the heterotrophic denitrification, nitrogen oxides serve as the terminal electron acceptor for organic carbon metabolism. Part of nitrate produced in anammox reaction can be removed by denitrification with the remaining organic carbon (COD) as electron donors, which will improve total nitrogen removal efficiency.

The performance of the SNAD process in treating wastewater would not only depend on anammox bacteria but also on the co-existence of the other important processes, mainly the aerobic ammonia and nitrite oxidation and heterotrophic denitrification via nitrate and nitrite. Previous studies indicated that also other mechanisms could occur: organic compounds can be utilized as supplementary carbon sources by some chemolithoautotrophic nitrifiers; anammox bacteria could consume acetate, propionate and could compete successfully over heterotrophic denitrifiers. However, the contribution of those other mechanisms can be considered secondary.

The SNAD process requires the simultaneous presence of aerobic and anaerobic conditions. Oxygen limited conditions are required to inhibit nitrite oxidizing bacteria (NOBs) over the ammonia oxidizing bacteria (AOBs) and further to avoid inhibition of anammox bacteria. In addition, some substances could act both as substrates

for some processes and inhibitors for other processes, requiring appropriate substrate concentrations and ratios. In particular, an appropriate C/N ratio of the influent wastewater should be established to ensure the co-existence of anammox and denitrifying bacteria. A maximum working Free Ammonia levels of 10 mg NH_3-N should be reached in order to avoid ammonia oxidizing bacteria (AOBs) inhibition while a minimum Free ammonia concentration of 0.04-0.08 mg NH_3-N should be ensured in order to achieve the nitrite oxidizing bacteria (NOBs) inhibition. High nitrite accumulation in the system should be avoid to prevent inhibition of anammox bacteria.

The control of other parameters (i.e., DO values, temperature, pH, C/N ratios, sludge structure) plays an important role in maintaining the co-existence of anammox with other processes (Kumar and Lin 2010; Jin et al. 2012) and in impacting on the activities of partial nitrification, anammox and denitrification. However, the impact on the microbial interaction has not been well documented (Zhang et al. 2012). The structure of the sludge also plays a key role on the resistance of the bacteria involved in the SNAD process. Chen et al. (2009), in a single, oxygen-limited, non-woven rotating biological contactor (NRBC) reactor, showed that in the outer layer of biofilm, biomass was mostly micrococcus, presumably AOBs, with a spot of aerobic heterotrophic bacteria. In the inner layer of biofilm, biomass was typical cauliflower like aggregates, presumably anammox organisms. The biomass structure was similar to that of CANON biomass, however, the authors also found other biomass in the inner layer of biofilm, presumably denitrifying organisms. Those results were confirmed later by other researches in granular systems (Li et al. 2011b).

Sequencing Batch Reactors (SBRs) are usually applied to develop the SNAD process (Lan et al. 2011; Daverey et al. 2012). SBRs provides efficient biomass retention (over 90% of biomass retention), ensuring the enrichment of very slow-growing microbial community, such as the aerobic and anaerobic autotrophic nitrifiers (AOB and anammox bacteria). Also other reactor configuration have been applied, such as a non-woven rotating biological contactor (NRBC) reactor (Chen et al. 2009), an aeration tank (Wang et al. 2010), a sequencing batch biofilm reactor (SBBR) (Zhang et al. 2012). The performance of SNAD process can be further enhanced by applying high sludge retention time. This could be achieved by applying carrier materials to develop biofilms or by self-aggregation in granules. Biofilm and granules also help to prevent inhibition by high concentration of oxygen, nitrite and other inhibitors in the bulk liquid.

The co-existence of nitrifying, anammox and denitrifying bacteria has also been proved by molecular techniques (Innerebner et al. 2007; Langone et al. 2013b). Investigations are further required to work out optimal conditions for such challenging co-existence.

4. Biological process modeling

Nowadays, in Wastewater Treatment Plants (WWTPs), mathematical models, based on *deterministic approaches*, are widely applied, especially to describe biological processes. Models usually express bulk biological processes which are not pure microbiologically correct due to the difficulty in modeling the several mechanisms involved and to the lack of a clear knowledge. The Activated Sludge Model No.1, **ASM1** (Henze et al. 1987)) can be considered as the reference deterministic model, describing autotrophic and heterotrophic reactions, developed with the aim of creating a word standard mathematical model. In 1995, updated versions have been proposed to incorporate biological phosphorous removal, **ASM2** (Henze et al. 1995) and **ASM2d** (Henze et al. 2000). In 1999, another version of ASM1, called the **ASM3** model (Gujer et al. 1999), has also been introduced which corrects a number of known defects present in the original model. The modular structure of **ASMx** is designed to be easily update by adding new chemical- biological modules. As consequence several

ASM-type models have been developed. Also *stochastic approaches* that model time series have been applied to WWTPs. Those methods can be developed from input and output monitoring data, without the use of a large number of coefficients required in common deterministic dynamic mathematical models (Novotny et al. 1990). In recent years, the application of *Artificial Neural Networks* to predict the performance of biological systems has been attempted (Civelekoglu et al. 2007; Khataee and Kasiri 2011) especially to capture the non-linear relationships that exist between variables in complex systems. However the mathematical modeling remains the most applied method to understand and predict phenomena in WWTPs, due to its large diffusion and relatively simplicity. At the same time, as the knowledge of the biological- physical- and chemical processes improves, the mathematical models are getting more and more complex with a large numbers of kinetic and stoichiometric parameters. The support of a mathematical model is fundamental to both research purposes, to evaluate experimental results and in testing hypotheses, in industrial applications, in the design of processes and management strategies.

Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process is a changeling choice to treat highly concentrated ammonium wastewaters with a low biodegradable carbon nitrogen ratio (CODbio/N), such as anaerobic digester effluents, landfill leachates, industrial wastewaters (Chen et al. 2009; Wang et al. 2010; Daverey et al. 2012). In order to explain, predict and control the complicated interactions and dynamics in SNAD processes, models and computer simulations are helpful tools. The developments of mathematical modeling, focusing mainly on recent ASM-type models and aerobic and anoxic nitrite route models in WWTPs are following reported.

4.1 Over the ASMx Models

The Activated Sludge Models (**ASMx**) are the most widespread and standard models for WWTPs. They were developed by a Task Group on Mathematical Modeling for design and Operation of Activate Sludge Processes established by the International Association on Water Pollution Research and Control (IAWPCR), as it was then called.

The ASMx family describes biological reactions introducing a matrix format (the Petersen or Gujer table). The ASMx structure is composed of a stoichiometric matrix with compounds and biological processes, and a composition matrix with the composition in terms of conserved balances (es. COD, N and Charge balance), a rate vector and extra information as units and names. In the model, the organic carbon and nitrogenous compounds are subdivided into a restricted number of fractions based on their biodegradability and solubility characteristic building a specific component. The chemical oxygen demand (COD) is usually adopted as measure of the concentration of organic matter. The effects of each component are described in the kinetic equations with a Monod saturation function, which includes two main parameters: the maximum rate parameter (μ) and the saturation constant (K). The saturation term (S/(K+S)) can have a value between 0 and 1. Inhibition effects are described similarly, using an inhibition constant. Some of the main assumptions of the first ASM1 – ASM2 and ASM3 models have several implications for practical model application. Some of these assumptions had been modified in later extended models (temperature and pH range, wastewater type influent, to name a few). In this contribution, notations according to Corominas et al., (2010) are adopted.

The Activated Sludge Model No.1, **ASM1**, was published in 1987 (Henze et al. 1987) and represents the first standard mathematical model. Many of the basic concepts of ASM1 were adapted from the activated sludge model described by (Dold et al. 1980). ASM1 was developed primarily for municipal activated sludge WWTPs

to model carbon oxidation, nitrification and denitrification, performed by autotrophic nitrogen organisms (ANO) and ordinary heterotrophic organisms (OHO), which use oxygen or nitrate as the electron acceptor. ASM1 was designed for applications at lower temperature (5-20°C), under which no significant accumulation of nitrite occurs. Thus, the nitrite production and consumption is not modeled and, for simplicity, both nitrification and denitrification were considered to be a single step process. The first step of nitrification (nitritation) is normally considered the limiting step in aerobic oxidation of ammonia (Downing et al. 1964). The model does not take into account temperature and pH variations (pH near neutrality). Further this model does not implement storage of organic substrates process. The model aims at achieving a good description of the sludge production.

The ASM1 has 13 components and 19 parameters, five of which are stoichiometry. In ASM1 basically four processes are considered: (*i*) *growth of biom*ass: aerobic growth of heterotrophs (X_{OHO}); anoxic growth of heterotrophs; aerobic growth of autotrophs (X_{ANO}); (*ii*) *decay* of heterotrophs and autotrophs biomass; (*iii*) *ammonification* of organic nitrogen ($XC_{B,N}$); (*iv*) *hydrolysis* of particulate organic carbon (X_S) and nitrogen ($XC_{B,N}$). The readily biodegradable COD (S_B) and the soluble total ammonium-nitrogen concentration (TAN), S_{NHx}, are considered as the only substrates for the growth of heterotrophs and autotrophs, respectively. The slowly biodegradable material (XC_B) is supposed to be converted into readily biodegradable (S_B) material by hydrolysis reaction, introducing a delay into the oxygen utilization. The aerobic growth of heterotrophs occurs at the expense of soluble organic matter (S_B) and oxygen (S_{O2}) while the anoxic growth of heterotrophs occurs at the expense of soluble organic matter (S_B) and nitrate (S_{NOx}) with oxygen as inhibitor using a switching function. The S_{NOx} is assumed to be both the sum of nitrate and nitrite and the sum of the ionized (NO_2^--N) and un-ionized (HNO_2) nitrogen forms, not considering pH variation and HNO_2/NO_2^- equilibrium. In the model, the growth rate expression ($\mu_{OHO,max}$) and the saturation coefficient ($K_{SB,OHO}$) used for heterotrophic aerobic growth and anoxic are the same, thus to simulate that the maximum specific rate of substrate removal under anoxic conditions is lower than under aerobic condition, an empirical coefficient ($\eta_{u,OHO,AX}$) is added to the anoxic rate expression. The aerobic growth of autotrophs is expressed as function of total soluble ammonium-nitrogen concentration (TAN), S_{NHx}, which is assumed to be the sum of the ionized (NH_4^+-N) and un-ionized (NH_3-N) nitrogen forms, not considering pH variation and NH_3/NH_4^+ equilibrium. The coefficients for nitrification are assumed to be constant and to incorporate any inhibitory effects. In all growth process TAN is considered removed from solution and incorporated in cell mass ($i_{N,XBio}$). The approach adopted for modeling decay of the heterotrophic and autotrophic biomass is the same and based on the growth - death – growth concept (*death-regeneration*) of Dold et al., 1980, defined as the decay of biomass followed by hydrolysis and growth on secondary substrate arising from decay (Van Loosdrecht and Henze 1999). A single decay process (decay, lysis, endogenous respiration, predation, etc) was introduced to describe the sum of all decay processes under all environmental conditions (aerobic, anoxic). The decay acts to convert biomass to a combination of endogenous particulate products ($X_{U,B}$ and $XC_{B,N}$) and slowly biodegradable material (XC_B), without loss of COD and electron acceptor utilization. Further the decay continues at a constant rate (b_{OHO} and b_{ANO}). The biomass decay implies a recycle of organic and nitrogen substrate through the system. As consequence the flow of COD in ASM1 is rather complex, with interaction between the decay of heterotrophs and of nitrifiers. The model considers the ammonification process of soluble biodegradable organic nitrogen ($XC_{B,N}$) into total ammonium nitrogen (S_{NHx}). Finally, the model puts emphasis on hydrolysis of organic matter, which is well described but the model couples together the hydrolysis of the slowly biodegradable substrate (XC_B) and particulate biodegradable organic nitrogen ($XC_{B,N}$) considering that occur simultaneously with equal rate. The alkalinity is introduced to guarantee

the continuity in ionic charge of biological processes and is taken into account in the stoichiometry but does not limit the kinetic rates. Main gaps in ASM1 are: the absence of any term in the kinetic rate expression to model nutrient (ammonia) limitation in the heterotrophic growth process and ammonia and nitrite inhibition in the autotrophic growth process; the lack of the component N_2 in the anoxic growth of heterotrophic biomass in order to close mass balances. Finally soluble non-biodegradable organic nitrogen and particulate non-biodegradable organic nitrogen do not appear.

In 1995, an updated version of ASM1 was introduced to include a simple model of biological phosphorous removal (BPR) performed by phosphorous-accumulating- organisms (PAO), **ASM2** (Henze et al., 1995). In addition to the phosphorous biological process, ASM2 includes the fermentation process and two chemical processes to model chemical precipitation of phosphorus. ASM2 replace the readily biodegradable substrate (S_B) of ASM1 by the sum of the end products of fermentation (S_{Ac}) and the truly readily biodegradable substrate, which is fermentable (S_F). In contrast to ASM1, where all particulate organic material is expressed as COD, in ASM2 the biomass has a cell internal structure where all organic storage products are lumped into one model component ($X_{PAO,PHA}$) and includes poly-phosphate ($X_{PAO,PP}$) as inorganic fraction of activated sludge, which does not exert any COD. PAOs can only grow on cell internal organic storage material ($X_{PAO,PHA}$); storage is not depending on the electron acceptor conditions, but is only possible when fermentation products (S_F) such as acetate are available. In ASM2 basically six processes are considered: (*i*) *growth of biomass*: aerobic growth of heterotrophs (X_{OHO}) on fermentable substrate (S_F) and fermentable products (S_{Ac}); anoxic growth of heterotrophs on fermentable substrate (S_F) and fermentable products (S_{Ac}); aerobic growth of phosphorous- accumulating organisms (X_{PAO}) on cell internal organic storage products ($X_{PAO,PHA}$), aerobic growth of autotrophic nitrifiers (X_{ANO}); (*ii*) *transformation process*: anaerobic fermentation of heterotrophs (*iii*) *storage processes*: storage of cell external fermentation products (S_{Ac}) in the form of cell internal organic storage material ($X_{PAO,PHA}$); storage of ortho-phosphate (S_{PO4}) in the form of cell internal poly-phosphate ($X_{PAO,PP}$); (*iv*) *decay* of heterotrophs, autotrophs and phosphorous-accumulating biomass and decay of PAO storage products; *(v*) *hydrolysis* of particulate organic carbon; (*vi*) *chemical precipitation of phosphate*.

The heterotrophic biomass (X_{OHO}) is responsible for the hydrolysis of slowly biodegradable substrate (XC_B), the aerobic degradation of fermentable organic substrate (S_F) and of fermentation products (S_{Ac}), anoxic oxidation of S_F and S_{Ac} and the reduction of nitrate (S_{NOx}) (denitrification), and anaerobic fermentation of S_F into S_{Ac}. Further, microorganisms are subject to decay. These processes are modeled as parallel processes and require nutrient uptake into the biomass. Nitrification is one step process and it is modeled as proposed in ASM1 with the exception of nutrient uptake into the biomass. In contrast to ASM1, ASM2 introduces phosphorous – accumulating microorganisms (X_{PAO}) which are able to accumulate phosphorous in the form of poly-phosphate (X_{PP}). ASM2 does not consider the ability of PAO to denitrify but they can only grow on cell internal stored organic materials ($X_{PAO,PHA}$). First, it is assumed that PAO, in anaerobic/anoxic/aerobic condition may release phosphate (S_{PO4}) from the internal inorganic storage products (poly-phosphate ($X_{PAO,PP}$)). This reaction release energy that is utilized in the hydrolysis of $X_{PAO,PP}$ in order to store cell external fermentation products (S_{Ac}) in the form of cell internal organic storage materials ($X_{PAO,PHA}$). Then the regeneration of poly-phosphate, necessary for the growth of PAO, occurs and the storage of ortho-phosphate (S_{PO4}), in the form of cell internal poly-phosphate ($X_{PAO,PP}$) happens. The ASM2 can describe the block of storage of poly-phosphate if the phosphorous content of PAOs becomes too high, introducing an inhibition term which is active as the ratio $X_{PAO\ PP}/X_{PAO}$ achieves the maximum allowable value of $K_{SF\ PP\_PAO.}$ Finally the growth of PAO is modeled as an

obligate aerobic process and it is assumed to occur only on the cell internal organic storage materials ($X_{PAO,PHA}$). ASM2 includes three decay process for the biological phosphorous process, taking into account the biomass X_{PAO} and the storage products $X_{PAO,PP}$ and $X_{PAO,PHA}$. The decay is modeled in analogy to ASM1. In ASM2 the process of ammonification and phosphatification are not directly included. In ASM2 it is assumed that the fermentable substrates (S_F) contain a constant fraction of nitrogen and phosphorous ($i_{N,SF}$ and $i_{P,SF}$). As the hydrolysis processes depends on the available electron acceptor, ASM2 introduce three hydrolysis processes of slowly biodegradable organic matter (XC_B), in aerobic, anoxic and anaerobic conditions. In contrast with ASM1, the hydrolysis of particulate biodegradable organic nitrogen is not integrated and it is assumed that the slowly biodegradable substrate (XC_B) contains a constant fraction of nitrogen ($i_{N,XCB}$) and phosphorous ($i_{P,XCB}$). Alkalinity is taken into account in both stoichiometry and kinetic rates.

In 1999, an extention of ASM2 was proposed, **ASM2d** (Henze et al. 2000). Based on research results, ASM2d considers that phosphorous accumulating organisms (PAO_S) can use cell internal organic storage products $X_{PAO,PAH}$ for denitrification. It means that the model assums that PAOs may grow in an anoxic as well as an aerobic environment, whereas in ASM2 only aerobic growth is considered. In order to includes denitrifying PAOs, ASM2d implements two additional processes: anoxic storage of poly-phosphate $X_{PAO,PP}$ and anoxic groth of PAOs.

At Delft University of Technology, a new model **TUDP** had been developed, combining the ASM1 model with the metabolic model for denitrifying and non-denitrifying bio-P proposed by Murnleitner et al., 1997 (Van Veldhuizen et al. 1999; Brdjanovic et al. 2000). The TUDP totally considers primary biochemical processes of phosphorus accumulating organisms and models all organic storage components explicitly.

Furthermore, the ASM2d model had been modified by the addition of the ammonification and hydrolysis of organic nitrogen processes of ASM1 to deal with the observation that the influent wastewater contains soluble and particulate organic nitrogen, **ASM2dN** (Insel et al. 2006)).

Both ASM2 and ASM2d have some typing errors and inconsistencies as reported by Corominas et al., (2010). In 1999, a new version of ASM1 called the **ASM3** model (Gujer et al. 1999), has also been introduced which corrects a number of known defects present in the original model with the goal to become the new standard model. One of the main features in ASM3 is the introduction, similarly to ASM2, of cell internal storage compounds ($X_{OHO,STOR}$) of heterotrophic bacteria (X_{OHO}), thus it includes the storage of organic substrates as a new process and furthermore lysis is replaced by an endogenous respiration process. As consequence, the biomass is modeled with cell internal structure, decay process (which includes predation) include both fractions of biomass (X_{OHO} and $X_{OHO,STOR}$) and growth process are linked to the ratio of $X_{OHO,STOR}$ /X_{OHO}. The death – regeneration concept of ASM1 has been converted in a growth – endogenous respiration model and the flow of COD in ASM3 results clearer than in ASM1. ASM3 includes only biological process, such as: (*i*) *growth of biomass*: aerobic growth of heterotrophs on stored organic materials ($X_{OHO,STOR}$); anoxic growth of heterotrophs on stored organic materials ($X_{OHO,STOR}$); (*ii*) *storage processes*: aerobic storage of readily biodegradable substrate (S_B) and anoxic storage of readily biodegradable substrate; (*iii*) *respiration of biomass:* aerobic endogenous respiration; anoxic endogenous respiration; aerobic respiration of storage products; anoxic respiration of storage products; (*iv*) *hydrolysis* of slowly biodegradable substrates (XC_B).

The heterotrophic biomass (X_{OHO}) is responsible for the hydrolysis of slowly biodegradable substrate (XC_B). The importance of hydrolysis process has been reduced in ASM3 compared to ASM1. Heterotrophs growth is entirely on stored organic materials ($X_{OHO,STOR}$) and biomass growth directly on external substrate as described

in ASM1 is not considered in ASM3. As in previous models, only a fraction of the heterotrophs is capable of denitrification and a reducing factor is introduced. The storage of readily biodegradable substrate (XC_B) in the form of cell internal products ($X_{OHO,STOR}$) can occur both in aerobic and anoxic conditions using the energy from aerobic respiration and denitrification respectively. The model considers that the anoxic storage of (XC_B) is lower than aerobic one, introducing a reducing factor. The model assumes that substrates (S_B), first become stored material ($X_{OHO,STOR}$) an then are assimilated to biomass for growth. This hypothesis is not true and a direct growth rather than storage followed by growth can be simulated using a low yield coefficient for storage ($Y_{SB,STOR\ Ox}$ and $Y_{SB,STOR\ Ax}$) and a higher one for subsequent growth ($Y_{stor,OHO\ Ox}$ and $Y_{stor,OHO\ Ax}$). In ASM3, the model of decay is completely different from ASM1. The endogenous respiration occur both in aerobic and anoxic conditions and describe all forms of biomass loss and energy requirements not associated with growth: decay, maintenance, endogenous respiration, lysis, predation, motility, death and so on. Further, the model assumes that also the storage products ($X_{OHO,STOR}$) decay with biomass, under aerobic and anoxic conditions. ASM3 makes some change in the matrix form, introducing a composition matrix that indicated the composition of each components of the stoichiometric matrix relative to ThOD, N, ionic charge, SS. ASM1 and ASM3 are both capable of describing the dynamic behavior in municipal WWTPs. Koch et al., 2000 concluded that ASM3 performs better in situations where the storage of readily biodegradable substrate is important such as for industrial wastewater or for WWTPs with significant non-aerated zones. Also a carbon- based model (**ASM3C**) was proposed where organic state variables are expressed in terms of organic carbon rather than COD (Henze et al. 2000). The ASM3 model has been extended with a bio-P removal module **ASM3+BioP** by EAWAG Institute (Rieger et al. 2001). The EAWAG Bio-P module requires four state variables in addition to the 13 components defined in ASM3 to model the biological phosphorus removal differing from the BPR proposed in ASM2 model.

The ASMx models family do not consider as a model component nitrite (NO_2^-), which is an intermediate of nitrification and denitrification. In the context of nitrification, modeling nitrite production and consumption would be relatively easy. However, the exact fate of nitrite during denitrification was not sufficiently known until the last decade. Until the ASM3, ASMx models with one step nitrification – denitrification always considering total soluble ammonium-nitrogen concentration (TAN), S_{NHX}, and total soluble nitrite-nitrogen concentration, (TNN), as actual substrates for ammoniaum and nitrite oxidizers, respectively. In the subsequent models some hypothesis have been advanced and tested to explain the limitation/inhibition effect of real substrates/inhibitors of nitrification process.

Several models describing nitrite build-up during nitrification ($NH_4^+/NH_3 \rightarrow NO_2^- \rightarrow NO_3^-$) have been developed as reviewed by Sin et al., (2008) (Hellinga et al. 1999; Hao et al. 2002; Wett and Rauch 2003; Carrera et al. 2004; Wyffels et al. 2004; Pambrun et al. 2006; Kaelin et al. 2009; Park and Bae 2009). Denitrification is considered as a four-step process ($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$). Usually, denitrification is modeled as two step process ($NO_3^- \rightarrow NO_2^- \rightarrow N_2$.) as the production of NO and N_2O does not contribute significantly to the total mass –flow of nitrogen in the biological system.

To date, some authors propone an extension of ASMx models for nitrite build-up in aerobic and anoxic conditions as nitrite can play an important role in microbial conversions. Under certain conditions nitrite concentrations can increase to a harmful level in WWTPs and aquatic environments (Philips et al. 2002a). Further, nitrite route is becoming a changeling opportunity in WWTPs as new processes have been discovered and developed: nitritation, simultaneous partial nitrification denitrification "via nitrite" route, anammox, SHARON/anammox, CANON, SNAD and so on (Van Hulle et al. 2010).

The Activated Sludge Model Nr. 1 (**ASM1**) was extended with a 2 step nitrification model (Wyffels et al. 2004). The authors preferred the ASM1 model with death–regeneration concept over the ASM3 model with the endogenous respiration concept from the fact that endogenous respiration process was not yet clearly documented for ammonium and nitrite oxidizing biomass. Further, the ASM1 described by Wyffels et al., 2004, has been implemented with submodels for anammox process to interpret the results of an anammox enrichment in an SBR system (Van Hulle et al. 2003; Dapena-Mora et al. 2004). Here, the death–regeneration concept was chosen as biomass behaviors under substrate-limiting conditions was not completely clear and no biodegradable substrate (BOD) was added with the influent, avoiding accumulation phenomena. The extension of ASM1 with anammox process and two-step nitrification and denitrification was implemented is in the commercial software WEST® (**ASM1.e**). In 2006, an evolution of an ASM2d-like model was proposed, **ASM2d2N** (Sin and Vanrolleghem 2006)). The ASM2d2N introduces hydrolysis of organics nitrogen, two-step nitrification and two-step denitrification. The Arrhenius equation was also included in the ASM2d2N to take into account temperature effects. Further, updated versions of ASM3 were introduced to incorporate the 2 step nitrification and denitrification process, **ASM3_N** (Iacopozzi et al. 2007) and the revised version of ASM3_N, **ASM3_NO2** (Kaelin et al. 2009; Giusti et al. 2011).

Recently N_2O modeling has received attention, as N_2O is a powerful greenhouse gas. Most models only consider N_2O production by denitrifying microorganisms. A three-step denitrification model including the reduction of nitrate, nitrite and nitrous oxide has been formulated (Von Schulthess et al. 1994). Samie et al., (2011) considered a four-step denitrification process, based on ASM1, to simulate soluble N_2O production by heterotrophic denitrifying biomass and estimate the gaseous emissions from the reactors. Nevertheless, N_2O can be produced both by heterotrophs (Richardson et al. 2009) and nitrifiers (Campos et al. 2009; Wunderlin et al. 2012; Xie et al. 2012). The N_2O production mechanisms by nitrifying microorganisms are not well known. Schreiber et al., (2009) have recently proposed a preliminary model for NO and N_2O emissions from both nitrification and denitrification occurring in a complex biofilm where during nitrification nitric and nitrous oxide can be emitted, respectively, from the reduction of NO_2^- and NO by ammonia-oxidizing bacteria.

A **ASMN** model (activated sludge model for nitrogen) based on the ASM1 with a two-step nitrification and a four-step denitrification has been developed (Hiatt and Grady CP 2008). The ASMN model incorporates two nitrifying populations-ammonia-oxidizing bacteria and nitrite-oxidizing bacteria-using free ammonia and free nitrous acid, respectively, as their true substrates and four-step denitrification (sequential reduction of nitrate to nitrogen gas via nitrite, nitric oxide, and nitrous oxide) using individual, reaction-specific parameters. The authors also showed that results determined by using ASMN seem to be to be comparable with ASM1 results for municipal-type influent. Magrí et al., (2007) developed a new model for the simulation of the treatment of wastewaters with high nitrogen concentration, containing organic matter and phosphorus based on activated sludge models ASMx and on specific models of the SHARON process. Finally, several modeling protocols suggest values or how to measure some kinetic and stoichiometric parameters to be used in mathematic models: WERF (Melcer et al., 2003), BIOMATH (Vanrolleghem et al., 2003) and HSG (Langergraber et al., 2004).

5. Conclusion

Anammox process was first proposed in wastewater treatment plants by Jetten et al. (1997), introducing the concept of a more sustainable WWTP. The concept consisted in a first sedimentation step for the complete removal of COD from wastewaters, maximizing the sludge production. Then, the sludge could be digested to

yield methane for energy generation while the nitrogen could be removed from the digester effluent in two step reactors, performing nitritation and anammox process. From 1997 until now the concept of *ZeroWasteWater* has been proposed (Verstraete and Vlaeminck 2011). ZeroWasteWater offers an approach to the short-cycle water, energy and valuable materials, while adequately abating pathogens, heavy metals and trace organic. At the entry of the sewage treatment plant, an advanced physico-chemical or/and biological concentration step should additionally concentrate sewage components into a sludge. Key technologies for further treatment include the anaerobic digestion, the air stripping and the pyrolysis for the concentrated stream, and heat pumps, membrane technologies and anammox process (or better SNAD process) for the water stream. Hence, SNAD process could been thought to be applied in the WWTPs for the treatment of both sludge (concentrated) streams (Jetten et al. 1997; Joss et al. 2009) and water streams (Verstraete and Vlaeminck 2011).

SNAD process is also a challenging treatment for some industrial wastewaters characterized by high ammonia concentrations and low organic carbon such as old landfill leachate, wastewater from digested animal waste, wastewater from high-tech industry (opto-electronic wastewater) and wastewater from monosodium glutamate industry.

Nevertheless, a better understanding and a better knowledge of the biochemistry of the involved microorganisms and modeling are the keys to efficiently design and operate a wastewater treatment system and to implement innovative configurations of chemical, physical and biological processes. The use of molecular tools is fundamental to evaluate the microbial composition and distribution especially in biological systems such as the SNAD process, where the nitrogen and carbon removal efficiency depend on the well-equilibrated co-existence of different bacteria. Coupling the advances of microbial techniques with the monitoring of the macroscopic performance may lead to more robust and conscious operating strategies for the SNAD process. Finally, mathematical models would be important tools for studying the biological processes behavior and would be strongly needed in order to increase the understanding on the biological interaction in the SNAD processes.

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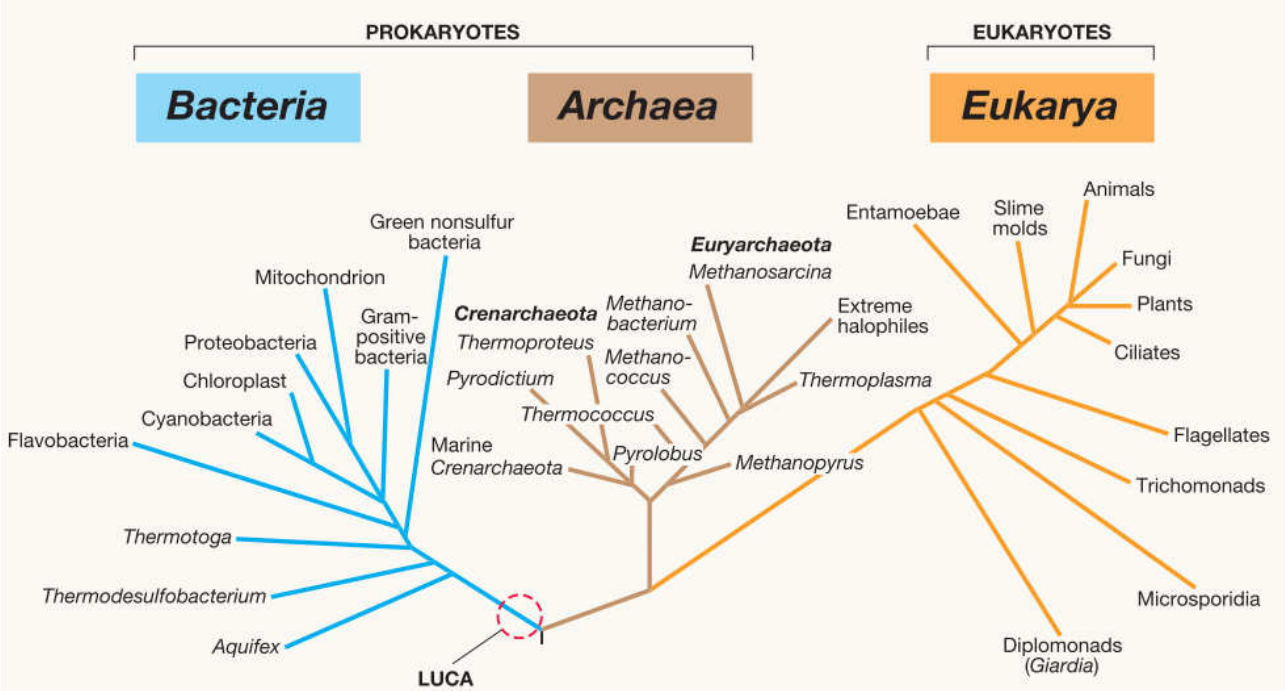
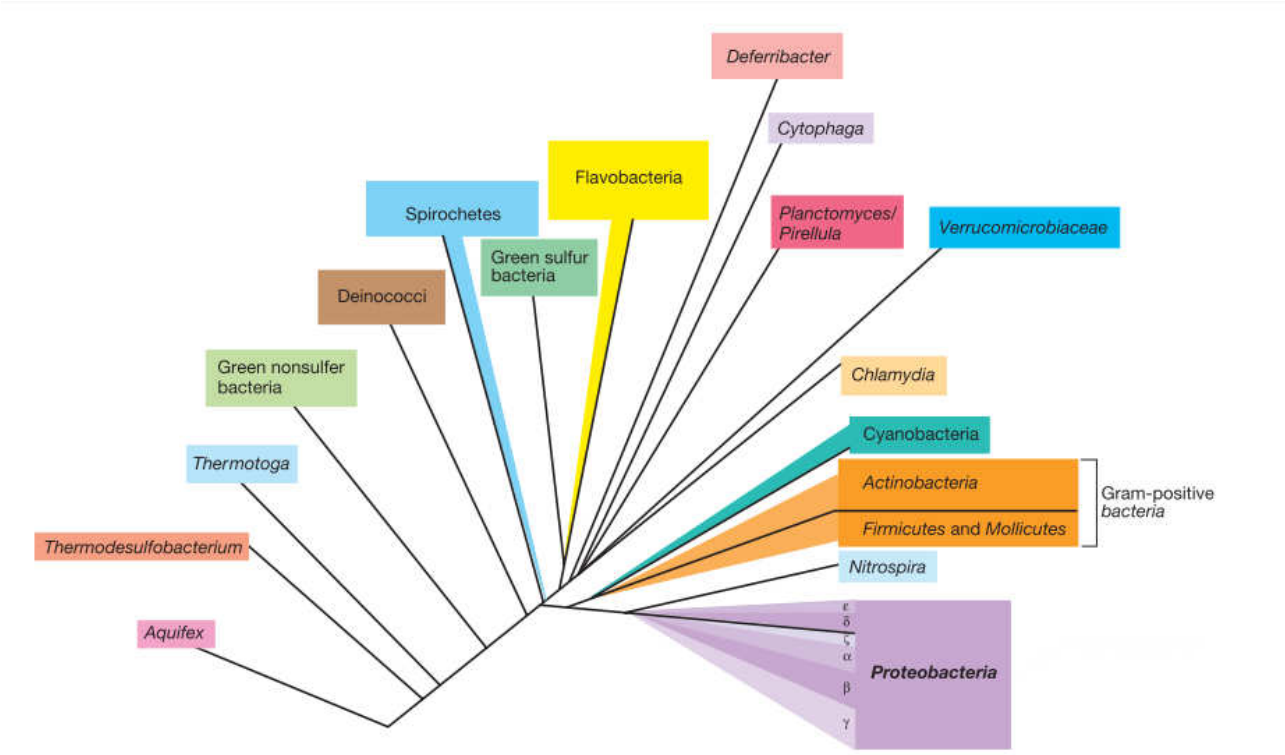
Author Contributions

All authors contributed extensively to the work presented in this review. M.L. wrote the paper with input from G.A, discussing the results, implications and commenting on the manuscript at all stages.

Supplementary Figures



Supplementary Fig. I-1 The phylogenetic tree of life as defined by comparative rRNA gene sequencing.
The tree shows the three domains of organisms and a few representative groups in each domain. All Bacteria and Archaea and most Eukarya are microscopic organisms; only plants, animals, and fungi contain macroorganisms. Figure from Madigan and Martinko (2006).



Supplementary Fig. I-2 Some major phyla of Bacteria based on 16S ribosomal RNA gene sequence comparisons.
Over 80 phyla of Bacteria are currently known, including many phyla known only from environmental sequences obtained in community sampling. Figure from Madigan and Martinko (2006).

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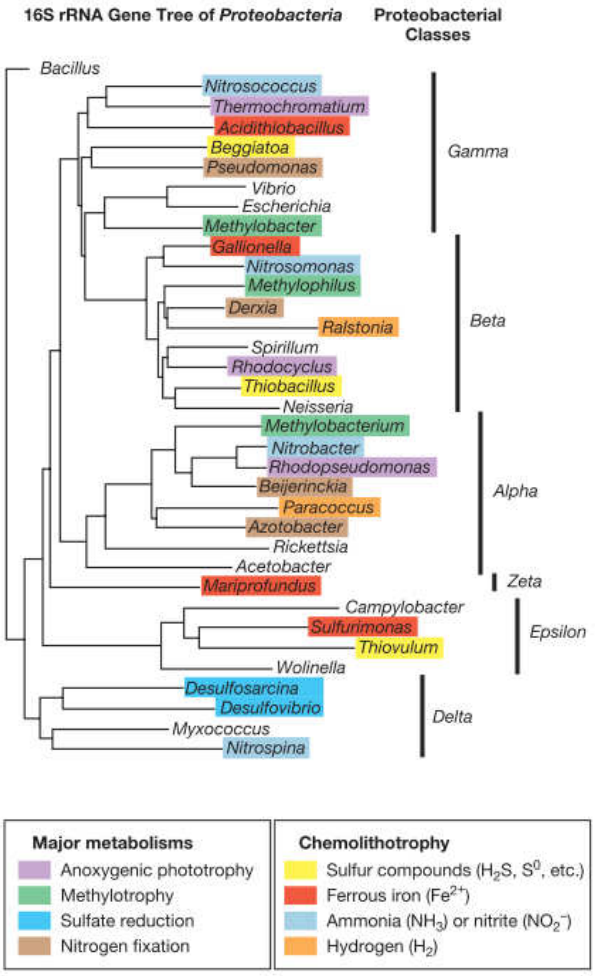

Supplementary Fig. I-3 Phylogenetic tree of some key genera of proteobacteria.
Phylogenetic tree constructed by comparative 16S rRNA sequencing. Identical metabolisms are often distributed in phylogenetically distinct genera. Some organisms listed may have multiple properties: some sulfur chemolithotrophs are also iron or hydrogen chemolithotrophs, and several of the organisms listed can fix nitrogen. Figure from Madigan and Martinko (2006).

II. Chapter

Co-existence of nitrifying, anammox and denitrifying bacteria in a sequencing batch reactor

This chapter was based on:

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Coexistence of nitrifying, anammox and denitrifying bacteria in a Sequencing Batch Reactor.

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ABSTRACT

High nitrogen and carbon removal efficiencies were observed in a sequencing batch reactor (SBR), treating reject waters (up to 650 mg NH_4^+-N L^{-1} and 300 mg COD L^{-1}) of the Zürich wastewater treatment plant (Switzerland). The Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process appeared to occur. The SNAD biomass, at room temperature, showed potential aerobic and anaerobic ammonia oxidation activities of 1.8 nmol N mg protein^{-1} min^{-1} and 0.34 nmol N mg protein^{-1} min^{-1}, respectively. The microbial community composition of the SNAD biomass was analyzed by 16S rRNA and functional gene-based analysis and further visualized by FISH. The 16S rRNA gene and functional gene approach suggested this SNAD biomass had a diverse microbial community. The main groups of microorganisms were found to be ammonia-oxidizing bacteria of the N*itrosomonas europaea/eutropha* group, anaerobic ammonium-oxidizing bacteria of the "*Candidatus* Brocadia fulgida" type and denitrifying bacteria related to the betaproteobacteria *Thauera*, *Pseudomonas*, *D. aromatica* and *Aromatoleum aromaticum*. Nitrite-oxidizing bacteria from the genus *Nitrobacter* were detected while no nitrite-dependent anaerobic methane oxidizing bacteria were found.

FISH analysis of the SNAD biomass samples clearly showed that long filamentous microorganisms were dominant in the outer layer of the granules, with single cells as well as aggregates trapped in a net of these filamentous bacteria. The spatial position of two of the major community constituents, aerobic and anaerobic ammonia oxidizing bacteria, were visualized within the biomass. Aerobic nitrifiers were located at the outside of the aggregates as single cells and in clusters, while anammox bacteria were abundant in the inner part of the granules in agreement with the expectation that the interior of the aggregates would be anoxic. PCR and FISH results correlated well and confirmed that the co-existence of aerobic ammonia-oxidizing, anammox and denitrifying bacteria.

Keywords: amoA, Brocadia, co-existence, Denitrifiers, FISH, n-damo, Nitrobacter, Nitrosomonas, NirS, PCR, phylogenetic, SNAD, 16 S rRNA

1. Introduction

Biological nitrogen removal in wastewater treatment plants (WWTPs) is carried out by various subsequent microbial processes. Traditionally, nitrogen removal from main-stream wastewaters has been performed by a combination of autotrophic nitrification and heterotrophic denitrification in activated sludge, biofilm and granular systems. Nitrification and denitrification processes can be developed either in separate tanks or in one single reactor. In conventional activated sludge processes, nitrification and denitrification processes are physically divided and the optimal operative conditions for each microorganism species are achieved in each reactor. In one-step systems, the co-existence of nitrifiers and denitrifiers has to be ensured by optimal design and operation of the bioreactor, biomass carrier and selection pressure. Due to its space-saving and operation flexibility, the Sequencing Batch Reactor (SBR) is a one-step system that has been widely used for nitrogen removal (Henze 1991; Obaja et al. 2003). However, high operational costs, due to the oxygen supply for nitrification and external organic carbon requirements for denitrification, have restricted wider applications of nitrification and denitrification for side-stream wastewater such as reject waters of urban and industrial plants and landfill leachates (Ahn 2006; van Hulle et al. 2010; Zhang et al. 2010a; Verstraete and Vlaeminck 2011).

Reject waters, the liquid fractions produced in the dewatering stage after anaerobic digestion, like landfill leachates, are characterized by high nitrogen (N) concentrations and low organic carbon (COD) content, resulting in a very low biodegradable COD:N ratio. The amount of total ammonium nitrogen is extremely high, with a high oxygen demand in nitrification, while organic matter is usually not enough for a complete denitrification and has to be provided externally (Obaja et al. 2003; Wiszniowski et al. 2006). New nitrogen removal pathways were recently discovered and new biological nitrogen removal processes have been developed in WWTPs as alternatives to the traditional nitrification–denitrification. For instance, the nitritation process followed by denitrification via nitrite is more energy and cost-effective than conventional full nitrification/denitrification process, due to the elimination of nitrite oxidizer activity. This process has been successfully implemented in full scale application (Hellinga et al. 1998; Mulder et al. 2001). Further innovation came from the enrichment of the anaerobic ammonium oxidizing bacteria (anammox), whose existence were predicted by Broda (Broda 1977) and activity first observed in a WWTP by Mulder et al. (1995), and finally identified by Strous et al. (1999b). The possibility to convert ammonium with nitrite under anaerobic conditions without the need for addition of electron donors has opened up possibilities to further minimize energy and other expenses within wastewater treatments (Jetten et al. 1997; Kartal et al. 2010). Combination of nitritation with the anammox process has been implemented in various wastewater treatment plants around the globe (Wett et al. 1998; Third et al. 2001; Philips et al. 2002b; Szatkowska et al. 2007b). Other nitrogen converting bacteria of possible use for wastewater treatment are the so-called n-damo bacteria which are capable of nitrite-dependent anaerobic methane oxidation. These bacteria were first discovered in a freshwater system (Raghoebarsing et al. 2006), enriched from a ditch in the “Ooijpolder”, The Netherlands, (Ettwig et al. 2009) and finally detected in nine activated sludges of WWTPs with an anaerobic digestion section (Luesken et al. 2011b).

The possible co-existence of anammox bacteria in a single reactor with either nitrifiers (Third et al. 2001), denitrifiers (Kumar and Lin 2010) or n-damo bacteria (Luesken et al. 2011c) is attractive and has been studied and tested. In particular, different combinations of nitrification, denitrification and anaerobic oxidation of ammonium, under appropriate environmental oxygen-limited conditions, have been reported for nitrogen removal both for main and side-stream wastewaters (Daverey et al. 2012; Winkler et al. 2012a). The engineering challenge of using one single reactor to achieve a complete nitrogen and carbon removal has led to studies on the co-existence and interaction between different bacteria (Yan et al. 2010, 2012; Luesken et al. 2011c). The use of a single tank has some advantages over a configuration with two or more reactors, as single-stage processes generally have a higher volumetric nitrogen removal rate and lower capital costs than multi-stage systems (Kalyuzhnyi et al. 2006; van Hulle et al. 2010; Wang et al. 2012). Full-scale implementations of the biological Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process in one single reactor, where autotrophic oxidation of ammonium to nitrite is followed by an anaerobic ammonium oxidation and denitrification process, were successfully achieved in the last decade (Chen et al. 2009; Wang et al. 2010; Lan et al. 2011; Daverey et al. 2012). Most SNAD systems are operated in granular Sequencing Batch Reactors (SBRs). Both SBR technology and granulation allow the co-existence of several bacterial populations. The SBR, widely used in WWTPs and in laboratory studies, is a single tank that serves as both a biological reactor and a settler in a temporal sequence (Artan and Orhon 2005). The dynamic operative conditions of SBRs can dictate different metabolic activity by sequencing each phase of the mixing, aeration and non-mixing/non-aeration pattern during each cycle, ensuring different macro-environmental conditions for bacteria (aerobic/anoxic conditions or high/low loading). On the other hand, bio-granulation technology (both aerobic and anaerobic) has attracted

great research interests for the removal of organic, nitrogen and phosphorus compounds from wastewater. Aerobic granulation is naturally developed in SBR systems (Liu and Tay 2004; Liu et al. 2005). Aerobic granules, compared to fluffy and irregular conventional activated-sludge flocs, have a compact microbial structure and a strong microbial stratification that develops different micro-environments from the outer to the inner layer of aggregates (Vlaeminck et al. 2010). Both SBR and granulation technology facilitate high biomass concentration and accumulation of slow growing organisms, such as nitrifying and anammox bacteria (Strous et al. 1998; de Kreuk and van Loosdrecht 2004; Liu et al. 2005). Moreover, both of these technologies improve the system resistance to shock and toxic loadings, preventing inhibition effects (Etterer and Wilderer 2001; Mohan et al. 2005). Therefore, coupling the SBR and granulation technology, creating different macro- and micro-environments both in the bulk liquid and in the biomass, is a sustainable way to improve the co-existence of nitrifying, anammox and heterotrophic denitrifying biomass in one single reactor for nitrogen and organic carbon removal.

A better understanding of the microbial community composition and concomitant knowledge of the physiology of the community members may lead to improved success for biological nitrogen removal in wastewater treatment. Recent applications of molecular biology have provided techniques to determine microbial presence, diversity and abundance in different environmental compartments and in WWTPs. A variety of molecular methods are used to estimate either the diversity or overall abundance of microbial communities. The nucleic acid isolation and extraction from environmental samples (total DNA isolation and extraction) is one of the first steps in molecular studies, as organisms can be detected by assaying genes that encode either ribosomal RNA (rRNA) or enzymes that support a specific physiology. The rRNA or functional genes can be amplified by the Polymerase Chain Reaction (PCR) technique. Following the amplification of a gene, there are a number of strategies for investigating the diversity, abundance and structure of that gene in a mixed community. In order to examine the community structure and phylogeny, a library of gene sequences is usually developed from the products of the PCR by cloning gene fragments and performing a sequencing analysis. The overall relative abundance of any particular gene fragment is frequently measured by a modified PCR (quantitative PCR - qPCR). Fluorescence in situ hybridization (FISH) is another molecular technique widely applied in environmental analysis, where fluorescent probes are used to identify organisms that contain a nucleic acid sequence complementary to the probe (Amann et al. 1990). Studies have investigated the diversity of specific bacteria in a complex microorganism community, as could be the SNAD biomass, using FISH (Third et al. 2001; Vázquez-Padín et al. 2010; Winkler et al. 2012a), phylogenetic analysis (Quan et al. 2008; Li et al. 2009; Xiao et al. 2009; Burgmann et al. 2011; Daverey et al. 2012) and both techniques (Date et al. 2009; Wang et al. 2010).

However, further research into both the microbial community composition and the activities of important community members remained to be performed. Therefore the objectives of this study were to assess the microbial community composition and the activities of major community members of SNAD biomass originating from a WWTP located in Zürich, Switzerland (Joss et al. 2009). To investigate the microbial community composition by polymerase chain reaction (PCR), amplifications using primers targeting 16S rRNA genes (phylogenetic marker) and primers targeting genes coding for parts of physiologically relevant enzymes (functional marker) were performed for relevant groups of microorganisms (anammox bacteria, aerobic ammonia oxidizers and denitrifiers). For each microorganism, clone libraries were constructed and clones sequenced and phylogenetic analyses were performed. In addition, the presence and diversity of bacteria involved in possible secondary processes were assessed: the aerobic nitrite-oxidizing bacteria and nitrite-dependent anaerobic

methane oxidation (n-damo) bacteria. Furthermore, the spatial structure within the biomass of two of the major community constituents, the aerobic and anaerobic ammonia-oxidizing bacteria, was visualized by FISH analyses using specific probes. The activities of these two groups were also assessed.

2. Materials and Methods

2.1. Sample collection

The SNAD seed sludge, investigated in this study, was collected from an SBR for the digester supernatant treatment at the WWTP of Zürich, Switzerland. In this reactor a complete partial nitrification, anammox and denitrification process occurred (Joss et al. 2009). Table II-1 and Table II-2 list SBR details. The SNAD sludge contained granules surrounded by a matrix of brownish flocs. Two kinds of granules (red and brown) could be distinguished. The granules had diameters between 0.1 and 2.0 mm. Prior to the microbial characterization, the sludge was stored at – 4°C and gently mixed to avoid the destruction of granules.

Table II-1 Influent and effluent characteristics of reject water of Zürich plant (CH) (Joss et al. 2009).

Parameters	Influent	Effluent	u.m
Temperature	27±1	30±3	°C
Conductivity	5.5±0.1	2.4±0.2	mS cm^{-1}
pH	7.8 ±0.1	7.1 ±0.2	
Total Ammonium Nitrogen (TAN)	650±50	30±10	mgN L^{-1}
Total Nitrite Nitrogen (TNN)	<0.2	<0.2	mgN L^{-1}
Nitrate Nitrogen	<0.2	5 ±5	mgN L^{-1}
Soluble COD	300±50	190±50	mg L^{-1}
Total COD	630±50	400±100	mg L^{-1}
SST	250±50	150±100	mg L^{-1}
DOC	80	-	mg L^{-1}

Table II-2 SBR operative conditions of Zürich plant (Joss et al. 2009).

Parameters	Values	u.m
Volume	1400	m^3
Dissolved Oxygen	< 0.8	mg L^{-1}
pH	7.1 ± 0.2	-
Temperature	30	°C
MLSS	~ 3.6	kg $_{SS}$ m^{-3}
Nitrogen Load	1250	kgN d^{-1}
Volumetric Nitrogen Load	0.44	kgN m^{-3} d^{-1}
HRT	1.45	d

2.2. Genomic DNA extraction and purification

To maximize DNA extraction efficiency a representative sample (0.3 mL) with red and brown granules and brownish flocs was taken and its granules and flocs were aseptically broken, by passing through a series of syringe needles (0.60-0.30 mm). Thereafter, DNA was extracted using a CTAB DNA isolation method adapted from the protocol published by Zhou et al. (1996) and described previously (Yan et al. 2010).

The method is a CTAB (cetyltrimethylammonium bromide) extraction buffer- and a SDS-lysis-based method with phenol chloroform purification, as it is a suitable method for samples with a high molecular weight. Biomass (0.3 mL) was harvested by centrifugation for 10 min, 13,000×g. Cells were re-suspended in 675 µL of 1% CTAB extraction buffer (1% CTAB, 100 mM Tris, 100 mM EDTA, 100 mM sodium phosphate buffer, and 1.5 M NaCl, adjust to pH 8 using HCl). After addition of 50 µL lysozyme (10 mg mL^{-1}; 66,200U mg^{-1}) and of 30µL Rnase A (10 mg mL^{-1}), biomass was incubated (30 min, 37°C). Then, 50 µL of proteinase K (10 mg mL^{-1}; 20 U mg^{-1}) was added, followed by incubation for 30 min at 37°C. Subsequently, 150µL 10% SDS was added and the mixture was incubated for 2h at 65°C. The DNA was separated from cell debris by addition of 500 mL Phenol/Chloroform/Isoamylalcohol (25:24:1) and 20 min of incubation at 65°C, followed by centrifugation for 20 min at 13,000×g. The upper water phase, containing the genomic DNA, was mixed with 1 volume of Chloroform/Isoamylalcohol (24:1) and centrifuged (20 min, 13,000×g) to remove traces of phenol. Again, the upper water phase was removed and 0.6 volume of isopropanol was added to the supernatant. After incubation at room temperature for 30 min and subsequent centrifugation for 20 min at 13,000×g, the precipitation of DNA was achieved. The genomic DNA was then washed with 0.5 mL of ice cold 70% ethanol (to remove co-precipitated salts) and air dried at room temperature for about 15 min. Finally, the DNA was suspended in 50 µL of ultrapure water (MilliQ, Millipore SA, Moisheim, France) and kept at 4°C until further analysis. DNA quality was checked by 1% agarose gel electrophoresis.

2.3. PCR amplification

After genomic DNA isolation, qualitative PCRs have been performed. PCR analyses were performed in a T gradient cycler (Whatman Biometra, Göttingen, Germany) using as buffer and enzyme formulation the GoTaq® Green Master Mix (Promega Benelux BV, Leiden, the Netherlands), according to the manufacturer's protocol instructions. Negative controls (no DNA added) were included in all sets of amplifications. To analyze the microbial community composition in the SNAD process, independent PCR assays were conducted, targeting both main and possible secondary biological processes (Fig. II.1). Reaction mixtures (25 µL) used for PCR amplification contained 0.5 µL of each primer forward and 0.5 µL of each primer reverse, 12.5 µL of GoTaq® Green Master Mix, including GoTaq® DNA Polymerase, dNTPs, $MgCl_2$ and reaction buffers, 1 µL of DNA diluted template corresponding to 25 ng of total DNA, and RNase-free water (DEPC) to complete the 25 µL volume. Specifications of all the primers used in this study are listed and described in Table II-3 including the annealing temperatures (T_a). All PCR's were run with an initial denaturation of the template DNA at 96 °C for 1 min, followed by 35 cycles of 1 min at 96°C, 1 min at a different annealing temperature (T_a), and 1 min at 72 °C. The reaction was completed after 10 min at 72 °C. In all cases, PCRs were performed both using 1:100 and 1:10 dilutions of the original template DNA solution.

Aerobic Ammonia oxidizing bacteria (AerAOB). To detect AerAOB of the beta-subclass of proteobacteria, the bacterial *amoA* gene (coding for the alpha subunit of the ammonia monoxygenase enzyme) was amplified

using forward primer *AmoA-1F* and reverse primer *AmoA-2R*, resulting in 453-bp fragments (Rotthauwe et al. 1997).

Anammox bacteria. To investigate the anammox bacterial community two different primer combinations were used. A standard PCR amplification of the 16S rRNA gene of members of the *Planctomycetales* phylum was performed with forward primer *Pla46F* targets a stretch corresponding to positions 46-63 and universal reverse primer 630R targets a stretch corresponding to positions 1529-1545, resulting in 1400 bp products (Schmid et al. 2003). Also, a specific primer combination of *Amx368F/Amx820R* (452 bp) was used for the direct preferential amplification of the 16S rRNA gene of the members of anammox bacteria (Schmid et al. 2003).

Denitrifying bacteria. To determine the presence of denitrifying bacteria, PCR amplification targeting the nitrite reductase (*nirS*) gene was performed, as the *nirS* gene is widely distributed in denitrifiers microorganisms (Braker et al. 1998). Forward primer *NirS1* targeting a stretch corresponding to positions 763–780 and reverse primer *NirS 6* targeting a stretch corresponding to positions 1638–1653 (890 bp products) were used (Braker et al. 1998; Throbäck et al. 2004).

Aerobic Nitrite oxidizing bacteria (AerNOB). Both *Nitrobacter* spp. and *Nitrospira* spp. AerNOB were targeted. To identify *Nitrobacter* spp. specific primer combinations targeting both 16S rRNA genes *Nitro1198F/Nitro1423R* (225 bp) (Graham et al. 2007), and *NxrA* functional genes, *NxrAF1370F/F2843R* (1473 bp) (Wertz et al. 2008) were used. *Nitrospira*-like nitrite-oxidizing bacteria were investigated by amplification of the 16S rRNA gene using primer combinations Nspra-*675F/Nspra-746R* (93 bp) (Graham et al. 2007) and *616F/ Ntspa1158R* (1168 bp) as previously described by Maixner et al. (2006).

Anaerobic nitrite-dependent methane oxidation bacteria (n-damo). In order to detect n-damo bacteria two different primer combinations were used. First, in order to identify *M. oxyfera*-like bacteria on a functional level, the bacterial *pmoA* gene was amplified using the specific forward primer *pmoA 47* (*cmo182*) (Luesken et al. 2011a) and the reverse primer A 682 (Holmes et al. 1995) in a direct PCR. In view of the fact that all enrichment cultures of n-damo bacteria were dominated by the same group of bacteria within the NC10 phylum, specific 'NC10' primers, targeting the 16S rRNA gene, were used (Holmes et al. 1995; Luesken et al. 2011b). Considering the presumably low concentration of DNA from the n-damo bacteria, the 16S rRNA of 'NC10' phylum bacteria was amplified through a nested PCR. The 16S rRNA primers used were an 'NC10' specific forward primer 202F (Ettwig et al. 2009) and a general bacteria reverse primer (Juretschko et al. 1998). The PCR product obtained was then used as template for the nested PCR using the 'NC10' specific primers qP1F and qP2R (Ettwig et al. 2009).

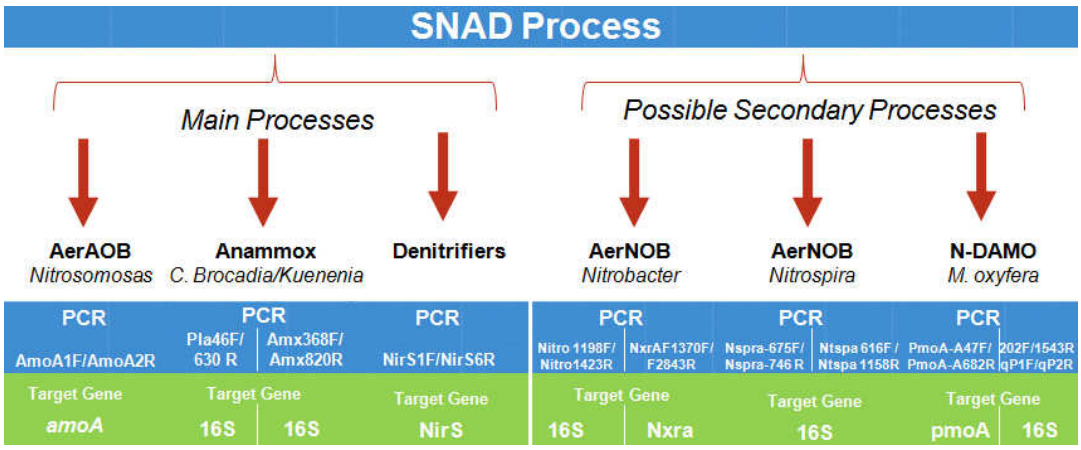

Fig. II.1 Schematic representation of main and possible secondary biological processes involved in the SNAD process with the corresponding genes targeted and primer sets used in this study.

Table II-3 Primers used for PCR amplifications and sequencing

Name primer	target organism(s)	target gene	sequence (5'-3') of primer	Position	T_a (°C)	reference
AmoA-1F	*Nitrosomonas* spp.	*amoA*	GGGGTTTCTACTGGTGGT	332-349 [a]	52	(Rotthauwe et al. 1997)
AmoA-2R			CCCCTCKGSAAAGCCTTCTTC	802-822 [a]		(McTavish et al. 1993)
Pla46-F	*Planctomycetes* Most Bacteria	*16S*	GGATTAGGCATGCAAGTC	46-63 [a]	56	(Neef et al. 1998)
630 R			CAKAAAGGAGGTGATCC	1529-1545 [a]		(Juretschko et al. 1998; Schmid et al. 2003)
Amx 368F	*Anammox bacteria Kuenenia/ Brocadia*	*16S*	CCTTTCGGGCATTGCGAA	368-385	56	(Schmid et al. 2003)
Amx 820R			AAAACCCCTCTACTTAGTGCCC	820-841 [b]		(Schmid et al. 2000, 2003)
NirS1F	*Denitrifiers*	*nirS*	CCTA(C/T)TGGCCGCC(A/G)CA(A/G)T	763-780 [b]	57	(Braker et al. 1998; Throbäck et al. 2004)
NirS6R			CGTTGAACTT(A/G)CCGGT	1638-1653 [b]		(Braker et al. 1998; Throbäck et al. 2004)
Nitro-1198F	*Nitrobacter* spp.	*16S*	ACCCCTAGCAAATCTCAAAAAACCG	1198 [a]	58	(Graham et al. 2007)
Nitro1423R			CTTCACCCCAGTCGCTGACC	1423 [a]		(Graham et al. 2007)
NxrA F1370 F	*Nitrobacter* spp.	*nxrA*	CAGACCGACGTGTGCGAAAG	1370	55	(Wertz et al. 2008)
F2843 R			TCCACAAGGAACGGAAGGTC	2843		(Wertz et al. 2008)
Nspra-675F	*Nitrospira* spp. (total)	*16S*	GCGGTGAAATGCGTAGAKATCG	675–696 [a]	58	(Graham et al. 2007)
Nspra-746 R			TCAGCGTCAGRWAYGTTCCAGAG	746–768 [a]		(Graham et al. 2007)
Ntspa 616F-	*Domain Bacteria*	*16S*	AGAGTTTGATYMTGGCTC	8-25 [a]	56	(Juretschko et al. 1998)
Ntspa 1158R	*Nitrospira* spp.		CCCGTTMTCCTGGGCAGT	1158–1176 [a]		(Maixner et al. 2006)
PmoA-A47F	*n-damo* bacteria	*pmoA*	TCACGTTGACGC CGA TCC	47	60	(Luesken et al. 2011a)
PmoA-A682R			GAAASGCNGAGAAGAASGC	682		(Holmes et al. 1995)
202F	*'N10' Phylum bact.*	*16S*	GACCAAAGG GGGCGAGCG	193 [c]	57	(Ettwig et al. 2009)
1543R	Most Bacteria		TCTCCACGCTCCCTTGCG	1027 [c]		(Juretschko et al. 1998)
qP1F	*M. oxyfera*		GGGCTTGACATCCCACGAACCTG	1016-	65	(Ettwig et al. 2009)
qP2R	Most Bacteria.		CTCAGCGACTTCGAGTACAG	1481		(Ettwig et al. 2009)
M13 Forward	sequencing	pGEM T easy vector	GTAAAACGACGGCCAG	Region flanking cloning site	-	-

[a] *Escherichia coli numbering* (Brosius et al. 1981).
[b] Positions in the nirS gene of *Pseudomonas stutzeri* ZoBell EMBL (X56813).
[c] Positions based on the gap-free sequence of the N10 bacterial clone D-BACT (DQ369742), starting at *Escherichia coli* position 8.

2.4. Cloning and sequencing

Presence and size of amplificates were checked by agarose (1%) gel electrophoresis of 5 µL aliquots of the PCR products. PCR products were cloned directly using the pGEM-T Easy cloning vector kit, following the instructions of the manufacturer (Promega Benelux BV, Leiden, the Netherlands). Plasmid-DNA of clones was isolated from randomly selected clones per library using a GeneJET^{TM} Plasmid Miniprep Kit (Fermentas GMBH, St. Leon-Rot, Germany). Clones were checked for inserts of the expected size by agarose (1%) gel electrophoresis after EcoRI digestion (5 U, Eco RI- buffer for 3 h at 37 °C). Sequencing was performed at the DNA Diagnostics Center of Nijmegen University Medical Center using the M13 forward primer.

2.5. Nucleotide sequence accession numbers

The 16S rRNA and the functional gene sequences determined during this study were deposited in the GenBank database using the the stand-alone software tool Sequin developed by the NCBI (http://www.ncbi.nlm.nih.gov/Sequin/). The partial *amoA* gene sequences are available under the accession numbers KC569470 - KC569477 (*Nitrosomonas* spp.), the partial *NirS* gene sequences are found under KC569496 - KC569503 (denitrifiers), while the partial 16S rRNA gene sequences are available under the accession number KC569478 - KC569483 (anammox species) and KC569484 - KC569495 (*Nitrobacter* spp.).

2.6. Phylogenetic analysis

Clone gene sequences were analyzed by BLASTN searches (http://www.ncbi.nih.nlm.edu/BLAST) (Altschul et al. 1997). The clone sequences were aligned using the MEGA5 software (Tamura et al. 2011). Sequences of related species and a selection of relevant clones sequences from different environments taken from the Genbank (NCBI) database were also included in the alignments. Phylogenetic trees were constructed based on nucleic acid (*16S rRNA, amoA, NirS*) and amino acid (*amoA, NirS*) sequence alignments using the same software, applying the neighbor joining statistical method (Saitou and Nei 1987) with the pairwise deletion option for gaps. The tree topology was based on bootstrap analysis. Evolutionary distances were computed using the Jukes-Cantor substitution model (Jukes and Cantor 1969) for nucleic acid analysis. Poisson correction model (Zuckerkandl and Pauling 1965) and the Dayhoff matrix based method (Schwarz and Dayhoff 1979) were used for amino acid analysis.

2.7. Fluorescence in situ hybridization (FISH)

The presence of two of the major community constituents of the SNAD process, the aerobic ammonia-oxidizing- and anammox bacteria, was verified by qualitative fluorescence *in situ* hybridization (FISH) analysis, using 16S rRNA- and 23S rRNA- genes targeted oligonucleotide probes. Two samples were analyzed: (*i*) biomass with entire granules and flocs and (*ii*) biomass with broken granules and flocs, where granules and flocs were aseptically broken by passing them through syringe needles as described before. Fixation and hybridization were performed as described by Schmid et al. (2000).

Fixation. Biomass (1 mL) was harvested from the SNAD sample by centrifugation. Samples were fixed in 4% (w/v) paraformaldehyde, incubated on ice (2 h) and centrifuged (10 min; 13,000×g). The resulting pellets were washed with phosphate buffered saline (PBS; 10 mM Na_2HPO_4/NaH_2PO_4 pH 7.5 and 130 mM NaCl) and finally suspended in 1 mL of PBS and 100% EtOH (1:1) mixture. Fixed samples were stored at −20°C until analysis. **Oligonucleotide probes**. To detect anammox bacteria, the probe S-*-Amx-0820-a-A-22 (AMX820), specific for "*Candidatus* Kuenenia stuttgartiensis" and "*Candidatus* Brocadia anammoxidans" (Schmid et al. 2001), was used. The phylogenetic group-specific probe for the identification of betaproteobacteria, L-C-bProt-1027-a-A-17 (BET42a), which targets the 23S rRNA gene, was labeled with Cy3 and used to assess the presence of betaproteobacteria (Manz et al. 1992). In order to avoid false positives probe GAM42a was always used as an unlabeled competitor for BET42a. Probe S-*-Neu-0653-a-A- 18 (NEU653), which targets the 16S rRNA of most halophilic/tolerant *Nitrosomonas* spp. (*N. europaea, N. eutropha, N. mobilis, N. halophila*), was used to identify *Nitrosomonas* spp. An equimolar amount of corresponding competitor oligonucleotide probe was used for NEU

653 (Wagner et al. 1995). Details of the probes used in this study, their sequences, hybridization conditions, and references are given in the Table II-4.

Immobilization of fixed cells on microscope slides. For individual hybridizations, 5 µL of paraformaldehyde fixed material was used and located together with 10 µL of PBS in microscopic slides. After air drying (30 min), to allow optimal attachment to the slides, the samples were dehydrated by successive passage through 50, 80 and 100% ethanol (3 minutes each) and finally air dried. **In situ hybridization.** All probes were purchased as Cy3- and FLUOS- (5(6)- carboxyfluorescein-N-hydroxysuccinimide ester) labeled derivatives from Thermohybaid (Ulm, Germany). To each well on the slide were added 10 µL of freshly prepared hybridisation buffer and 1 µL of each probe. All hybridizations were performed at 46°C for 90 min in hybridization buffer, containing 180 µL 5 M NaCl, 20 µl 1 M Tris-HCl (pH 8.0), formamide at the percentage shown in the Table II-4, and 2 µL 10% (w/v) SDS. Hybridization was followed by a stringent washing step at 48°C for 20 min in washing buffer, containing 1 mL 1 M Tris-HCl (pH 8.0), 1.02 mL 5 M NaCl and 0.5 mL 0.5 M EDTA. Washing buffer was removed by rinsing the slides with precooled distilled water. The slides were air dried with compressed air or briefly in the 46°C oven. Samples were counterstained using Vectashield (Vector Laboratories, CA, USA) mounting medium with DAPI (4,6-diamidino-2-phenylindole, 1.5 µg mL^{-1}) in order to enhance the fluorescent signal and stain all DNA. **Image acquisitions.** Image acquisitions were performed with a Zeiss Axioplan II epifluorescence microscope (Zeiss, Jena, Germany) together with the standard software package delivered with the instrument. The relative abundance of *Nitrosomonas* spp. and anammox bacteria was estimated by visual inspection because the large flocs and granules in which these organisms are present makes difficult counting.

Table II-4 Oligonucleotide probes for FISH

Probe	Labeling dye	Formamide	Sequence of probe (Accession number pB)[+]	target organism	Reference
BET-42a L-C-bProt-1027-a-A-17	Cy3	30	GCCTTCCCACTTCGTTT (pB-00034)	ß-proteobacteriën (23S rRNA gene)	(Manz et al. 1992)
Competitor for BET-42a Gam-42a L-C-gProt-1027-a-A-17	-	30	GCCTTCCCACATCGTTT (pB-00174)	γ-proteobacteriën (23S rRNA gene)	(Manz et al. 1992)
AMX-0820 S-*-Amx-0820-a-A-22	*FLUOS*	30	AAAACCCCTCTACTTAGTGCCC (pB-00409)	"*Ca.* Kuenenia stuttgartiensis", "*Ca.* Brocadia anammoxidans" (16S rRNA)	(Schmid et al. 2001)
NEU -0653 S-*-Nsm-0653-a-A-18	Cy3	30	CCCCTCTGCTGCACTCTA (pB-00382)	Most halophilic/tolerant *Nitrosomonas* spp. (16S rRNA)	(Wagner et al. 1995)
Competitor for NEU -0653 Cte	-	30	TTCCATCCCCCTCTGCCG (pB-00378)	*Comamonas* spp., Acidovorax spp., *Hydrogenophaga* sp., *Aquaspirillum* sp. (16S rRNA)	(Wagner et al. 1995)
AMX-0820 S-*-Amx-0820-a-A-22	*FLUOS*	30	AAAACCCCTCTACTTAGTGCCC (pB-00409)	"*Ca.* Kuenenia stuttgartiensis", "*Ca.* Brocadia anammoxidans" (16S rRNA)	(Schmid et al. 2001)

[+]Information about all probes can be found in probeBase.net (Loy et al. 2007)

2.8. Potential activity analyses

Activity assays were conducted in order to determine the potential activities of aerobic ammonia oxidizing bacteria (AerAOB) and anaerobic ammonium oxidizing bacteria (anammox). The assays were performed under aerobic and anaerobic conditions, respectively. Sludge (10 mL) was washed 3-5 times with tap water in order to remove residual substrates, mainly ammonia for AerAOB and ammonia and organic carbon for anammox bacteria. The last wash was performed using HEPES 20 mM as a buffer solution (pH 8). All incubations were performed at room temperature, pH 8 and under continuous mixing (150 rpm). Activity was determined by

measurement of the rates of depletion of ammonium and nitrite. To this end, measurements were performed as described below over a time span of 3h with a sampling interval of 30 min.

AerAOB activity assays. The washed SNAD biomass (10 mL) was transferred to 30 mL conical glass flasks covered with a wad of cotton wool. To measure the aerobic ammonium oxidation activity, the flasks were incubated aerobically, aerobic conditions were maintained under active mixing and substrate was added from a sterile 100 mM NH_4Cl stock solution to initial concentrations of 9-10 mM total ammonium nitrogen.

Anammox activity assays. The washed SNAD biomass (10 mL) was transferred to 30 mL glass serum bottles. Bottles were sealed with butyl rubber stoppers and soluble substrates added from sterile 100 mM stock solutions (NH_4Cl and $NaNO_2$). Directly hereafter, the bottles were made anoxic by alternatingly applying under-pressure and flushing with N_2 gas, as no growing effect was investigated. An overpressure of 1 bar was maintained in the bottles. Initial concentrations of 5 mM total ammonium nitrogen and 1-3 mM total nitrite nitrogen were used.

2.9. Analytical methods

To monitor the depletion of total ammonium and nitrite in the activity assays, samples (1 mL) were centrifuged (5 min, 10,000 x g) and the resulting supernatants analyzed. Ammonium, as total ammonium nitrogen (TAN), and nitrite, as total nitrite nitrogen (TNN), were measured colorimetrically at 420 nm (adapted from Taylor et al. (1974)) and 540 nm (adapted from Griess-Romijn van Eck (1996)), respectively. Total solids (TS) were quantified weighing samples after drying at 105 ˚C for 24 hours and subsequent cooling in a desiccator. The Biuret method was used to determine the protein concentration (Layne 1957).

3. Results

3.1 PCR and phylogenetic analysis

In previous studies on SNAD bioreactors, it has been confirmed that different bacteria can co-exist in biofilms (Xiao et al. 2009; Zhang et al. 2012), flocs and granules (Third et al. 2001; Vázquez-Padín et al. 2010), but further phylogenetic analysis are necessary. As described in the materials and methods section, genomic DNA extracted from the SNAD sludge from the Zürich wastewater treatment plant was used as a template for PCR reactions targeting respectively: bacterial *amoA* genes which are diagnostic for aerobic ammonia oxidizing bacteria (AerAOB), *nxrA* genes diagnostic for *Nitrobacter* spp. (AerNOB), *pmoA* genes diagnostic for methane-oxidizing bacteria and *nirS* genes diagnostic for denitrifying bacteria. In addition to functional gene targeting PCR reactions also 16S rRNA amplifications were performed using primers specific for *Nitrobacter* spp., *Nitrospira* spp., *Planctomycetes,* anammox and NC10 phylum bacteria were performed. PCR products were checked for size on 1% (w/v) agarose gel (Supplementary Fig. II-1).

Phylogenetic diversity of the AerAOB

Primers specific for subunit A of the ammonium monooxygenase (*amoA*) enzyme, key enzyme in aerobic ammonium oxidation, were used to detect AerAOB. Correct-sized products of 453 nt were obtained. After transformation and cloning, eight clones were randomly selected for plasmid DNA isolation and sequencing. Phylogenetic analysis of amino acid sequences deduced from the DNA sequences showed that the AerAOBs in the samples belonged to the betaproteobacteria and were affiliated to environmental clone sequences from

wastewater and other engineered nitrogen treatment systems. Clone sequences closely related to different members of the multiple *Nitrosomonas* genera (*N. europaea, N. eutropha* and *N. oligotroph*a) were detected, suggesting a high AOB species diversity (Fig. II.2). One clone SNAD_amoA_7 was closely related to *Nitrosomonas oligotropha* (AF272406, 92% identity), one clone SNAD_amoA_4 to *Nitrosomonas eutropha* (AY177932, 100% identity), and the other six clones were closely related to *Nitrosomonas europaea* (L08050, 97- 99% identity) (Fig. II.2).

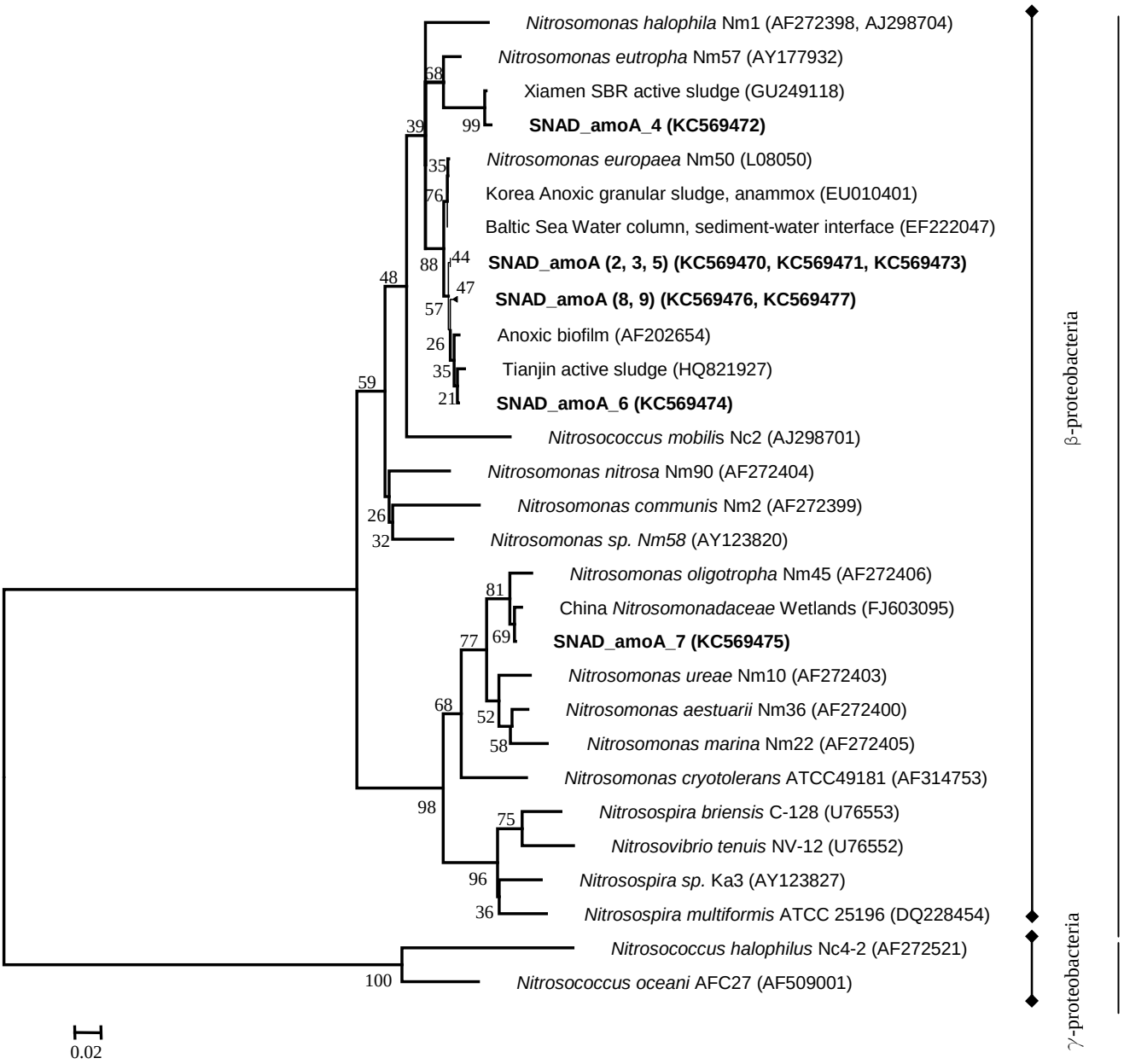

Fig. II.2 Phylogenetic tree, reflecting *amoA g*ene diversity in SNAD sludge.
Names of ammonia-oxidizing bacteria are printed in italics and names of clone sequences are printed in bold. The phylogenetic tree was constructed using the Neighbour – Joining method (Saitou and Nei 1987) with the Poisson correction model (Zuckerkandl and Pauling 1965). Distances are in the units of the number of amino acid substitutions per site. The analysis involved 32 amino acid sequences. Evolutionary analyses were conducted in MEGA.5 (Tamura et al. 2011). The numbers at the nodes indicate the levels of bootstrap support (based on data for 500 replicates) for groups to the right of the nodes. The scale bar represents 2% estimated sequence divergence.

Phylogenetic diversity of the anammox bacteria.

To determine the diversity of anammox bacteria, two different primer combinations were used. The *Planctomycete* 16S rRNA was amplified using *Pla46F* and *630R* primers and correct-sized (1483 nt) products cloned. Nine clones were randomly selected for sequencing and almost full-length 16S rRNA sequences (average length 1,490 bases) were obtained. Subsequent phylogenetic analysis showed that the clones were associated with the phylum *Planctomycetes* but none of the sequences were similar to the known anaerobic ammonium oxidizing (anammox) bacteria. When the anammox specific primer combination (*368F- 820R*) was applied, a clear PCR product of 452 bp was obtained. Six clones were randomly selected and sequenced. Phylogenetic analysis of the nucleotide sequences revealed that the clone sequences were highly similar to each other (more than 99 % sequence similarity), forming a cluster SNAD_Amx (4, 5, 9, 11, 20, 33). Bootstrap analysis confirmed that the cluster SNAD-Amx (4, 5, 9, 11, 20, 33) was closely related (99%) to the known anammox bacteria of the "*Candidatus* Brocadia fulgida" (bootstrap value, 99%) (Fig. II.3).

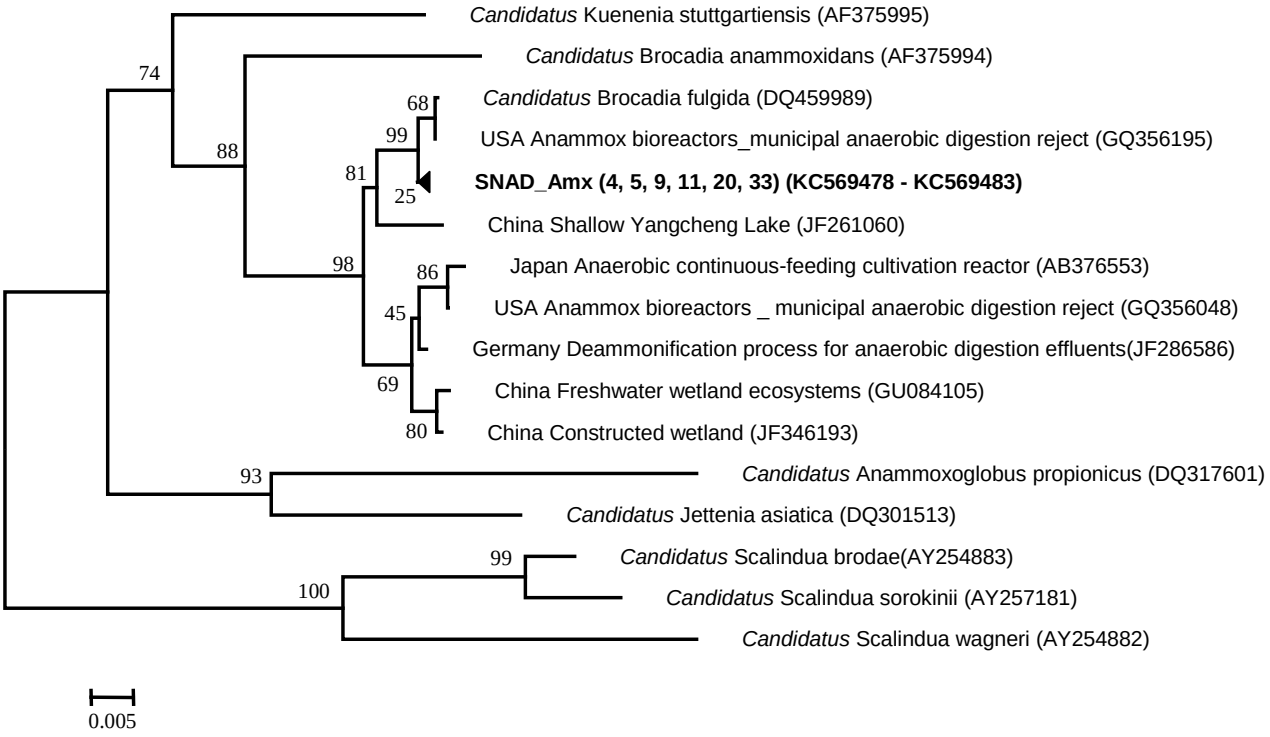

Fig. II.3 Phylogenetic tree, based on 16S rRNA gene sequences using anammox-specific primers
The tree shows the phylogenetic position of the clone sequences within the anammox bacterial genera. The clone sequences are in bold. Names of anammox species are printed in italics. The phylogenetic tree was constructed using the Neighbour – Joining method (Saitou and Nei 1987) with the Jukes-Cantor model (Jukes and Cantor 1969). The analysis involved 21 nucleotide sequences. Evolutionary analyses were conducted in MEGA.5 (Tamura et al. 2011). The numbers at the nodes indicate the levels of bootstrap support (based on data for 500 replicates) for groups to the right of the nodes. The scale bar represents 0.5% estimated sequence divergence.

Phylogenetic diversity of the denitrifiers

In addition to the aerobic ammonia oxidizing and anammox bacteria, the diversity of denitrifiers was assessed by using the dissimilatory nitrite reductase *(nirS)* gene as a proxy. Until now, no detailed studies, investigating the diversity of the functional *NirS* gene of heterotrophic bacteria in the SNAD sludge, have been carried out. The correct-sized (875 nt) amplificated were cloned and eight clones were randomly selected and further analyzed by comparative sequencing. Phylogenetic analysis of the deduced amino acid sequences showed that the denitrifiers in the SNAD sample belonged to the betasubdivision of the proteobacteria. The phylogenetic tree of nitrite reductase genes (*nirS*) is presented in Fig. II.4.

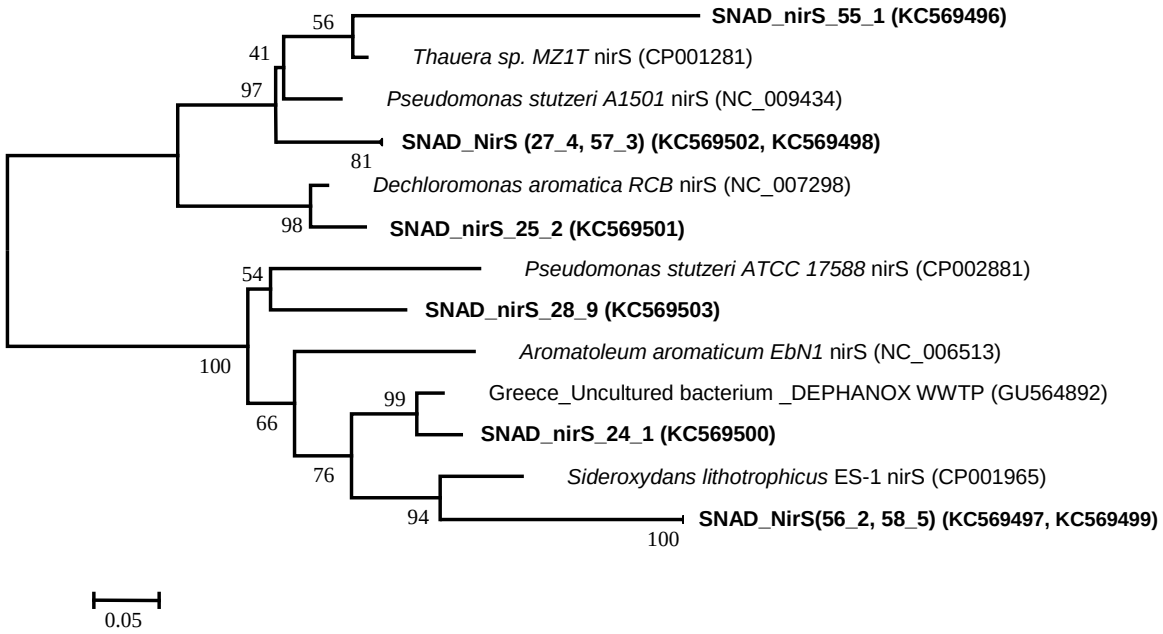

Fig. II.4 Phylogenetic tree, based on nitrite reductase (*Nir*S) functional gene sequences obtained with the *nirS1F/6R* primers.
Names of denitrifiers are printed in italics, while the *Nir*S clones are printed in bold. The phylogenetic tree was constructed using the Neighbour – Joining method (Saitou and Nei 1987) with the Dayhoff matrix based method (Schwarz and Dayhoff 1979). The analysis involved 14 amino acid sequences. Evolutionary analyses were conducted in MEGA.5 (Tamura et al. 2011). The numbers at the nodes indicate the levels of bootstrap support (based on data for 500 replicates) for groups to the right of the nodes. The scale bar represents 5% estimated sequence divergence.

Phylogenetic diversity of the AerNOB

The AerNOB diversity was analyzed by using multiple primer sets. The primer combination *Nitro1198F/Nitro1423R,* targeting the 16S rRNA genes of *Nitrobacter*-like bacteria (Graham et al. 2007), yielded products of the correct size (225 nt), while no PCR products were obtained using primers targeting the *NxrA* gene of *Nitrobacter* spp., nor the 16S rRNA gene of *Nitrospira* spp.. Although recent metagenomic-based studies (Daims et al. 2011) indicated that *Nitrospira*-like bacteria are more abundant AerNOB in sewage treatment systems, our PCR analysis showed that in our SNAD biomass only *Nitrobacter* spp. could be detected. The PCR targeting the 16S rRNA of *Nitrospira* spp. did not yield any specific products. A 16S rRNA gene clone library was constructed from the aggregate samples to identify the nitrite oxidizers in SNAD sludge. Twelve clones were selected at random and used for comparative sequence analysis. Phylogenetic analysis of the nucleotide sequences showed that the nitrite oxidizers belonged to the alphasubdivision of the proteobacteria (Fig. II.5). Five clone sequences SNAD_16SrRNA_NOB_1_1, SNAD_16SrRNA_NOB1_3 and a cluster of sequences SNAD_16SrRNA_NOB(1_2, 1_4 and 1_7) were related (98% identity) to *Nitrobacter hamburgensis*. Five of the remaining clone sequences (SNAD_16SrRNA_NOB(1_5, 2_5), SNAD_16SrRNA_NOB(1_6, 1_9,

2_2) and SNAD_16SrRNA_NOB_1_8) were closely related to non–nitrifying bacteria of the alphasubclass: *Bradyrhizobium* spp. *Shinshu-th2* (Hamaki et al. 2005) and *Rhodoblastus acidophilus* (Kempher and Madigan 2012). The remaining partial sequence (SNAD_16SrRNA_2_3) was related to the 16S rRNA gene of a non–nitrifying bacterium (*Myxococcales*) of the deltasubclass of proteobacteria.

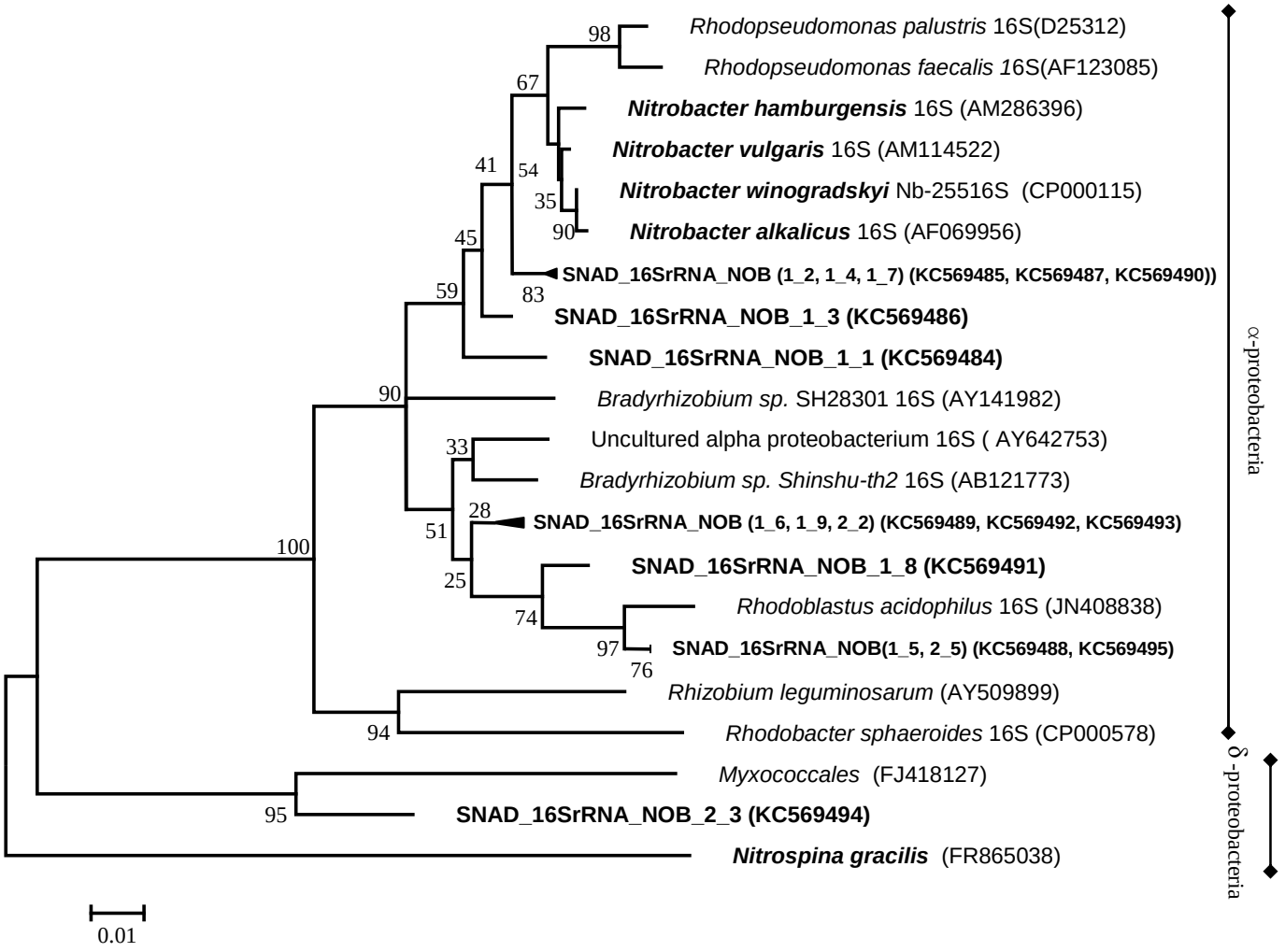

Fig. II.5 Phylogenetic tree, based on 16S rRNA gene sequences of aerobic nitrite oxidizing bacteria.
The tree shows the relationship of the clone sequences within the α – and σ - protobacteria, including both nitrite-oxidizing and non-nitrifying bacteria. Names of nitrite oxidizers are printed in bold and italics while clone sequences are in bold. The phylogenetic tree was constructed using the Neighbour – Joining method (Saitou and Nei 1987) while the distances were computed using the Jukes-Cantor method (Jukes and Cantor 1969). The analysis involved 26 nucleotide sequences. Evolutionary analyses were conducted in MEGA.5 (Tamura et al. 2011). The numbers at the nodes indicate the levels of bootstrap support (based on data for 500 replicates) for groups to the right of the nodes. The scale bar represents 1% estimated sequence divergence.

Detection n-damo bacteria in SNAD sludge

As dissolved methane from anaerobic digester effluent can be carried over to the SNAD reactor, we also investigated the presence of the nitrite - dependent anaerobic methane oxidation bacteria (n-damo) bacteria. In order to detect "*Ca*. Methylomirabilis oxyfera", first a direct PCR with *pmoA* primers was applied. PCR products were not of the expected size and therefore not further analyzed for sequencing. The results indicate that either the amount of n-damo bacteria in the SNAD sludge were too low for PCR detection or that the primer combination used for the direct PCR was not targeting the n-damo bacteria present in the sludge (Luesken et al. 2011a). The nested PCR approach and the sequences retrieved with this did not yield any n-damo bacterial sequences either. The clones clustered with an uncultured alpha proteobacterium from environmental DNA

sampled from oligotrophic environments (obtained from 15mdepth in the Northern Atlantic Gyre) (AM706683 (Mühling et al. 2008) (100% identity).

3.2 Fluorescence in situ hybridization (FISH)

To investigate the microbial spatial distribution of bacteria, and in particular the community structure of two of the main processes (aerobic and anaerobic ammonia oxidation) in the SNAD system, qualitative FISH measurements with specific probes for *Nitrosomonas* spp. and "*Candidatus* Brocadia anammoxidans" were performed. The presence of both aerobic ammonia-oxidizing and anammox cells was confirmed in these analyses. FISH analysis applied to the whole biomass, containing both granules and flocs, gave a high background signal. However, a stratification in the granules was observed with a clear division of different bacteria in the inner (anammox bacteria) and outer layers (AerAOB). (Supplementary Fig. II-2 A,B,C). Smaller aggregates and single cells exhibited less background and therefore clearer results (Supplementary Fig. II-2 D). A distinct distribution of aerobic ammonia-oxidizing bacteria (outer layers) and anaerobic ammonium-oxidizing bacteria (inner layers) within the aggregates was observed (Fig. II.6, A and B). While, the aerobic ammonia oxidizers were located at the surface layer of the aggregate, where oxygen is expected to be available, anammox bacteria tended to occupy the inner (anoxic) zones of the aggregates, probably to avoid oxygen exposure which is reported to be inhibitory to anammox activity. Pictures taken of DAPI-stained SNAD sludge showed a high morphological diversity of the microorganisms which included spherical-, rod- and long filamentous-shaped microorganisms (Supplementary Fig. II-3 A). In particular, long filamentous-shaped microorganisms were the dominant bacteria in the outer layer of granules, while single cells as well as aggregates appeared fixed in a net of filamentous bacteria.

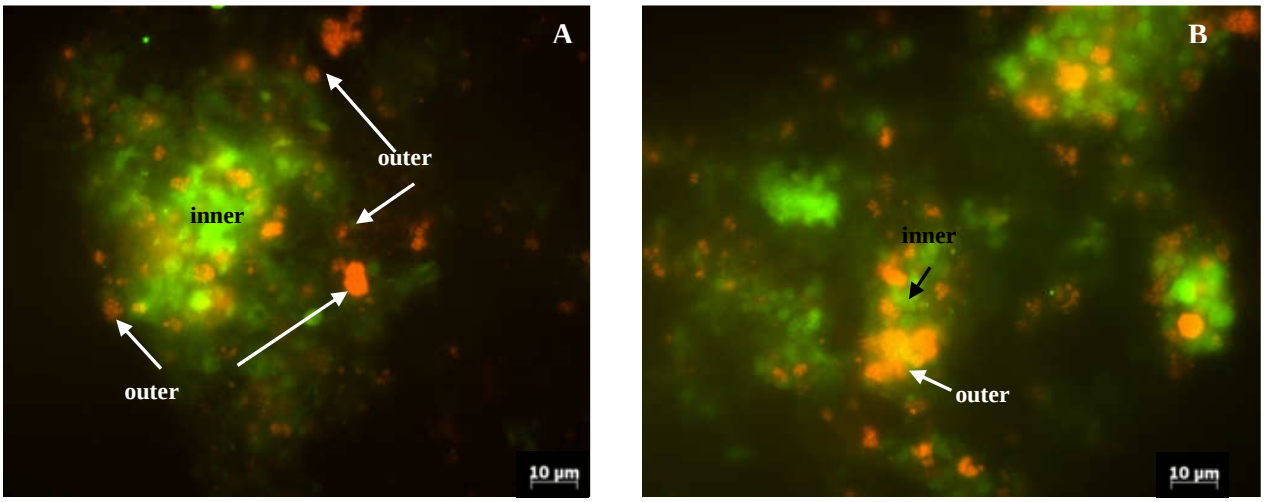

Fig. II.6 Fluorescence images of homogenized SNAD biomass.
Nitrosomonas spp. were labeled with Cy3 (red) (probe Neu 653, targeting most halophilic/tolerant *Nitrosomonas* spp.) while anammox bacteria were labeled with FLOUS (GREEN) (Probe AMX820, targeting "*Candidatus* Brocadia anammoxidans"). Scale bar: 10 um.

3.3 AerAOB and anammox activity assays

In the Sequencing Batch Reactor (SBR), treating the anaerobic digested effluent at the wastewater treatment plant in Zürich (Switzerland), the SNAD process was full achieved (Joss et al. 2009). Data reported by Joss et al. (2009) demonstrated that total ammonium nitrogen (95,4%), total nitrogen (94,6 %) and COD removal (36,7 %) were achieved simultaneously in the SBR. Our activity assays confirmed that both aerobic and anaerobic ammonia oxidation occurred in the SNAD sludge (Fig. II.7 A and B).

The total protein concentration and total solids content of the SNAD sludge were determined prior to the activity assays as described in the analytical methods section. The protein concentration was about 2.85 mg protein mL^{-1}, while the total solids content was 10 g TSS L^{-1}. This showed that the protein concentration was approximately a third of the measured dry weight (0.3 g protein/g biomass), similarly to the data previously reported for a CANON biomass (Third et al. 2001).

The potential aerobic ammonia oxidizer activity was 1.8 nmol N mg $protein^{-1}$ min^{-1}, corresponding to about 4.3 mg TAN $gTSS^{-1}$ h^{-1}. The molar ratio of $TNN_{produced}/TAN_{consumed}$ was about 0.9, in accordance to the nitritation stoichiometry performed by AerAOB (1.0), demonstrating a low activity of nitrite oxidizing bacteria (AerNOB). The potential anammox activity was 0.34 nmol N mg $protein^{-1}$ min^{-1}, corresponding to 0.82 mg TAN$gTSS^{-1}$ h^{-1}. The molar ratio of $TNN_{consumed}/TAN_{consumed}$ was calculated to be 1.7. This value was higher than the theoretical anammox stoichiometry (1.32), indicating that part of the nitrite is converted by heterotrophic denitrifiers (Kumar and Lin 2010) or anammox bacteria (Kartal et al. 2007b; Kartal 2008) which use organic carbon substrates present in the sludge, mainly cell lysis products.

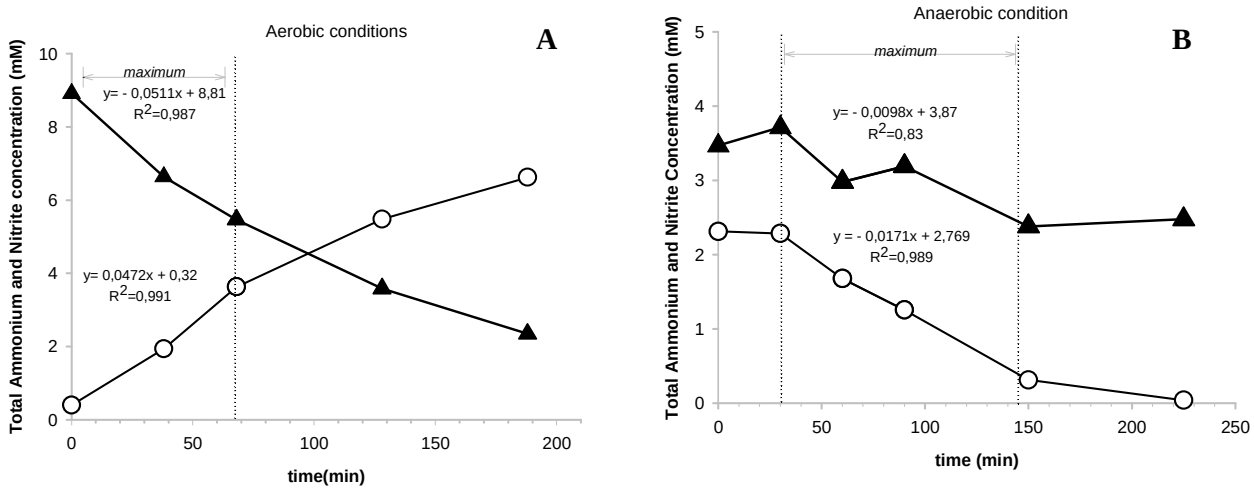

Fig. II.7 Potential AOB and anammox activity
Potential aerobic ammonia-oxidizing activity (A) and potential anaerobic ammonia-oxidizing activity (B) in the SNAD sludge. Concentration of total ammonium (▲) and nitrite (○) in batch tests.

4. Discussion

4.1 Microbial community of the SNAD biomass

In this study an analysis of the microbial community of the SNAD biomass was performed, using molecular tools to detect the bacteria involved in the biological process. In the SNAD system, ammonium and carbon removal is achieved by the concerted action of aerobic ammonia-oxidizing, anammox and denitrifying bacteria under oxygen limitation. We showed that the SNAD sludge of the SBR of the WWTP of Zürich (CH) did contain different bacteria (aerobic and anaerobic ammonia oxidizers, aerobic nitrite oxidizers, denitrifiers). The SNAD sludge harbored a high microbial diversity. Both the main (aerobic and anaerobic ammonium oxidation and denitrification), and possible secondary biological processes (aerobic nitrite oxidation and n-damo) were assessed.

Our results showed that different members of *Nitrosomonas* genera coexisted in the SNAD sludge. As expected on basis of the high total ammonium load (0.3 – 1.1 kgTAN m^{-3} d^{-1}) of the SBR, *amoA* gene sequences were affiliated with those of the ammonia oxidizers of the genus *Nitrosomonas europaea* (97-98% similarity). Clone sequences were highly similar to environmental sequences previously recovered in an anoxic granular sludge (EU010401), an anoxic biofilm (AF202654 (Schmid et al. 2000)) and an activated sludge, treating ammonium rich wastewater (HQ821927). Similar results were observed by Pynaert et al. (2003) in a highly loaded Rotating Biological Contactor (RBC). We observed one sequence belonged to the *Nitrosomonas-eutropha*-like cluster (SNAD_AmoA_4), usually detected and demonstrated to have activity in oxygen-limited environments (Li et al. 2009). Both *Nitrosomonas europea* and *Nitrosomonas eutropha* are capable of tolerating high-ammonia concentration (Holmes et al. 1995; Szatkowska et al. 2007a). The sequence SNAD_AmoA_7 was distantly related to all of the previously recognized sequences and closely linked to the *Nitrosomonas-oligotroph*a-like cluster, which have been found usually in pools with low ammonia/ammonium concentration (Park et al. 2002). The clone sequence was extremely similar to environmental sequences previously recovered in a constructed wetland for wastewater treatment (FJ603095). Our results supported the hypothesis that in the SNAD process, carried out in an SBR, different macro- and micro-environments strongly affected the AerAOB population structure. The different aerobic and anoxic phases and the presence of dense biomass-flocs and granules can create physical and chemical gradients of substrates. The high ammonia concentration in the bulk liquid is a critical factor for the involvement of *Nitrosomonas europea* and *Nitrosomonas eutropha* relatives in the flocs and in the outer part of granules, while the low ammonia concentration in the inner part of granules might allow the development of *Nitrosomonas-oligotropha*-spp. A high AOB species diversity may be beneficial to the stability of wastewater treatments (Daims et al. 2001) and in particular for the SNAD process.

Phylogenetic analysis revealed that all the clone sequences obtained from the anammox 16S rRNA analysis were highly similar to eachother and affiliated with the known anammox bacteria of the "*Candidatus* Brocadia fulgida" (99% similarity). The cluster was most closely related to clones retrieved from a USA anammox reactor, treating anaerobic digestion effluents (GQ356195, (Park et al. 2010)). Our results were in line with the previous studies that found "*Ca.* Brocadia" as the abundant anammox species in WWTPs, where organic acids are present (Hu et al. 2010). *Brocadia*-like bacteria are able to oxidize organic substrates to CO_2 with the reduction of nitrate and nitrite to di-nitrogen gas (Kartal et al. 2007b; Kartal 2008).

The amplification of the nitrite reductase gene (*nirS*) fragments revealed that the SNAD biomass was characterized by a high diversity of denitrifiers of the betasubdivision of the proteobacteria. Phylogenetic

analysis showed that the clone sequences were related to the *nirS* sequences from different anaerobic denitrifying bacteria, *Thauera* spp., *Pseudomonas stutzeri*, *Dechloromonas aromatic* and *Aromatoleum aromaticum*. The presence of so many denitrifying bacteria indicated that denitrification may have an important role in nitrogen removal from high nitrogen streams which contain organic substrate. Among the denitrifiers here identified, *Thauera* MZ1T, *Pseudomonas stutzeri* and *Dechloromonas aromatica* have been shown to degrade several aromatic compounds (Yan et al. 2005; Heylen et al. 2006; Salinero et al. 2009). *Thauera* MZ1T is unique among the *Thauera* spp. in its production of abundant extracellular polymeric substances (EPS) which may contributes to the granular process (Heylen et al. 2006), while *Dechloromonas aromatica* strain RCB appears to support a highly complex lifestyle which might involve biofilm/granules formation and interaction with a eukaryotic host (Salinero et al. 2009). Both *Pseudomonas stutzeri* and *Dechloromonas aromatica* encode proteins, suggestive of the ability to fix nitrogen and CO_2 (Yan et al. 2005; Salinero et al. 2009). The sequence SNAD_nirS 24 was closely related to clones from biological anoxic phosphorus removal reactor (DEPHANOX-type) fed with municipal wastewater, where denitrifying polyphosphate accumulating organisms (DPAOs) have been observed (Zafiriadis et al. 2011). This observation is probably mirrored by the fact that in the SBR anaerobic/anoxic and aerobic phases alternate, favoring phosphorus release/uptake. Finally, the cluster SNAD_NirS (56.2, 58.5) was 88% similar to the lithoautotrophic, microaerophilic Fe(II)-oxidizing betaproteobacterium *Sideroxydans lithotrophicus* ES-1, which may play a role for Fe(II) oxidation in the enrichment culture (Blöthe and Roden 2009).

Bacteria involved in the possible secondary biological processes such as aerobic nitrite oxidizing and n-damo bacteria were investigated. Although recent metagenomic-based studies (Daims et al. 2011) indicated that *Nitrospira*-like bacteria are more abundant AerNOB in sewage treatment systems, our PCR analysis showed that *Nitrobacter*-like bacteria, rather than *Nitrospira* spp., were the dominant AerNOB in the SNAD biomass. Those finding are in agreement with results of previous studies, where significant amounts of *Nitrobacter* spp. have been detected by FISH in high-loaded WWTPs (Mobarry et al. 1996; Gieseke et al. 2003). The PCR, targeting the 16S rRNA gene of *Nitrospira* spp., did not yield any specific products, which may indicate either that the *Nitrospira* spp. cell numbers are too low for PCR detection or that a *Nitrospira* species not targeted by the primer set are present. The 16S rRNA sequences of two of the twelve clones (SNAD_16SrRNA_NOB_1_1), (SNAD_16SrRNA_NOB_1_3) and the cluster SNAD_16SrRNA_NOB(1_2, 1_4,1_7) were related to the members of nitrite oxidizers of the *Nitrobacter hamburgensis* (98% identity). In addition, in our study, some sequences related to *Nitrobacter* spp. closest non-nitrifying relatives, *Bradyrhizobium sp. Shinshu-th2* (Hamaki et al. 2005) and *Rhodoblastus acidophilus* (Kempher and Madigan 2012), were detected. The detection of non-nitrifying relatives may be explained considering both the low phylogenetic diversity of *Nitrobacter* spp. (Daims et al. 2011) and their high 16S rRNA similarities to their closest non-nitrifying relatives. Further, recent research reported the ability of other non-nitrifying bacteria (purple non-sulfur bacteria (strain LQ17)) related to the *Rhodopseudomonas* spp. to use nitrite phototrophically as an electron donor as well (Schott et al. 2010). Some studies reported that the 16S rRNA method seems to be too conserved to unequivocally determine evolutionary lineage within the genus *Nitrobacter* (Vanparys et al. 2007; Daims et al. 2011). However the presence of AerNOB does not seem to compromise the SNAD process efficiency, probably due to the selective pressures in the SBR management (low dissolved oxygen, high ammonia concentration) and consequently to the low AerNOB activity, as also confirmed by the AerAOB activity assays.

As dissolved methane from anaerobic digester effluent can be carried over to the SNAD reactor in the Zürich WWTP, the nitrite - dependent anaerobic methane oxidation bacteria (n-damo) bacteria were also screened in our study, using both the 16S rRNA and the functional level approach (Luesken et al. 2011b). No n-damo bacterial amplificates were obtained suggesting the absence of these microorganisms in the SNAD sludge. Further analysis would be required to definitively prove the absence of n-damo bacteria in the SNAD sludge.

4.2 Microbial spatial distribution in the SNAD biomass

Our FISH results correspond to the experimental findings of other researchers (Guo et al. 2009; Cho et al. 2010; Wang et al. 2011b), who revealed that long filamentous- shaped microorganisms were dominant bacteria in the outer layer of granules, while single cells as well as aggregates were trapped in a net of filamentous bacteria. Filamentous microorganisms are considered to be responsible for the construction of web-like structures (Li et al. 2008) and were also detected in some anammox reactors co-existing with the AerAOB (Li et al. 2009; Park et al. 2010; Zhu et al. 2011). We delved deeper into the spatial distribution of microorganisms responsible for two of the main biological processes (aerobic and anaerobic ammonia oxidation) involved in the SNAD process by FISH, with specific probes for *Nitrosomonas* spp. and "*Candidatus* Brocadia anammoxidans" and hereby confirmed the presence of both aerobic ammonia-oxidizing- and anammox cells. Furthermore, this revealed that a distinct microbial distribution from the outer to the inner layer of aggregates existed, as was observed in previous studies by other researchers (Gong et al. 2007; Vázquez-Padín et al. 2009c, 2010; Vlaeminck et al. 2010). Although anammox bacteria are sensitive to oxygen, our visual estimations, based on FISH pictures, demonstrated that nitrifying and anammox bacteria co-existed in the SNAD reactor, which was operated at micro-aerobic (oxygen-limited) conditions. This suggests that the aerobic ammonia oxidizers are able to effectively protect the anammox bacteria from inhibiting substances such as oxygen. In an anammox granular reactor, Li et al. (2011b) observed that in the outer layer of a granule, the biomass was mostly micrococcus shaped, presumably aerobic ammonia-oxidizing bacteria. In the inner layer of a granule, the biomass consisted of typical cauliflower-like aggregates, presumably anammox organisms. Anammox bacteria tends to form aggregates like biofilm (Gong et al. 2007; Szatkowska et al. 2007a; Zhang et al. 2010b) or granules (Quan et al. 2008; Ni et al. 2009; Li et al. 2011b; Volcke et al. 2012; Winkler et al. 2012a). Recently, Chen et al. (2010) confirmed the complex anammox granules morphology. The authors detected lots of cavities in the granules, which can enhance substrate transfer from the bulk to granules and intermediates or by-products transfer from inside granules to the bulk, allowing the co-existence of partial nitrification and anammox process. Moreover, the biofilm of non-woven rotating biological contactor (NRBC) reactor, performing the SNAD process, was characterized by a outer layer of biofilm, where the biomass was mostly micrococcus shaped, apparently AerAOB, with a spot of aerobic heterotrophic bacteria. In the inner layer of biofilm, anammox organisms were detected, but presumably denitrifying organisms were also present (Chen et al. 2009). The identity of the aerobic nitrite oxidizers and denitrifiers in our SNAD sludge has not yet been investigated through FISH analysis in this study. *Nitrobacter* spp., *Nitrospira* spp. and denitrifiers were not specifically targeted in our approach. PCR analysis did demonstrate the presence of *Nitrobacter* spp. nitrite oxidizers as well as denitrifiers. *Nitrospira* spp. targeting PCR did not yield any specific products, which may indicate that either the *Nitrospira* spp. cell numbers are too low for PCR detection or that a *Nitrospira* species not targeted by the primers used in this study are present. The SNAD sludge was characterized by the presence of both brown and red granules. De Clippeleir et al. (2009), in their experimental study, verified that brown granules were dominated by aerobic ammonia-

oxidizing bacteria (AerAOB), resulting in a high nitrite accumulation compared to red granules, where the net nitrite production by AerAOB and the net anoxic nitrite consumption by anammox bacteria were in equilibrium. Moreover, when a carbon source is present in the system, aerobic heterotrophic bacteria, performing aerobic carbon oxidation and denitrification via nitrite or nitrate, can contribute to the formation of different types of aggregates with different characteristics. Further research is necessary to study the microbial diversity of different types of granules. Finally, the presence of compact aggregates in the sludge made it difficult to determine the absolute quantity of AerAOB and anammox bacteria in the SNAD biomass with FISH microscopy. Enumeration of both aerobic ammonia-oxidizing and anammox bacteria by quantitative real-time PCR (qPCR) may be of use.

4.3 Activity analyses

The activity analyses performed on aerobic and anaerobic ammonia-oxidizing bacteria showed that these microorganisms coexist. Even with the activity assays designed and optimized to measure aerobic and anaerobic ammonia potential rate only, the effects of other biological processes were detected. The molar ratio of $TNN_{produced}/TAN_{consumed}$ of aerobic ammonia oxidation was about 0.9, in accordance to the nitritation stoichiometry performed by AerAOB, demonstrating the low activity of nitrite-oxidizing bacteria. On the other hand, the molar ratio of $TNN_{consumed}/TAN_{consumed}$ of anaerobic ammonia oxidation was calculated to be 1.7, higher than the theoretical anammox stoichiometry (1.32). This indicates that part of the nitrite is consumed faster than ammonium. Nitrite may be converted by other pathways, that involve carbon removal. In fact, carbon was clearly present in the batch test due to the starvation conditions of the analyzed sample. Heterotrophic denitrifiers could use organic carbon substrates present in the sludge (Kumar and Lin 2010). Further, the anammox process contribution should be take into account, as anammox bacteria may oxidize organic carbon to CO_2, reducing nitrate/nitrite to di-nitrogen gas (Kartal et al. 2007b; Kartal 2008). The anammox process contributed up to 76% of total nitrite consumption in the anammox activity assay while the other processes overall account for 24%. The aerobic and anaerobic ammonia oxidizer activities measured for the SNAD sludge were low compared to previously reported rates for CANON or SNAD systems (Strous et al. 1998; Third et al. 2001; Sliekers et al. 2002; Xu et al. 2010). The lower aerobic and anaerobic ammonia oxidizer activities observed in this study may be explained by the fact that the SNAD sludge was stored for 5 months at 4°C prior to analysis.

5. Conclusions

In conclusion, the data in this study gave insights into the phylogenetic diversity and relationships in the SNAD microbial community, providing a clear evidence for a high species diversity. The findings reported in this study may be of relevance for wastewater treatments. The identification of specific groups of bacteria may contribute to better understanding design, and enable stable operation of complicated but challenging biological processes, where different bacteria may co-exist, such as the SNAD process for the treatment of ammonium-rich wastewaters, with a low C/N ratio. Further research to evaluate the diversity of heterotrophic denitrifiers in SNAD systems responsible for the COD reduction, may use different functional genes or enzymes involved in denitrification as molecular markers looking at both the nitrite and nitrate reduction routes. The n-damo bacteria were not detected in our current study but further research, e.g by designing and using less specific primers, is needed to conclusively validate the absence of these bacteria. Moreover, other possible secondary biological processes may be investigated, such as the presence of the sulfate reducing bacteria (SRB) which reduce sulfate (SO_4^{2-}) to hydrogen sulfide (H_2S) using organic compounds as electron donors and the sulfide-oxidizing autotrophic denitrifiers (SOAD) which may utilize the produced dissolved sulfide with oxidized nitrogen compounds (Lau et al. 2006). In addition, the quantification of bacteria in the granules and flocs and the detection of the active fraction (mRNA) of the community for monitoring the activity in the SNAD process could be useful tools to control the process in WWTPs and require future researches.

Acknowledgements

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Author Contributions

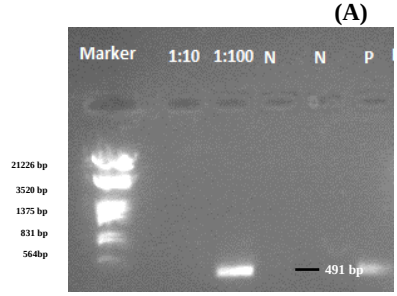
All authors contributed extensively to the work presented in this paper. Phylogenetic analysis were conducted by M.L and J.Y. H.J.M.O.d.C. provided support with alignments and trees. Experiments for microbial activity were designed by J.Y., S.C.M.H. and M.L. M.S.M.J. an G.A. conceived the research. M.L. wrote the paper with input from all other authors, discussing the results, implications and commenting on the manuscript at all stages.

Supplementary Material

Further details concerning the PCR products and FISH analysis as well as nucleotide and amino acid sequences.

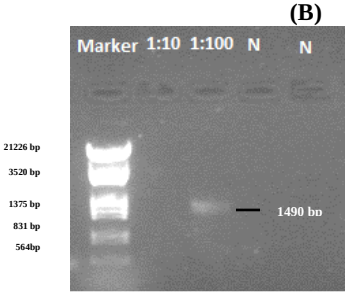
Supplementary Figures

(A)



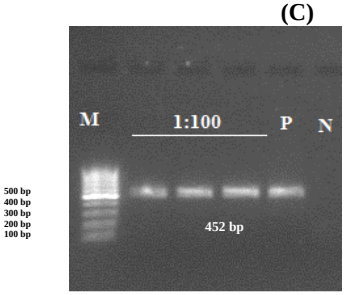
AOB
AmoA-1F/AmoA-2R
AmoA
(A) PCR amplification of a 491-bp fragment of the *amoA* gene DNA extracted from an aerobic ammonia-oxidizing cultures served as a template for the positive control.

(B)



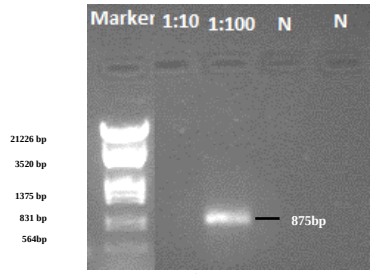
Planctomycete spp.
Pla46F/369R
16S RNA
(B) PCR amplification of a 1490-bp fragment of the 16S rRNA gene of *Planctomycete* bacteria. *C.* Brocadia anammoxidans DNA was used for the positive control.

(C)



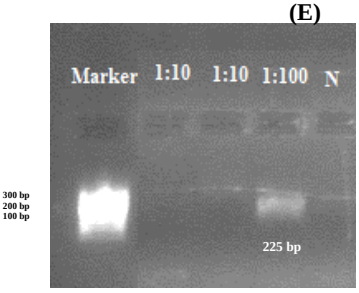
Anammox bacteria specific
368F/820R
16S RNA
(C) PCR amplification of a 452-bp fragment of the 16S rRNA gene of anammox bacteria. *C.* Brocadia anammoxidans DNA was used for the positive control.

(D)



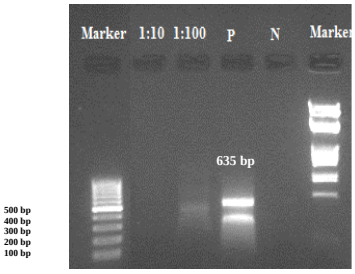
Denitrifiers
Nirs 1F/Nirs 6R
nitrite reductase gene
(D) PCR amplification of a 875-bp fragment of the *nir*S gene of denitrifiers.DNA froman aerobic ammonia-oxidizing culture was used for the negative control.

(E)



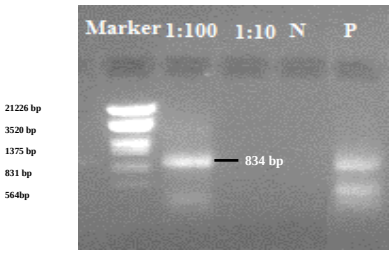
NOB
1198F/1423R
16S rRNA *Nitrobacter* spp.
(E) PCR amplification of a 225-bp fragment of the 16S rRNA gene of *Nitrobacter* spp.

(F)



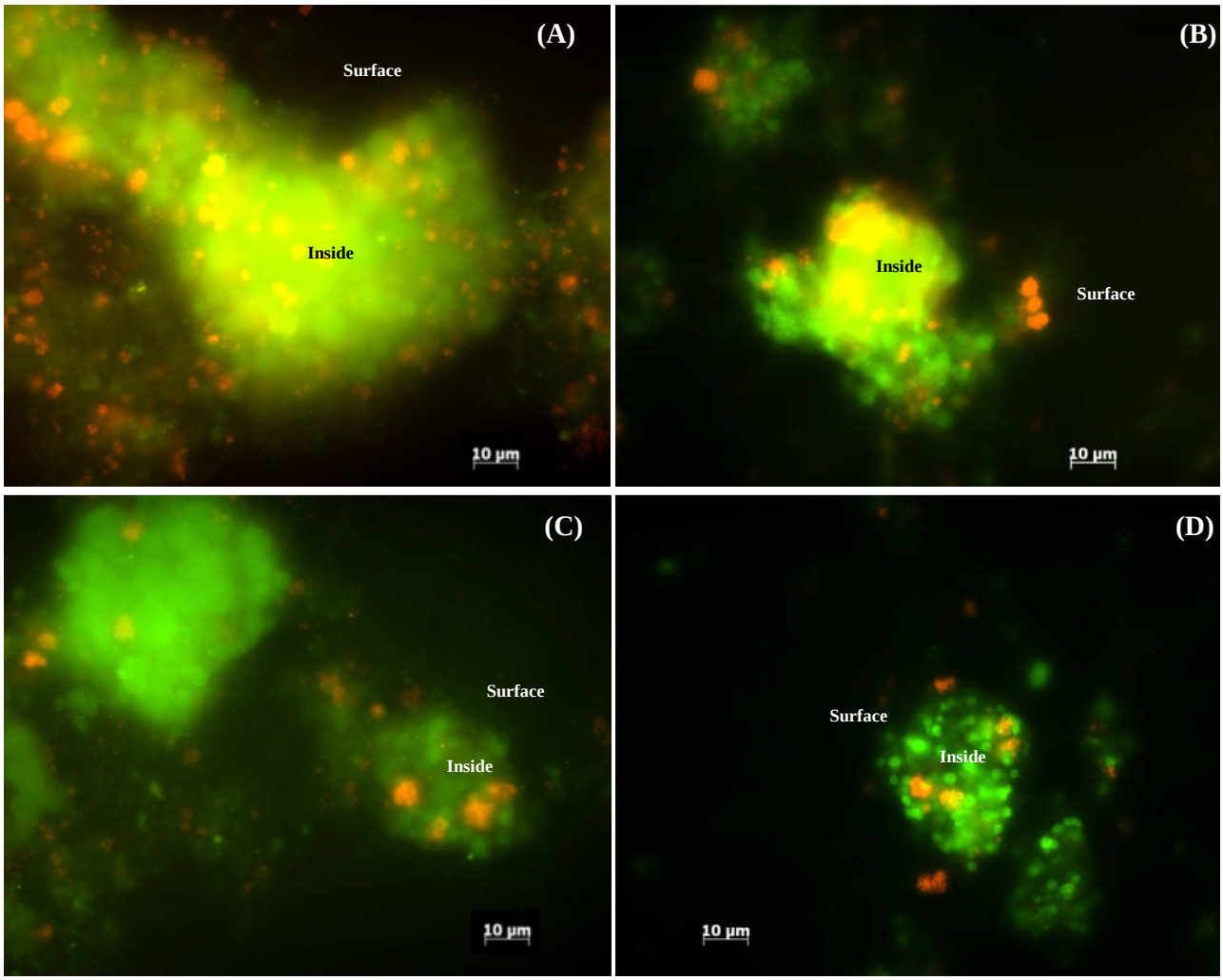
n-damo
A47F/A682R
pmoA gene
(F) PCR amplification of a 635-bp fragment of the *pmo*A gene. DNA from an n-damo culture served as the template for the positive control.

(G)

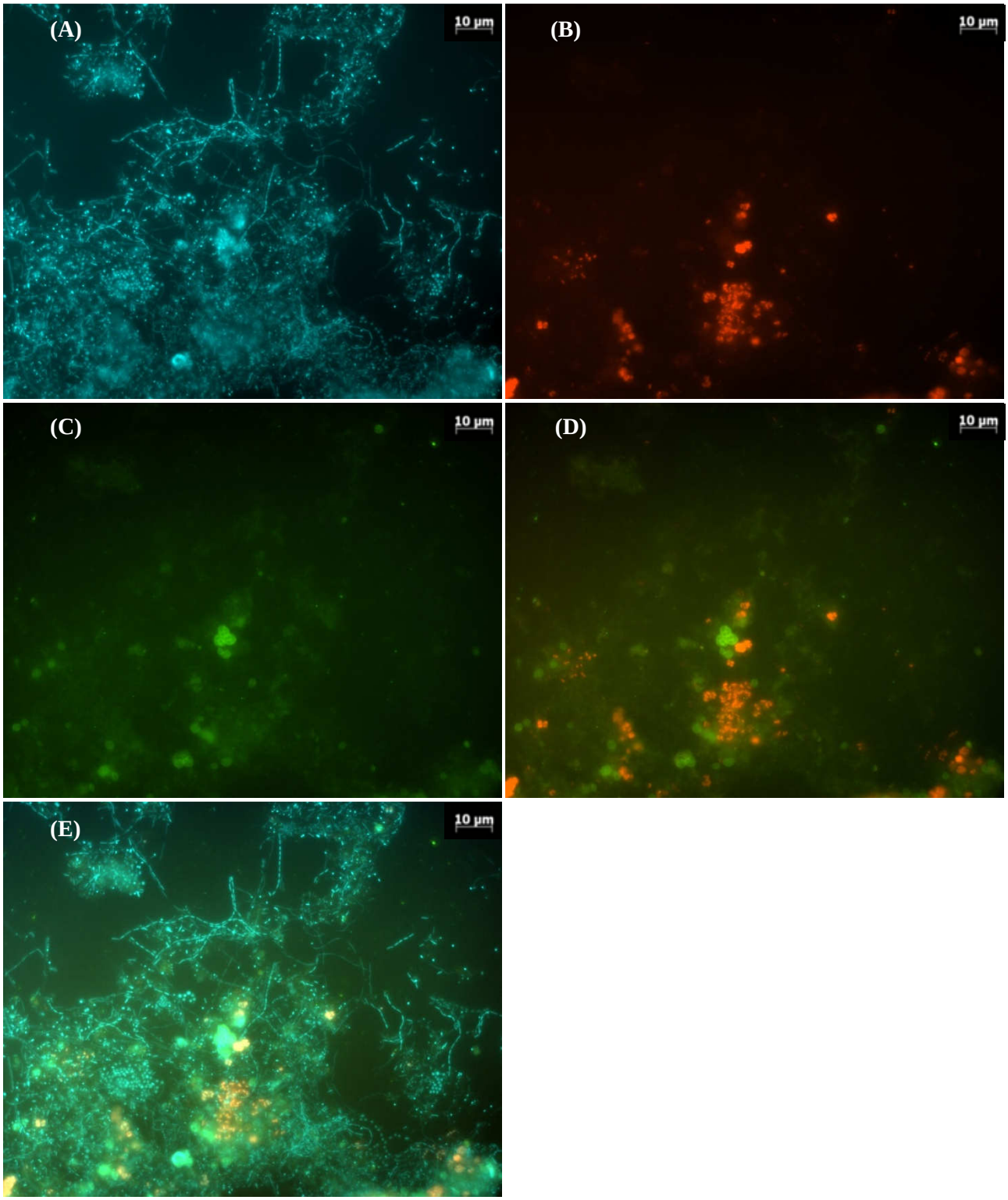


n-damo
202F/1543R
16S rRNA *"NC10" phylum*
(G) PCR amplification of a 834-bp fragment of the 16S rRNA gene of '*NC10*' phylum bacteria. DNA from an n-damo culture served as the template for the positive control

Supplementary Fig. II-1 Results of 1% agarose gel electroforesis of PCR amplificates.
1:10 and 1:100 indicates how much the template DNA extracted from the SNAD sludge was diluted prior to use in PCR. N and P indicate negative (no DNA) and positive control reactions. Lambda DNA/EcoRI+HindIII Marker (A, B, D, G) and Gene Ruler TM 100bp+DNA Ladder (C, E, F).



Supplementary Fig. II-2 Fluorescence microscopy images of SNAD sludge.
Nitrosomonas spp. were labeled with Cy3 (red) (probe Neu 653, targeting most halophilic/tolerant *Nitrosomonas* spp.) while anammox bacteria were labeled with FLUOS (green) (Probe AMX820, targeting "*Candidatus* Brocadia anammoxidans" and "*Candidatus* Kuenenia stuttgartiensis"). (A,B). SNAD Biomass with entire granules; C). Biomass with entire granules; (D) broken SNAD biomass with a small aggregate. A to D were pictures from different locations of the sample. Scale bar: 10 µm.



Supplementary Fig. II-3 SNAD biomass stained with DAPI and labeled with different specific probes.
(A) all DNA stained with DAPI (cyan). (B) *Nitrosomonas* spp. visualized using Cy3 (red) labeled probe Neu653. (C) anammox bacteria visualized using Fluos (green) labeled probe AMX820. In picture D the probe signal of Neu653 and AMX820 were combined, and in picture E both probe signals are combined with the DAPI stain. Scale bar: 10 µm.

Supplementary data: nucleotide and amino acid sequences.

Aerobic Ammonia oxidizing bacteria

Nucleotide Sequences

>SNAD_amoA_2 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_amoA_2] [environmental_sample=TRUE] AmoA SBR clone SNAD_amoA_2 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTGGACTTCAC
GCTGTATCTGACACGCAACTGGCTGGTGACGGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATTT
TTGGACCAACCCATTTGCCAATCGTTGTAGAAGGCACATTGCTGTCGATGGCTGATTACATGGGACATCTGTATGTTCGTACAGGTACA
CCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGT
ATCAATGTTGATGTTCACCGTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTATCG
TACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGCTTTCCCGAGGGGAATCA

>SNAD_amoA_3 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_amoA_3] [environmental_sample=TRUE] AmoA SBR clone SNAD_amoA_3 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTGGACTTCAC
GCTGTATCTGACACGCAACTGGCTGGTGACGGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATTT
TTGGACCAACCCATTTGCCAATCGTTGTAGAAGGCACATTGCTGTCGATGGCTGATTACATGGGACATCTGTATGTTCGTACAGGTACA
CCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGT
ATCAATGTTGATGTTCACCGTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTATCG
TACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGCTTTCCCGAGGGGAATCA

>SNAD_amoA_4 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_amoA_4] [environmental_sample=TRUE] AmoA SBR clone SNAD_amoA_4 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACATTACCCGCTTAACTTTGTAACACCGGGTACAATGCTCCCAGGCGCACTGATGCTGGACTTCAC
AATGTACCTGACACGCAACTGGCTGGTAACAGCACTGGTTGGTGGTGCATTCTTTGGTCTGTTGTTCTACCCTGGAAACTGGGCAATCT
TTGGTCCAACCCATCTGCCAATCGTTGTAGAAGGAACCCTGTTATCAATGGCTGACTACATGGGTCACCTGTATGTCCGTACAGGTACA
CCAGAATATGTTCGTCATATTGAACAAGGCTCATTACGTACCTTCGGTGGTCATACAACTGTGATTGCAGCGTTCTTCGCATCATTCGT
ATCCATGTTGATGTTTGCAGTCTGGWGGTATCTTGGAAAAGTATATTGCACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTGTTG
TACAACGCAATGACGTTACAGCATTTGGTGAAGAAGGCTTTGCAGAGGGGAATCA

>SNAD_amoA_5 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_amoA_5] [environmental_sample=TRUE] AmoA SBR clone SNAD_amoA_5 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTGGACTTCAC
GCTGTATCTGACACGCAACTGGCTGGTGACGGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATTT
TTGGACCAACCCATTTGCCAATCGTTGTAGAAGGCACATTGCTGTCGATGGCTGATTACATGGGACATCTGTATGTTCGTACAGGTACA
CCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGT
ATCAATGTTGATGTTCACCGTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTATCG
TACATCGCAATGATGTTACCGCATTCGGTGAAGAAGGCTTTCCCGAGGGGAATCA

>SNAD_amoA_6 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_amoA_6] [environmental_sample=TRUE] AmoA SBR clone SNAD_amoA_6 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTGGACTTCAC
GCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTGGAGGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCT
TTGGTCCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTCGTACAGGTACA
CCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGT
ATCAATGTTGATGTTCACCGTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTATCG
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>SNAD_amoA_7 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_amoA_7] [environmental_sample=TRUE] AmoA SBR clone SNAD_amoA_7 ammonia monooxygenase (amoA) gene, partial cds
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CCTGTTGTTGACGGGTAACTGGCTGATCACAGCCCTGTTAGGCGGCGGCTTCTTCGGATTATTTTTCTACCCGGGCAACTGGCCCATTT
TTGGCCCAACCCACTTACCGGTGGTAGTAGAAGGCGTCCTGTTATCAGTAGCTGACTACACAGGCTTCCTGTATGTACGTACAGGTACA

CCTGAATATGTTCGCCTGATTGAGCAAGGTTCACTGCGTACTTTCGGTGGACACACCACGGTAATTGCAGCGTTCTTCGCAGCGTTCGT
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>SNAD_amoA_8 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria;
environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating
anaerobic digester effluent] [clone=SNAD_amoA_8] [environmental_sample=TRUE] AmoA SBR
clone SNAD_amoA_8 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTGGACTTCAC
GCTGTATCTGACACGCAACTGGCTGGTGACAGCTCTGGTTGGAGGCGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATCT
TTGGTCCAACGCATCTGCCAATCGTTGTAGAAGGAACACTGTTGTCGATGGCTGACTACATGGGCCATATGTATGTTCGTACAGGTACA
CCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGT
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>SNAD_amoA_9 [organism=uncultured Bacteria, Proteobacteria; Betaproteobacteria;
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anaerobic digester effluent] [clone=SNAD_amoA_9] [environmental_sample=TRUE] AmoA SBR
clone SNAD_amoA_9 ammonia monooxygenase (amoA) gene, partial cds
GATTGGGGTTTCTACTGGTGGTCACACTACCCCATCAACTTCGTAACACCGGGCATTATGCTTCCGGGTGCGCTGATGCTGGACTTCAC
GCTGTATCTGACACGCAACTGGCTGGTGACGGCTCTGGTTGGAGGTGGATTCTTCGGTCTGCTGTTCTATCCGGGTAACTGGCCGATTT
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CCCGAGTATGTTCGTCATATTGAGCAAGGTTCACTGCGTACCTTTGGTGGTCATACCACAGTTATTGCAGCATTCTTCTCTGCGTTCGT
ATCAATGTTGATGTTCACCGTATGGTGGTATCTTGGAAAAGTTTACTGTACAGCCTTTTTCTACGTTAAAGGTAAAAGAGGTCGTATCG
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Amino Acid Sequences

>SNAD_amoA_2 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas europaea ammonia monooxygenase] ammonium monooxygenase subunit A
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PEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEGN

>SNAD_amoA_3 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas europaea ammonia monooxygenase]ammonium monooxygenase subunit A
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PEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEGN

>SNAD_amoA_4 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas eutropha ammonia monooxygenase] ammonium monooxygenase subunit A
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PEYVRHIEQGSLRTFGGHTTVIAAFFASFVSMLMFAVWXYLGKVYCTAFFYVKGKRGRVVQRNDVTAFGEEGFAEGN

>SNAD_amoA_5 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas europaea ammonia monooxygenase] ammonium monooxygenase subunit A
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PEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEGN

>SNAD_amoA_6 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas europaea ammonia monooxygenase] ammonium monooxygenase subunit A
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PEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFPEGN

>SNAD_amoA_7 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas oligotropha ammonia monooxygenase] ammonium monooxygenase subunit A
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PEYVRLIEQGSLRTFGGHTTVIAAFFAAFVSMLMFCVWWYFGKLYCTAFYYVKGERGRISMKNDVTAFGEEGFPEGN

>SNAD_amoA_8 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas europaea ammonia monooxygenase] ammonium monooxygenase subunit A
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PEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFAEGN

>SNAD_amoA_9 [gene=amoA] [protein=ammonium monooxygenase] [comment=similar to Nitrosomonas europaea ammonia monooxygenase] ammonium monooxygenase subunit A
DWGFYWWSHYPINFVTPGIMLPGALMLDFTLYLTRNWLVTALVGGGFFGLLFYPGNWPIFGPTHLPIVVEGTLLSMADYMGHLYVRTGT
PEYVRHIEQGSLRTFGGHTTVIAAFFSAFVSMLMFTVWWYLGKVYCTAFFYVKGKRGRIVHRNDVTAFGEEGFAEGN

Anammox bacteria

Nucleotide Sequences

>SNAD_Amx_4 [organism=uncultured planctomycete, anammox bacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Amx_4] [environmental_sample=TRUE] Uncultured planctomycete clone SNAD_Amx_4 16S ribosomal RNA gene, partial sequence
TTCGCAATGCCCGAAAGGGTGACGAAGCGACGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGC
AAGGATGTTAATAGCATCTTTGTTTGAGTACGGCTCCGGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGG
CAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCTGTGTAAGTCGGTTGTGAAAGCCTTCCGCTTAACGGAAGAACGG
CATCCGATACTGCATAGCTCGAGTGCGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGG
CGGCGAAGGCGACTCTCTGGCCCGTAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGC
CGTAAACGATGGGCACTAAGTAGAGGGGTTTT

>SNAD_Amx_5 [organism=uncultured planctomycete, anammox bacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Amx_5] [environmental_sample=TRUE] Uncultured planctomycete clone SNAD_Amx_5 16S ribosomal RNA gene, partial sequence
TTCGCAATGCCCGAAAGGGTGACGAAGCGACGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGC
AAGGATGTTAATAGCATCTTTGTTTGACTAAGGCTCCGGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGG
CAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCTGTGTAAGTCGGTTGTGAAAGCCTTCCGCTTAACGGAAGAACGG
CATCCGATACTGCATAGCTCGAGTGCGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGG
CGGCGAAGGCGACTCTCTGGCCCGTAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGC
CGTAAACGATGGGCACTAAGTAGAGGGGTTTT

>SNAD_Amx_9 [organism=uncultured planctomycete, anammox bacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Amx_9] [environmental_sample=TRUE] Uncultured planctomycete clone SNAD_Amx_9 16S ribosomal RNA gene, partial sequence
TTCGCAATGCCCGAAAGGGTGACGAAGCGACGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGC
AAGGATGTTAATAGCATCTTTGTTTGACTAAGGCTCCGGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGG
CAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCTGTGTAAGTCGGTTGTGAAAGCCTTCCGCTTAACGGAAGAACGG
CATCCGGTACTGCATAGCTCGAGTGCGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGG
CGGCGAAGGCGACTCTCTGGCCCGTAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGC
CGTAAACGATGGGCACTAAGTAGAGGGGTTTT

>SNAD_Amx_11 [organism=uncultured planctomycete, anammox bacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_Amx_11] [environmental_sample=TRUE] Uncultured planctomycete clone SNAD_Amx_11 16S ribosomal RNA gene, partial sequence
TTCGCAATGCCCGAAAGGGTGACGAAGCGACGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGC
AAGGATGTTAATAGCATCTTTGTTTGACTAAGGCTCCGGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGG
CAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCTGTGTAAGTCGGTTGTGAAAGCCTTCCGCTTAACGGAAGAACGG
CATCCGATACTGCATAGCTCGAGTGCGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGG
CGGCGAAGGCGACTCTCTGGCCCGTAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGC
CGTAAACGATGGGCACTAAGTAGAGGGGTTTT

>SNAD_Amx_20 [organism=uncultured planctomycete, anammox bacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_Amx_20] [environmental_sample=TRUE] Uncultured planctomycete clone SNAD_Amx_20 16S ribosomal RNA gene, partial sequence
TTCCCAATGCCCGAAAGGGTGACGAAGCGACGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGC
AAGGATGTTAATAGCATCTTTGTTTGACTAAGGCTCCGGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGG
CAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCTGTGTAAGTCGGTTGTGAAAGCCTTCCGCTTAACGGAAGAACGG
CATCCGATACTGCATAGCTCGAGTGCGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGG
CGGCGAAGGCGACTCTCTGGCCCGTAACTGACGCTGAGTGTGTGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGC
CGTAAACGATGGGCACTAAGTAGAGGGGTTTT

>SNAD_Amx_33 [organism=uncultured planctomycete, anammox bacteria; environmental samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Amx_33] [environmental_sample=TRUE] Uncultured planctomycete clone SNAD_Amx_33 16S ribosomal RNA gene, partial sequence
TTCGCAATGCCCGAAAGGGTGACGAAGCGACGCCGCGTGCGGGAAGAAGGCCTTCGGGTTGTAAACCGCTGTCGGGAGTTAAGAAATGC
AAGGATGTTAATAGCATCTTTGTTTGACTAAGGCTCCGGAGGAAGCCACGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGCGG
CAAGCGTTGTTCGGAATTATTGGGCGTAAAGAGCACGTAGGCGGCTGTGTAAGTCGGTTGTGAAAGCCTTCCGCTTAACGGAAGAACGG
CATCCGATACTGCATAGCTCGAGTGCGGGAGGGGAGAGTGGAACTTCTGGTGGAGCGGTGAAATGCGTAGATATCAGAAGGAACACCGG
CGGCGAAGGCGACTCTCTGGCCCGTAACTGACGCTGAGTGTGCGAAAGCTAGGGGAGCAAACGGGATTAGATACCCCGGTAGTCCTAGC
CGTAAACGATGGGCACTAAGTAGAGGGGTTTT

Denitrifying bacteria

Nucleotide Sequences

>SNAD_Nirs_55_1 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Nirs_55_1] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_Nirs_55_1 dissimilatory nitrite reductase (nirS) gene, partial cds
TGATCTCGACGCCGCATGACAATCCCAAGTTCGCCAAGTACAAGAGCAAGAACTGGAAGGTTGTCCAGGAACTGCCGATGCCCGGCGCC
GGCAACCTGTTCGTCAAGACCCACCCGAAGTCGAAGCACCTGTGGGCCGACATGCCGATGAACCCCGAGCGCGAGAACGCCGAGTCGAT
GGTGGTGTTCAATATGAACGACCTGACCAAGCCGCCGGTCAAGATCGACGTGGCCAAGGATTCGGGCCTGCCGATGACCAAGGCGCTGC
GCCGTGCGGTGCACCAGGAGTTCAGCCAGGACGGCACGGAAGTGTGGGTCTCACTGTGGGGCGGCAAGACGGACCAGTCGGCCATCGTG
GTGTATGACGACAAGACGCTCAAGGTCAAGAAGGTGATCACCGATCCGAAGATGATCACCCCGACCGGCAAGTTCAACG

>SNAD_Nirs_56_2 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Nirs_56_2] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_Nirs_56_2 dissimilatory nitrite reductase (nirS) gene, partial cds
CGGCACCGATCCGAAGGGGCACCCGAAGAGCGCCTGGAAAGTCGTGCGCTCGCTCAAGGCGCAGGGCGGCGGCAACCTGTTCATCAAGA
CCCATCCGAAGTCCACGAACCTCTGGGTCGACACGCCGCTGAACCCCGACGCCAAGATCGCCCAGTCGGTCGCGGTGTTCGACATCAGG
AACCTCGACAAGCCGTTCGAAGTGCTGCCGATCGCCGACTGGGCCCAGGTTGGCGAAGGCGCCAAGCGGATCACCCAGCCTGAGTACAA
CAAGGCGGGCGACGAGGTCTGGTTCTCGGTGTGGAGCGCGAAGAACCAGGAGTCCGCGATCGTGGTCGTCGACGACAGGACGCGCAAGC
TGAAAGCCGTGATCAAAGACCCCAAGCTGATCACGCCTACCGGCAAGTTCAACG

>SNAD_Nirs_57_3 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Nirs_57_3] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_Nirs_57_3 dissimilatory nitrite reductase (nirS) gene, partial cds
CGCCGCAGTACTCGATCATGGAAGGCGAGACGCTCAAGCCGCTGAAGGTGGTCTCGACCCGCGGCAACACGGTGGATGGCGACTACCAC
CCCGAGCCGCGCGTGGCCTCGATCGTCTCCTCGATGATCAAGCCCGAGTGGGTGGTGAATGTGAAGGGGACCGGCCAGATCATGCTGGT
GGACTACTCCGACATCAAGAACCTGAAGACGACCACGATCGAATCGGCCAAGTTCCTCCATGACGGCGGCTGGGACGCCTCCAAGCGCT
ACTTCCTGGTGGCAGCCAACGCGTCCAACAAGATCGCTGCGGTCGACACGAAGACGGGCAAGCTGGCGGCGCTGGTCGACACCAAGAAG
ATCCCGCACCCGGGCCGCGGTGCCAACTTCACGCACCCGACGTTCGGTCCCGTGTGGGCCACCGGCCACTTGGGCGACGCCGTGGTCAC
GCTGATCTCGACGCCGTCGGAGAAGGCTTCCGATGCCAAGTTCAAGACGCACAATTGGAAGGTCGTGCAGGAGCTGCCGATGCCTGGCG
CGGGCAACCTGTTCGTGAAGACGCATCCGAAGTCCACCAACCTGTGGGCCGACATGCCGATGAACCCCGAGCGCGAGAACGCCGAATCG
GTGTACGTGTTCAGCCTCAAGGACCTGAACAAGGCGCCGGTGAAGATCGACGTCGCCAAGGACTCCGGCCTGCCGCAGACCAAGGCGCT
GCGCCGCGCAACGCATCCCGAGTACAACGAGGCCGGGGACGAGGTGTGG

>SNAD_Nirs_58_5 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_Nirs_58_5] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_Nirs_58_5 dissimilatory nitrite reductase (nirS) gene, partial cds
CGGCACCGATCCGAAGGGGCATCCGAAGAGCGCCTGGAAAGTCGTGCGCTCGCTCAAGGCGCAGGGCGGCGGCAACCTGTTCATCAAGA
CCCATCCGAAGTCCACGAACCTCTGGGTCGACACGCCGCTGAACCCCGACGCCAAGATCGCCCAGTCGGTCGCGGTGTTCGACATCAAG
AACCTCGACAAGCCGTTCGAAGTGCTGCCGATTGCCGACTGGGCCCAGGTTGGCGAAGGCGCCAAGCGGATCACCCAGCCCGAGTACAA
CAAGGCGGGCGACGAGGTCCGGTTCTCGGTGTGGAGCGCGAAGAACCAGGAGTCCGCGATCGTGGTCGTCGACGACAAGACGCGCAAGC
TGAAAGCCGTGATCAAGGACCCCAAGCTGATCACGCCTACCGGCAAGTTCAACG

>SNAD_Nirs_24_1 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_Nirs_24_1] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_Nirs_24_1 dissimilatory nitrite reductase (nirS) gene, partial cds
CATGAAGGGCGACACGCTCGAGCCGCTGAAGATCCAGGCGACCCGCGGCATGGCGGTCGACACGCAGGAGTTCCACCCCGAGCCGTGCG
TGGCCTCGATCGTCGCCGTGCACGACAAGCCGCAGTTCATCGTGAACGTGAAGGAAACCGGCAAGATCCTGCTGGTTGACTATGGCGAT
ATCAACGCTCTGAAGGTCACCGAAATCGGCGCCGCCCGCTTCCTGCACGACGGCGGTTTGGATTCGAGCCACCGCTACTTCATGGTGGC
CGCCAATCAGTCCAACAAGATCGCGGTGGTGGACACCAAGGAAGGCAAGCTGGCGGGGCTGGTGGATGTCGGCAAGATTCCGCATCCGG
GCCGTGGGGCCAACTTTGTGGATCCCAAGTTTGGACCGGTCTGGGCGACGGGTCACCTGGGCGATGAAACCATCTCGTTGATCGGCACC
GACCCAGAGAAACACAAGGATCAGGCCTGGAAGGTGGTGCGTACCCTCAAGGGTCAGGGCGGCGGTTCGCTGTTCATCAAGACCCATCC
CACATCCAAGAACGTGTATGTGGACACCACTCTGAATCCGGACCCCAAGATCAGCCAGTCGGTGGCGGTGTACAATGTGGATGATCTGG
ACAAGCCCTATCAGGTGCTGCCAATCGCCGAATGGGCCGGTTTGAAAGACGATAGCGCCAAGCGGGTGGTGCAACCGGAGTACAATAAG
GCCGGCGACGAGGTTTGGTTCTCGGTGTGGAGCGCCGAGGATAAGGAATCGGCTATCGTGGTGGTGGACGACAAGACCCTGAAGCTGAA
GACCGTCATCAAGGATCCGAAGCTGATCACCCCGACCGGCAAGTTCAACG

>SNAD_Nirs_25_2 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating

anaerobic digester effluent] [clone=SNAD_Nirs_25_2] [environmental_sample=TRUE]
Uncultured bacterium SNAD_Nirs_25_2 dissimilatory nitrite reductase (nirS) gene, partial
cds
CCTACTGGCCGCCGCAGTACGTGATCATGGATGGCGACACATTGAAGCCGCGCAAGGTCGTGTCCACCCGTGGCATGACGGTCGCTGGC
GAATACCATCCGGAGCCGCGCGTCGCTTCCATCGTCGCTTCCTTCATCAAGCCGGAATGGGTTATCAACATCAAGGAAACCGGCCAGAT
TCTGCTGGTCGATTACTCTGACATCGAGAACCTGAAGACGACGACGGTCGGTTCAGCCAAGTTCCTGCATGATGGCGGCTGGGATGCTT
CCAAGCGTTACTTCCTGGTGGCAGCGAATGCCTCCAACAAGATCGCTGCGGTTGATACCAAGACCGGCAAGCTGGCCGCTCTGGTCGAT
ACGGCCAAGATCCCGCACCCGGGTCGCGGCGCCAACTTCACCCATCCGAAGTTTGGTCCGGTATGGACGACGGGTCACCTCGGTGCCGA
TGTCCTGACCCTGATCAGCACGCCGTCGGAGAAGAAGTCGGATGCCAAGTTCAAGGAATACAACTGGAAGGTCGTTCAGGAAGTGAAGC
ACGTGCCGGGCAACCTGTTCGTCAAGACCTATCCGAAGTCCAAGCACCTGTGGGCTGACTCGCCGCAGAATCCGGACAAGACGCTGGCT
GAATCCGTTGCCATCTGGGACGTGGCTGATCTGTCCAAGCCGGTCAAGGTG

>SNAD_Nirs_27_4 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria;
Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating
anaerobic digester effluent] [clone=SNAD_Nirs_27_4] [environmental_sample=TRUE]
Uncultured bacterium clone SNAD_Nirs_27_4 dissimilatory nitrite reductase (nirS) gene,
partial cds
CTACTGGCCGCCGCAATACTCGATCATGGAAGGCGAGACGCTCAAGCCGCTGAAGGTGGTCTCGACCCGCGGCAACACGGTGGATGGCG
ACTACCACCCCGAGCCGCGCGTGGCCTCGATCGTCTCCTCGATGATCAAGCCCGAGTGGGTGGTGAATGTGAAGGAGACCGGCCAGATC
ATGCTGGTGGACTACTCCGACATCAAGAACCTGAAGACGACCACGATCGAATCGGCCAAGTTCCTCCATGACGGCGGCTGGGACGCCTC
CAAGCGCTACTTCCTGGTGGCAGCCAACGCGTCCAACAAGATCGCTGCGGTCGACACGAAGACGGGCAAGCTGGCGGCGCTGGTCGACA
CCAAGAAGATCCCGCACCCGGGCCGCGGTGCCAACTTCACGCACCCGACGTTCGGTCCCGTGTGGGCCACCGGCCACTTGGGCGACGCC
GTGGTCACGCTGATCTCGACGCCGTCGGAGAAGGCTTCCGATGCCAAGTTCAAGACGCACAATTGGAAGGTCGTGCAGGAGCTGCCGAT
GCCTGGCGCGGGCAACCTGTTCGTGAAGACGCATCCGAAGTCCACCAACCTGTGGGCCGACATGCCGATGAACCCCGAGCGCGAGAACG
CCGAATCGGTGTACGTGTTCAGCCTCAAGGACCTGAACAAGGCGCCGGTGAAGATCGACGTCGCCAAGGACTCCGGCCTGCCGCAGACC
AAGGCGCTGCGCCGCGCAACGCATCCCGAGTACAACGAGGCCGGGGACGAGGTGTGG

>SNAD_Nirs_28_9 [organism=uncultured Bacteria; Proteobacteria; Betaproteobacteria;
Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating
anaerobic digester effluent] [clone= SNAD_Nirs_28_9] [environmental_sample=TRUE]
Uncultured bacterium clone SNAD_Nirs_28_9 dissimilatory nitrite reductase (nirS) gene,
partial cds
CCTACTGGCCGCCACAATTCACGATCATGGACGGCGCCACGCTCGAGCCGCTCAAGGTGGTCTCGACGCGCGGCATGACGGTGGACACG
CAGGAGTTCCACCCGGAGCCGCGCGTGGCCGCCATCGTGGCCTCGCACCAGCATCCCGAGCTCATCGTCAACGTCAAGGAGACCGGGCA
GGTCATGCTGGTCAACTACGAGGACATCGATAACCTCCGGACCACCACGATCGGCGCGGCCCGCTTCCTGCACGACGGCGGCTGGGACG
TGACCAAGCGCTACTTCCTCACCGCGGCGAACCAGTCGAACAAGATCGCCGTGATCGATTCGAAGGACCAGAAGCTCACCGCACTCGTC
GACGTGGACAAGATCCCGCACCCGGGCCGCGGCGCGAACTTCATCGATCCGAAGTACGGGCCGGTGTGGGCGACGAGCGCGCTCGGCAA
CGAGAAGGTGACGCTGGTCGCGACCGACCCCGAGAACTACCCGCAGCACGCCTGGAAGGCCGTGCGCGTGCTGAAGGGCCAGGGTGGCG
GGTCGTTGTTCGTGAAGACGCACCCGAAATCGAAGCACCTGTACGTGGATACCACGCTGAACCCCGATCCGAAG

Amino Acid Sequences

>SNAD_Nirs_55_1 [gene=NirS] [protein= dissimilatory nitrite reductase] [comment=similar to Thauera dissimilatory nitrite reductase] dissimilatory nitrite reductase
ISTPHDNPKFAKYKSKNWKVVQELPMPGAGNLFVKTHPKSKHLWADMPMNPERENAESMVVFNMNDLTKPPVKIDVAKDSGLPMTKALR
RAVHQEFSQDGTEVWVSLWGGKTDQSAIVVYDDKTLKVKKVITDPKMITPTGKFN

>SNAD_Nirs_56_2 [gene=NirS] [protein= dissimilatory nitrite reductase] dissimilatory nitrite reductase
GTDPKGHPKSAWKVVRSLKAQGGGNLFIKTHPKSTNLWVDTPLNPDAKIAQSVAVFDIRNLDKPFEVLPIADWAQVGEGAKRITQPEYN
KAGDEVWFSVWSAKNQESAIVVVDDRTRKLKAVIKDPKLITPTGKFN

>SNAD_Nirs_57_3 [gene=NirS] [protein= dissimilatory nitrite reductase] [comment=similar to Thauera dissimilatory nitrite reductase] dissimilatory nitrite reductase
PQYSIMEGETLKPLKVVSTRGNTVDGDYHPEPRVASIVSSMIKPEWVVNVKGTGQIMLVDYSDIKNLKTTTIESAKFLHDGGWDASKRY
FLVAANASNKIAAVDTKTGKLAALVDTKKIPHPGRGANFTHPTFGPVWATGHLGDAVVTLISTPSEKASDAKFKTHNWKVVQELPMPGA
GNLFVKTHPKSTNLWADMPMNPERENAESVYVFSLKDLNKAPVKIDVAKDSGLPQTKALRRATHPEYNEAGDEVW

>SNAD_Nirs_58_5 [gene=NirS] [protein= dissimilatory nitrite reductase] dissimilatory nitrite reductase
GTDPKGHPKSAWKVVRSLKAQGGGNLFIKTHPKSTNLWVDTPLNPDAKIAQSVAVFDIKNLDKPFEVLPIADWAQVGEGAKRITQPEYN
KAGDEVRFSVWSAKNQESAIVVVDDKTRKLKAVIKDPKLITPTGKFN

>SNAD_Nirs_24_1 [gene=NirS] [protein= dissimilatory nitrite reductase] dissimilatory nitrite reductase
MKGDTLEPLKIQATRGMAVDTQEFHPEPCVASIVAVHDKPQFIVNVKETGKILLVDYGDINALKVTEIGAARFLHDGGLDSSHRYFMVA
ANQSNKIAVVDTKEGKLAGLVDVGKIPHPGRGANFVDPKFGPVWATGHLGDETISLIGTDPEKHKDQAWKVVRTLKGQGGGSLFIKTHP
TSKNVYVDTTLNPDPKISQSVAVYNVDDLDKPYQVLPIAEWAGLKDDSAKRVVQPEYNKAGDEVWFSVWSAEDKESAIVVVDDKTLKLK
TVIKDPKLITPTGKFN

>SNAD_Nirs_25_2 [gene=NirS] [protein= dissimilatory nitrite reductase] [comment=similar to Dechloromonas aromatica dissimilatory nitrite reductase] dissimilatory nitrite reductase
YWPPQYVIMDGDTLKPRKVVSTRGMTVAGEYHPEPRVASIVASFIKPEWVINIKETGQILLVDYSDIENLKTTTVGSAKFLHDGGWDAS
KRYFLVAANASNKIAAVDTKTGKLAALVDTAKIPHPGRGANFTHPKFGPVWTTGHLGADVLTLISTPSEKKSDAKFKEYNWKVVQEVKH
VPGNLFVKTYPKSKHLWADSPQNPDKTLAESVAIWDVADLSKPVKV

>SNAD_Nirs_27_4 [gene=NirS] [protein= dissimilatory nitrite reductase] [comment=similar to Thauera dissimilatory nitrite reductase] dissimilatory nitrite reductase
YWPPQYSIMEGETLKPLKVVSTRGNTVDGDYHPEPRVASIVSSMIKPEWVVNVKETGQIMLVDYSDIKNLKTTTIESAKFLHDGGWDAS
KRYFLVAANASNKIAAVDTKTGKLAALVDTKKIPHPGRGANFTHPTFGPVWATGHLGDAVVTLISTPSEKASDAKFKTHNWKVVQELPM
PGAGNLFVKTHPKSTNLWADMPMNPERENAESVYVFSLKDLNKAPVKIDVAKDSGLPQTKALRRATHPEYNEAGDEVW

>SNAD_Nirs_28_9 [gene=NirS] [protein= dissimilatory nitrite reductase] [comment=similar to Pseudomonas stutzeri dissimilatory nitrite reductase] dissimilatory nitrite reductase
YWPPQFTIMDGATLEPLKVVSTRGMTVDTQEFHPEPRVAAIVASHQHPELIVNVKETGQVMLVNYEDIDNLRTTTIGAARFLHDGGWDV
TKRYFLTAANQSNKIAVIDSKDQKLTALVDVDKIPHPGRGANFIDPKYGPVWATSALGNEKVTLVATDPENYPQHAWKAVRVLKGQGGG
SLFVKTHPKSKHLYVDTTLNPDPK

Aerobic nitrite oxidizing bacteria bacteria

Nucleotide Sequences

>SNAD_NOB_1_1 [organism=uncultured Nitrobacter; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_NOB_1_1] [environmental_sample=TRUE] Uncultured Nitrobacter clone SNAD_NOB_1_1 16S ribosomal RNA gene, partial sequence
TCCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGGGCTCTGCAACTCGAGCCCATGAAGTTGGAATCGCTAGTAATCGTGGAT
CAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCCGAAGGCGTTTCGCTAA
CCGCAAGGAGGCAGGCGACCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_1_2 [organism= uncultured Nitrobacter; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_NOB_1_2] [environmental_sample=TRUE] Uncultured Nitrobacter clone SNAD_NOB_1_2 16S ribosomal RNA gene, partial sequence
ACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGGGCTCCGCAACTCGAGCCCATGAAGTTGGAATCGCTAGTAATCGTGGAT
CAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCTGAAGACGGTGCGCTAA
CCCGCAAGGGAGGCAGCCGGCCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_1_3 [organism=uncultured Nitrobacter; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone=SNAD_NOB_1_3] [environmental_sample=TRUE] Uncultured Nitrobacter clone SNAD_NOB_1_3 16S ribosomal RNA gene, partial sequence
TACCCMTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGGACTCTGCAACTCGAGTCCATGAAGTTGGAATCGCTAGTAATCGTGGA
TCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCTGAAGGCGGTGCGCTA
ACCCGCAAGGGAGGCAGCCGACCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_1_4 [organism=uncultured Nitrobacter; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_1_4] [environmental_sample=TRUE] Uncultured Nitrobacter clone SNAD_NOB_1_4 16S ribosomal RNA gene, partial sequence
TACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGGGCTCTGCAACTCGAGCCCATGAAGTTGGAATCGCTAGTAATCGTGGA
TCAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCTGAAGACGGTGCGCTA
ACCCGCAAGGGAGGCAGCCGGCCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_1_5 [organism=uncultured bacterium; Rhodoblastus; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_1_5] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_1_5 16S ribosomal RNA gene, partial sequence
TACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCGCAGA
TCAGCACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCCGAAGGCGTTTCGCTA
ATCGCAAGGAGGCAGGCGACCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_1_6 [organism=uncultured bacterium; Bradyrhizobium; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_1_6] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_1_6 16S ribosomal RNA gene, partial sequence
ACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGTGCTCTGCAACTCGAGCACATGAAGTCGGAATCGCTAGTAATCGCAGAT
CAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCTGAAGGCGGTGCGCTAA
CCAGCAATGGAGGCAGCCGACCACGGTAGGGTCAGCGACTGGGGTGAAGA

>SNAD_NOB_1_7 [organism=uncultured Nitrobacter; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_1_7] [environmental_sample=TRUE] Uncultured Nitrobacter clone SNAD_NOB_1_7 16S ribosomal RNA gene, partial sequence
ACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGGGCTCTGCAACTCGAGCCCATGAAGTTGGAATCGCTAGTAATCGTGGAT
CAGCATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCTGAAGACGGTGCGCTAA
CCCGCAAGGGAGGCAGCCGGCCACGGTAGGGTCAGCGACTGGGGTGAAGA

>SNAD_NOB_1_8 [organism=uncultured bacterium; Bradyrhizobium; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_1_8] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_1_8 16S ribosomal RNA gene, partial sequence
TACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCGCAGA
TCAGCACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCTGAAGACGGTGCGCTA
ACCGCAAGGAGGCAGCCGGCCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_1_9 [organism=uncultured bacterium; Bradyrhizobium; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_1_9] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_1_9 16S ribosomal RNA gene, partial sequence
ACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGTGCTCTGCAACTCGAGCACATGAAGTCGGAATCGCTAGTAATCGCAGAT
CAGCACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCTGAAGGCGGTGCGCTAA
CCCGCAAGGGAGGCAGCCGACCACGGTAGGGTCAGCGACTGGGGTGAAGA

>SNAD_NOB_2_2 [organism=uncultured bacterium; Bradyrhizobium; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_2_2] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_2_2 16S ribosomal RNA gene, partial sequence
TACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGTGCTCTGCAACTCGAGCACATGAAGTTGGAATCGCTAGTAATCGCAGA
TCAGCATGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCTGAAGACGGTGCGCTA
ACCGCAAGGAGGCAGCCGGCCACGGTAGGGTCAGCGACTGGGGTGAAG

>SNAD_NOB_2_3 [organism=uncultured bacterium, Proteobacteria; Deltaproteobacteria; Environmental Samples a] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_2_3] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_2_3 16S ribosomal RNA gene, partial sequence
ACCCCTAGCAAATCTCAAAAAACCGGCCTCAGTACAGATAGGAGTCTGCAACTCGACTCCTTGAAGTCGGAATCGCTAGTAATCGCTGA
TCAGCAGGCAGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGCGGACTGCTACAGAAGTCGGTGCGCCA
ACCGCAAGGAGGCAGCCGCCTAAGAAGTGGTCAGCGACTGGGGTGAAGA

>SNAD_NOB_2_5 [organism=uncultured bacterium; Rhodoblastus; Proteobacteria; Alphaproteobacteria; Environmental Samples] [mol_type=genomic DNA] [isolation_source=SNAD sludge treating anaerobic digester effluent] [clone= SNAD_NOB_2_5] [environmental_sample=TRUE] Uncultured bacterium clone SNAD_NOB_2_5 16S ribosomal RNA gene, partial sequence
TACCCCTAGCAAATCTCAAAAAACCGTCTCAGTTCGGATTGTACTCTGCAACTCGAGTGCATGAAGGTGGAATCGCTAGTAATCGCAGA
TCAGCACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGCTTTACCCGAAGGCGTTTCGCTA
ACCGCAAGGAGGCAGGCGACCACGGTAGGGTCAGCGACTGGGGTGAAG

III. Chapter

Old landfill leachate characterization using respirometric and physical-chemical methods

This chapter was based on:

Langone M., Andreottola G. (2013). *Old landfill leachate characterization using respirometric and physical-chemical methods.* Submitted

Old landfill leachate characterization using respirometric and physical-chemical methods

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ABSTRACT

Old landfill leachates have a complex chemical composition, characterized by the presence of inorganic compounds which make difficult a proper characterization. In this study, a thorough characterization of an old leachate from a municipal landfill located in Trento, Italy, has been performed. The interferences due to the complex leachate composition were taken into account during sampling, experimental analysis and data processing procedures. The COD fractions were divided based on solubility and biodegradability in order to perform a proper leachate COD fractionation, useful for biological process simulation models. Further, looking at the leachate composition, the applicability of a suitable treatment was discussed.

Independent physical - chemical methods (filtration at 0.45 µm, coagulation using $ZnSO_4$ at pH 10.5 and sequential filtration) were conducted and compared to evaluate the truly total ammonia nitrogen (TAN) and the soluble organic carbon (COD) concentrations. Both respirometric techniques (multi-OUR and single OUR) and total biological oxygen demand method (TbOD) have been applied in order to assess the biodegradable organic fractions of the leachate.

Sequential filtration was the most appropriate method for the determination of both the TAN and the soluble COD content in leachates. The total COD was fractionated in two component: truly soluble (83.9%) and particulate (16.1%). The biodegradability depended on the biomass type utilized for the characterization. However, the slowly biodegradable COD was predominant in the old leachate, while the readily biodegradable COD was only a small fraction of the total organic content. The presence of both slowly biodegradable COD fractions and inhibiting compounds suggests the use of technologies such as Sequential Batch Reactors (SBRs) and granular biomass systems, while the low biodegradable COD/TAN ratio (0.2) makes the leachate suitable for the application of the Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process, an innovative cost-effectiveness nitrogen biological process.

Keywords: biodegradable COD fractions, C/N, filtration, old landfill leachate, respirometry, soluble COD

1. Introduction

Wastewaters characterization is essential for design, modeling and control purposes of wastewater treatment plants (WWTP). The interest for a thorough characterization is increased after the development of the simulation models for the biological processes that do not use traditional parameters, i.e. COD and BOD_5, such as the Activated Sludge Models of IAWPRC Task Group (Henze et al. 2000), which require a proper organic matter fractionation. In the case of old landfill leachates, the complex chemical composition, the low biodegradability, the presence of biorefractory and inhibiting compounds make necessary to further improve the characterization procedures. Further, recent innovative and performing biological processes, among them the Simulatneous partial Nitritation, Anammox and Denitrification (SNAD) process in one single reactor, under oxygen limitation, has been applied for treating wastewaters characterized by low biodegradability and low COD_{bio}/N ratios, such as old landfill leachates (Wang et al. 2010). The SNAD system design and modeling require specific information about the wastewater composition, explecially in terms of nitrogen and organic matter content (Kumar and Lin 2010). The SNAD process comprises three simultaneous, interacting and sequential processes operated by different bacteria, with opposite operative condition, co-existing in the same environment (Lan et al. 2011; Langone et al. 2013b). The determination of the truly biodegradable COD content and the biodegradable COD_{bio}/N ratio is one of the most critical aspects in order to evaluate the applicability of the SNAD process and to further model the biological processes. In particular the COD_{bio}/N ratio directly affects microorganism

populations, including autotrophic ammonium and nitrite oxidizer bacteria (AOB and NOB), heterotrophic denitrifying and anammox bacteria (Kumar and Lin 2010). In a SNAD system, high concentrations of biodegradable organic carbon in the influent wastewater may either cause a heterotrophic denitrifying bacteria competition with anammox bacteria for nitrite (Molinuevo et al. 2009) or the activation of different anammox metabolic pathways, which could inhibit the anammox bacteria activity, i.e., using organic matter rather than ammonium and nitrite as a substrate (Güven et al. 2005; Kartal et al. 2007b). The biodegradable organic carbon content (COD_{bio}) of the system and the influent biodegradable COD_{bio}/N ratio should be low in order to establish a coupling between anammox and denitrifying bacteria (Ruscalleda et al. 2008).

The organic carbon and nitrogen content are usually determined using the traditional approach, which consists in evaluating the COD, BOD_5 and TAN after filtration at 0.45 µm, in order to distinguish the soluble and particulate fractions. However, the soluble and particulate fractions evaluated using filtration at 0.45 µm not always correspond to the truly soluble and particulate parts. Further, the soluble and particulate content do not necessarily correspond to the readily and slowly biodegradable organic fractions (Henze et al. 2000). This makes wastewater characterization by the filtration at 0.45 µm less suitable for design, model and control of biological processes, expecially for SNAD process.

Further, attention must be paid to the characterization of some industrial wastewaters, among them old landfill leachates, where the presence of chemical compounds may compromize the results of procedures usually adopted for the wastewater characterization. An old leachate is generated from landfills, which are operating since more than 10 years (Neczaj et al. 2005; Kurniawan et al. 2006b; Yan and Hu 2009). It generally contains a complex mix of inorganic and organic pollutants in dissolved, colloidal or dispersed form: high ammonium nitrogen, low biodegradable organic (COD_{bio}), refractory compounds, heavy metals, alkalinity, hardness, salinity, chlorinated organic xenobiotic compounds such as benzene and toluene and numerous other substances that can be considered as pollutants (Kurniawan et al. 2006b). The composition and the ratio of various fractions organics, nutrients, and inert materials in old landfill leachates is not similar to sewage and has been previously investigated by several studies (Cecen and Gursoy 2000; Kjeldsen et al. 2002; Ozkaya et al. 2006; Ziyang and Youcai 2007; Bilgili et al. 2008; Viet Yen 2009; Vedrenne et al. 2012). However, no studies are reported in literature that specifically evaluate a complete COD fractionation characterization of old landfill leachates, considering the influences of some inorganic electron donors, such Fe_2^+, Mn (II) and S^2, on the carbon and nitrogen analytical methods.

Abbreviations			
BOD	Biological Oxygen Demand	AOB	Ammonia-Oxidizing Bacteria
COD	Chemical Oxygen Demand	NOB	Nitrite-Oxidizing Bacteria
CODtot	Total Chemical Oxygen Demand	SNAD	Simultaneous partial Nitritation, Anammox, Denitrification
CODfiltr	Filtrate Chemical Oxygen Demand	N	Nitrogen
CODsoluble	Soluble Chemical Oxygen Demand	TAN	Total Ammonia Nitrogen
CODbio	Biodegradable Chemical Oxygen Demand	TNN	Total Nitrite Nitrogen
RBCOD	Readily biodegradable substrate	NO_3^--N	Total Nitrate Nitrogen
SBCOD	Slowly biodegradable substrate	TKN	Total Kjeldahl Nitrogen
S	Total soluble substrate	OUR	Oxygen Uptake Rate
X	Total particulate substrate	VSS	Volatile Suspend Solid
S_B	Soluble biodegradable substrate	TSS	Total Suspend Solid
X_B	Particulate biodegradable substrate	TbOD	Total biological Oxygen Demand
S_U	Soluble unbiodegradable substrate	WWTP	Wastewater Treatment Plant
X_U	Particulate unbiodegradable substrate		

The main aim of this study was to characterize an old landfill leachate from a municipal landfill located in Trento, Italy, in order to provide an influent composition that could be used in biological simulation models.

Further, looking at the leachate composition the applicability of a suitable treatment was discussed mainly to verify the applicability of the SNAD process. Three independent physical – chemical methods (filtration at 0.45 µm, sequential filtration at 0.45 and 0.2 µm and coagulation using $ZnSO_4$ at pH 10.5) were performed in order to find out the proper analytical method for the determination of the total soluble (S) and particulate (X) COD and N fractions of leachates. Chemical–biological methods, respirometric methods and total biological oxygen demand methods, were used to fractionate the readily biodegradable (S_B, RBCOD) and the slowly biodegradable COD (X_B, SBCOD). The interference on the biological oxygen consumption, due to chemical compounds present in leachates, were taken into account.

2. Materials and Methods

2.1. Landfill leachate collection

In this study, the old landfill leachate was collected from a municipal landfill located in Trento, Italy. The samples were collected for approximately one year in polyethylene bottles washed with deionized water before use. Dissolved oxygen (DO) and oxidation-reduction potential (OUR) of the raw leachate were measured. A careful sampling was performed in order to minimize the exposition of the samples to oxygen, preventing the Fe (II) oxidation to Fe (III) and its following precipitation during sampling. The leachate samples were transported to the laboratory and stored at 4°C prior to the characterization, in order to prevent the COD and nitrogen degradation.

2.2. Analytical methods

Raw leachate samples were analyzed for total suspended solids (TSS) and volatile suspended solids (VSS) according to the Standard Methods (APHA-AWWA-WPCF 2005). Ferrous ions (FeII) were determined using a UV/Vis spectrophotometer, while Ferric ions (FeIII) through colorimetric analysis. Manganese was measured using the inductively coupled plasma mass spectrometry (ICP-MS) (EN ISO 17294-2:2004). Dissolved sulphide (S^{2-}) and hydrogen sulfide (H_2S) were measured according to APAT CNR IRSA, 2003.

In order to eliminate the chemical interference of inorganic constituents (i.e. ferrous ions Fe(II), manganese ions Mn(II) and dissolved sulphide (S^{2-})) on the COD measurement, leachate samples were partially exposed to oxygen prior to further characterizations. In this way the inorganic constituents may be oxidized, i.e. Fe (II) to Fe (III), and precipitate. The resulting samples were then analyzed for the total chemical oxygen demand (COD_{tot}) according to the Standard Methods (APHA-AWWA-WPCF 2005), correcting chloride interferences. Later, leachate samples were analyzed using the traditional approach, filtration at 0.45µm. The filtrate was analyzed colorimetrically for total ammonium as nitrogen (TAN) and total nitrite as nitrogen (TNN), according to APAT CNR IRSA, 2003. The nitrate as nitrogen (NO_3-N), total Kjeldahl Nitrogen (TKN) and chemical oxygen demand (COD_{filt}) were measured according to the Standard Methods (APHA-AWWA-WPCF 2005). The concentrations of the metallic ions were measured using a spectrometry ICP-MS (EN ISO 17294-2:2004). The filtrate samples were checked again for dissolved sulphide (S^{2-}), hydrogen sulfide (H_2S), Ferrous, Ferric (Fe_2^{+}) and Manganese Mn (II) ions, as previously described. Alkalinity determinations were conducted using the kit USEPA Buret Titration Method 8221 (Hach Company). The means and standard deviations have been determined for each parameter.

2.3. Determination of CODsoluble and TAN

The leachate samples, previously partially exposed to oxygen, were analyzed for the truly soluble COD (COD_{sol}) and the total ammonia concentration (TAN) of the leachate. Different physical-chemical procedures were used and compared for the separation of soluble (S) and particulate (X) compounds: filtration (0.45 µm nominal pore size); sequential filtration (0.45 µm and 0.2 µm nominal pore size) (Roeleveld and Van Loosdrecht 2002); $ZnSO_4$ coagulation at pH 10.5±0.3 (Mamais et al. 1993; Hu et al. 2002). The supernatant of each method was analyzed for COD and TAN as described in the analytical methods section. The tests were carried out on three samples of leachate, to representativeness of the leachate of the municipal landfill used in this study.

2.4. Determination of biodegradable COD fractions

The COD was divided into two fractions: biodegradable and undiodegradable COD. Further, based both on biodegradability and on solubility four fractions were measured: the readily biodegradable COD (RBCOD, S_B), the slowly biodegradable (X_B) COD, the soluble unbiodegradable COD (S_U), the particulate unbiodegradable (X_U) COD.

The respirometric methods, based on the oxygen uptake rate (OUR) of heterotrophic biomass in batch vessels were used to evaluate the biodegradable COD fractions (readily biodegradable, RBCOD and total biodegradable, COD_{bio}) of the leachate samples. The calculation of the other COD fractions was made according to the following equations:

$$S_B = RBCOD \quad \text{(Eq. III-1)}$$

$$S_U = COD_{soluble} - S_B \quad \text{(Eq. III-2)}$$

$$X_B = SBCOD = COD_{bio} - S_B \quad \text{(Eq. III-3)}$$

$$X_U = \left(COD_{tot} - COD_{soluble}\right) - X_B \quad \text{(Eq. III-4)}$$

As the biodegradability is biomass-specific, the respirometric experiments were performed using both an activated sludge from the municipal WWTP of Trento, Italy, and a SNAD biomass from a SBR of the WWTP of Zürich, Switzerland (Joss et al. 2009), where aerobic ammonium oxidizing-, anammox and denitrifying bacteria co-existed (Langone et al. 2013b). The respirometric tests were carried out using the same three leachate samples used for $COD_{soluble}$ and TAN determination. In addition, the total biological oxygen demand (TbOD) method had been applied on respirometric assay in order to verify the total biodegradable organic matter fraction (COD_{bio}) of leachate measured by using the respirometric technique.

2.4.1. Respirometers

Respirometric experiments using leachate were conducted separately to obtain the DO profile with both the activated sludge of the urban WWTP and the SNAD biomass. The respirometers consisted of 1.2 L reactors with mechanical stirring and an air diffuser for experiments performed on the activated sludge, and of 0.2 L reactors for experiments with the SNAD biomass. DO and temperature were measured in the liquid through the DO probe. The pH was also monitored. The DO probe was connected through a RS-232 port to a computer, which was used for storing and monitoring all data. Temperature was kept constant at 20°C and the pH between 7.5 and 8. The total solids concentration was in the range between 3-5 g $TSSL^{-1}$. For all the experiments, thiourea (20 mg L^{-1}) was added to inhibit the nitrification process. The DO depletion in the vessel, due to the substrate (organic carbon) utilization, was monitored over the time. The OUR values, expressed as $mgO_2\ L^{-1}h^{-1}$, were

evaluated by using the dynamic method, based on the respiratory activity of organisms. The OURs were determined by the slope of the plot of DO concentration versus time after stopping for few minutes the air flow inlet.

2.4.2. Readily biodegradable COD (RBCOD)

Respirometric method. According to Ziglio et al., (2001), single-OUR tests were conducted in batch vessels to measure the readily biodegradable COD. The tests were conducted separately, using the activated sludge and the SNAD biomass. For each sludge, first increasing pulses of sodium acetate were added in the batch tests in order to obtain the sludge-specific calibration curve (RBCOD vs ΔDO) and the slope coefficient (m), which relates the DO consumed to the COD removed (acetate). Then, the RBCOD of the leachate samples was calculated by adding 100 mL of leachate in the respirometers with activated sludge and 50 mL in the respirometers with SNAD biomass. The volume of the spikes was chosen in order to better appreciate the DO slope variation. The DO depletion in the vessel due to the substrate utilization was monitored over the time and the oxygen consumption (ΔDO) was determined. Then, from the calibration curve, the RBCOD of the leachate equivalent to acetate was determinate according to the following equation:

$$RBCOD = \frac{\Delta O}{m} \cdot \frac{V_{mix}}{V_{leach}} \qquad \text{(Eq. III-5)}$$

where

V_{mix}= mixed liquor volume (L)
V_{leach}= leachate volume (L)
ΔO=oxygen consumption (mg L^{-1})
m= slope coefficient of the plot DO consumed to COD removed ($\Delta O_{consumed}$ $\Delta COD_{removed}^{-1}$)

Further, for each sludge, the heterotrophic yield coefficient referred to the acetate, Y_{OHO}, was estimated according to the following equation:

$$Y_{OHO} = 1 - m = 1 - \frac{\Delta O}{\Delta COD} = \frac{\Delta COD - \Delta O}{\Delta COD} \qquad \text{(Eq. III-6)}$$

where

Y_{OHO}= Yield coefficient ($mgCOD_{cell}$ $mgCOD_{oxidized}^{-1}$)
ΔO=oxygen consumption (mg L^{-1})
ΔCOD=COD consumption (mg L^{-1})
m= slope coefficient of the plot DO consumed to COD removed ($\Delta O_{consumed}$ $\Delta COD_{consumed}^{-1}$)

2.4.3. Total biodegradable COD (CODbio)

Respirometric method. The assessment of the total biodegradable COD (COD_{bio}) was performed using the multi-OUR respirometric technique, which relies on the assumption that the biological oxygen consumption rate of heterotrophic biomass is related to the total biodegradable COD consumed during the tests, including the readily biodegradable COD (RBCOB) and the slowly biodegradable COD (SBCOD). The respirometric assays were conducted according to Andreottola et al., (2002), including modifications on the data processing procedure to considerate the presence of some inorganic compounds, which affect the biological oxygen consumption. Modifications on the OUR data processing were introduced as the complex composition of leachate may induce to overestimate the OUR, which would lead to an erroneous leachate characterization.

Respirometric experiments using leachate were conducted separately to obtain the OUR profile with both the activated sludge and the SNAD biomass, with an initial substrate on biomass ratio (F_0/X_0) < 0.1. Biomass (both

activated sludge and SNAD biomass) was left in starvation for several hours and then the endogenous OURs were evaluated from data obtained during endogenous respiration. Next, a pulse of leachate was added in the tests, 400 mL in the experiments with the activated sludge and 100 mL in the experiments with the SNAD biomass. OUR values from this point to the substrate depletion comprised endogenous and exogenous respiration. Hence, the exogenous oxygen consumption (ΔO) was evaluated subtracting the contribution of the endogenous respiration from the whole oxygen consumption (the area under the OUR curve). The exogenous oxygen consumption was then converted to equivalent COD using the following expression based on a COD mass balance (Ekama et al. 1986):

$$COD_{bio} = \left[\frac{1}{1 - f_{cv} \cdot y_{OHO}}\right] \cdot \Delta O \cdot \frac{V_{mix}}{V_{leach}} = \left[\frac{1}{1 - Y_{OHO}}\right] \cdot \Delta O \cdot \frac{V_{mix}}{V_{leach}} \quad \text{(Eq. III-7)}$$

where

V_{mix}= mixed liquor volume (L)
V_{leach}= leachate volume (L)
f_{cv}= COD_{cell}/VSS ratio (1.42)
y_{OHO} = Yield coefficient (mgVSS $mgCOD_{oxidized}^{-1}$)
Y_{OHO}= Yield coefficient ($mgCOD_{cell}$ $mgCOD_{oxidized}^{-1}$)
ΔO=oxygen consumption (mg L^{-1})

Total biological oxygen demand (TbOD) method. According to Bilgili et al., (2008), the total biological oxygen demand (TbOD) method was applied on the respirometric tests to evaluate the total biodegradable COD and verify the respirometric results. The method assumes that the TbOD is equal to the biodegradable COD, including soluble and particulate degradable COD, as the biodegradability particulate organic materials are completely hydrolyzed when the biological oxidation process is completed. The COD concentration was measured on the raw leachates (COD_{tot}) and on the filtrate leachates (COD_{filt}), passing through a 0.45-µm filter. Samples of the sludge-leachate mixture were taken at the beginning and at the end of the respirometric assay and analyzed for total and filtrate COD. The particulate COD was always calculated as difference of total and filtrate COD. The TbOD values was calculated as follows:

$$TbOD = COD_{bio} = \left(\left(\text{initial mixture total COD - initial biomass COD}\right) \text{ - final mixture soluble COD}\right) \cdot \frac{V_{mix}}{V_{leach}} \quad \text{(Eq. III-8)}$$

where:

$$\text{initial biomass COD} = \left(\text{initial mixture particulate COD - raw leachate particulate COD} \cdot \frac{V_{leach}}{V_{mix}}\right)$$

2.4.4. Chemical OUR

In order to consider the interference of the leachate composition on the biological oxygen consumption, chemical OUR tests were performed. The experiments were conducted using raw leachate samples, partially oxidized and used also in the respirometric assays, and tap water rather than sludge in a reactor of 1.2 L. The tap water was oxygenated, achieving oxygen saturation levels. Next, a pulse (400 mL) of leachate was added. The DO profile was monitored and the OURs were calculated.

3. Results and Discussion

3.1 Conventional leachate characterization

In literature studies, the leachate samples are usually characterized by a set of traditional parameters, also measured in this study and reported in Table III-1, together with relevant concentrations data from the literature on old landfill leachates. Raw samples were analyzed using the traditional approach (filtration at 0.45 µm). The average COD and BOD_5 concentrations were 1307±193 mgCOD_{filt} $L^{-1}$and 70±17 mgO_2 L^{-1}, respectively. Therefore, the leachate was characterized by a low BOD_5/COD_{filt} (0.05) ratio, indicating a poor biodegradability.

Low BOD_5/COD_{filt} ratios are characteristic of old landfill leachates, where mainly refractory compounds are responsible for the high COD value. Old leachates are generally characterized by low concentrations of volatile fatty acids and relatively higher amounts of humic and fulvic-like compounds. The high pH (pH 8.1±0.1) was also indicative of the old landfill age (Kulikowska and Klimiuk 2008).

The COD values measured may be influenced by the presence of some inorganic constituents that could be chemically oxidized. For instance, Kylefors et al., (1999) verified that Ferrous Fe (II) and Manganese Mn (II) ions and dissolved sulphides (S^{2-}) contributed up to one-third of the COD measured in the leachate used in their experiments. Nevertheless, in our study, both prior and after the pre-oxidation step only trace level of Fe (II), Mn (II) and H_2S were detected, not influencing the organic matter analysis.

Table III-1 also indicates that ammonium was the prevalent nitrogen inorganic species. The leachate was characterized by a high total ammonium nitrogen (TAN) concentration (1241±136 mg TAN L^{-1}), resulting in a very low COD_{filt}/N ratio (around 1.1). The low COD_{filt}/N measured indicates that conventional biological processes are not appropriate to treat the landfill leachate, used in our study, while SNAD process could represents a suitable process. Heavy metals were present at typical range concentrations for municipal landfill leachates operating in methanogenic phase, as they were precipitated as sulphides and remained inside the landfill (Morling 2010). Low contents of Fe (3.76±0.44 mgL^{-1}), B (5.6±0.79 mgL^{-1}), Al (0.42±0.09 mgL^{-1}), Ba (0.39±0.04 mgL^{-1}) and Zn (0.18±0.14 mgL^{-1}) were detected, while As and Cd were detected at trace levels below 0.08 mg/L and 0.005 mgL^{-1}, respectively. Low amounts of mercury were present (<3.0 mgL^{-1}). Lead did not appear. Cr (0.21±0.04mg L^{-1}) and Ni (0.10±0.015 mg L^{-1}), which are toxic and carcinogenic, were detected at trace levels.

Table III-1. Conventional old landfill leachates characterization.

Parameter	This study	old landfill leachate **
CONDUCTIVITY [mS/cm]	10.7±1	-
REDOX [mV]	30±10	-
pH	8.1±0.1	7.5 - 9
TSS [mg L^{-1}]	123 ± 129	-
COD_{filt} [mg L^{-1}] (filtration at 0.45 µm) [mg L^{-1}]	1307 ±193*	5.000-20.000
BOD_5 [mg L^{-1}]	70±17	20-1.000
TOTAL AMMONIUM NITROGEN (TAN_{filt}) [mg L^{-1}]	1241±136*	400-5000
TOTAL NITRITE NITROGEN (TNN) [mg L^{-1}]	<0.04*	-
NITRATE (NO_3-N) [mg L^{-1}]	10±2*	-
TOTAL KJELDAHL NITROGEN (TKN) [mg L^{-1}]	1288±169	-
ORGANIC NITROGEN (Norg) [mg L^{-1}]	57±39	-
TOTAL PHOSPHORUS (P) [mg L^{-1}]	14±2	6
SULFATE [mg L^{-1}]	1200*	10-420
ALKALINITY ($CaCO_3$) [mg L^{-1}]	7.000 – 15.000*	-
HYDROGEN SULFIDE (H_2S^-) [mg L^{-1}]	0.04*	-
Fe(II) [mg L^{-1}]	2,1*	-
Fe(III) [mg L^{-1}]	1,8*	
Mn(II) [mg L^{-1}]	0,9*	-
ALUMINIUM [mg L^{-1}]	0.42 0.1*	-
ARSENIC (As) [mg L^{-1}]	<0.08*	0.01-1
BARIUM (Ba) [mg L^{-1}]	0.39±0.036*	-
BORON (Bo) [mg L^{-1}]	5.6±0,79*	-
CADMIUM (Cd) [mg L^{-1}]	<0.005*	0.005
CHROMIUM (Cr) [mg L^{-1}]	0.21±0.04*	0.28
IRON (Fe) [mg L^{-1}]	3.8±0.44*	3 - 280
LEAD (Pb) [mg L^{-1}]	<0.04*	0.09
MANGANESE (Mn) [mg L^{-1}]	0.1±0.007*	0.03-45
MERCURY (Hg) [mg L^{-1}]	<3.0*	0.00005-0.16
NICKEL (Ni) [mg L^{-1}]	0.1±0.015*	0.17
ZINC (Zn) [mg L^{-1}]	0.18±0.15*	0.03-4
COPPER (Cu) [mg L^{-1}]	0.01±0.002*	0.065
SELENIUM (Se) [mg L^{-1}]	<0.003*	-
STANNUM (Sn) [mg L^{-1}]	0.06±0.006*	-

* values after filtration 0.45 µm, ** (Kjeldsen et al. 2002; Kurniawan et al. 2006b)

3.2 Truly CODsoluble and TAN

Different independent physical-chemical techniques were conducted and compared for the determination of the truly soluble COD and the TAN of the landfill leachate: coagulation by $ZnSO_4$, sequential filtration and filtration at 0.45µm.

The independent physical–chemical techniques applied to estimate the $COD_{soluble}$ showed a high content of the soluble organic matter in the old landfill leachate used in our study (Table III-2). The $COD_{soluble}$ was estimated to be 1211 ± 36 mg L^{-1} (mean value of sequential filtration and coagulation methods), which constituted ~84% of the total COD. The particulate COD, calculated as difference between total and soluble COD, was 233 ± 93 mg L^{-1}. Nevertheless, differences can be observed applying one method over the others. The $COD_{soluble}$ quantified by sequential filtration method was higher than that measured using the coagulation method (the percentage difference was ~ 5%). However our results showed that both techniques may be adequate for the determination of the truly soluble COD fraction in landfill leachate. The main difference was detected using the filtration at 0.45 µm. The truly soluble COD concentration estimated using the filtration at 0.45 µm was higher by 8,3% compared to sequential filtration, indicating the overestimation of $COD_{soluble}$ often occurring in research studies.

Table III-2. Soluble COD.

Component	**Concentrations [mg L^{-1}]**
TOTAL COD	1444 ± 86
SOLUBLE COD ($ZnSO_4$ coagulation method)	1185 ± 69
SOLUBLE COD (sequential filtration method)	1237 ± 125
FILTERED COD (filtration at 0.45 µm)	1291 ±42

Further, the application of the different physical-chemical techniques highlights some important aspects on the appropriate technique to chose for the determination of the truly ammonia nitrogen concentration (TAN) of leachates. Our results showed that the most appropriate technique to determine the TAN was the sequential filtration, that better remove the micro-colloids and macromolecules present in the leachate, without changing the composition of leachates. The $ZnSO_4$ method would not be appropriate for determining the soluble ammonium nitrogen. The high pH, on which the $ZnSO_4$ method operates, can lead to the nitrogen loss via ammonia volatilization. For instance, in our $ZnSO_4$ tests, the concentration of total ammoniom nitrogen (TAN) was 13% lower than the concentration of total ammonium nitrogen in sequential filtration tests (Table III-3). We also confirmed that the 0.45 µm filtration method, frequently used in research studies, overestimated the total ammonium nitrogen concentration in leachate by 14%, probably due to the interferences of colloids of iron, magnesium, calcium and sulphur with the Nessler reagent.

Table III-3. Soluble TAN

Component	**Concentrations [mg L^{-1}]**
TOTAL AMMONIOM – NITROGEN (TAN) (sequential filtration method)	1181 ± 43
TOTAL AMMONIOM – NITROGEN (TAN) ($ZnSO_4$ coagulation method)	1023± 19
TOTAL AMMONIOM – NITROGEN (TAN) (filtration at 0.45 µm)	1348 ± 181

3.3 Biodegradable COD fractions using the activated sludge of the WWTP

In order to investigate the influence of the leachate composition on the observed oxygen respiration of the heterotrophic biomass, and consequently on the COD measurement, chemical respirometric tests were performed, using tap water and raw leachate in aerobic conditions. When the leachate was added to the water an initial DO depletion was observed, but after 3-5 minutes, the DO values were spontaneously reported to the initial DO value and no DO depletions were observed during the test (not showed).

Considering the low inorganic compounds concentrations, and considering that the possiblie interferences of inorganic electron donors such as Fe_2^+, Mn (II) and S^2, were partially avoided using raw leachate samples previously exposed to oxygen, in order to make aerobic condition and allow the oxidation of chemical compounds before the respirometric tests, we concluded that no chemical oxidation was obtained during respirometric tests using the old landfill leaachate. However, the low biodegradable COD content in the raw leachate requires a high volume of leachate (400 mL) to be added to the respirometric assays (800 mL). As consequence the dilution effects on respirometric tests may induce to an erroneous calculation of the OUR values in the first minutes of the respirometric tests, overestimating the DO depletion.

In order to characterize the old landfill leachate, respirometric analysis were carried out using the activated sludge. At the beginning of the respirometric tests for the determination of both COD_{bio} and RBCOD, a slope variation in the DO curves was detected.

Fig. III.1 shows the single-OUR test, performed to measure the RBCOD using the activated sludge from the municipal the WWTP of Trento. In Fig. III.1 is clearly illustrated the slope variation obtained measuring the RBCOD. In this case, the slope variation was probably due to the dilution effect rather than the inorganic compounds oxidations. Further, we also attributed the slope variation to the dilution effect rather than the presence of rapidly hydrolysable organic fraction as previously reported by Ziglio et al. (2001). Thus, the respirometric method described by Ziglio et al. (2001) for RBCOD calculation was adapted and the OUR values at the beginning of the test were calculated not considering the first DO depletion slope. The single-OUR test confirmed that only a little part of COD was readily biodegradable ($21 mgL^{-1}$), which corresponded to ~1.5 % of the total COD and to ~ 1.7 % of the $COD_{soluble}$.

The conversion yield of heterotrophs (Y_{OHO}) measured in the activated sludge from the single-OUR tests was 0.73 $gCOD_{cell}gCOD_{oxidized}$ at 20 °C, higher than the default value reported in literature for urban wastewater (0.67 $gCOD_{cell}$ $gCOD_{oxidized}$) (Henze et al. 2000). The default value is valuable for domestic wastewater, where a large variety of carbon source (carbohydrates, proteins, alcohols, carboxylic acids) are present, while the Y_{OHO} calculated in this study can be considered substrate-specific, as it was obtained with a single carbon source (acetate). Its value is comparable to other study where acetate was used (Jiang et al. 2005).

Fig. III.2 illustrates the OUR profile of the multi–OUR respirometric experiment performed using the activated sludge with the unfiltered leachate in order to determine the total biodegradable COD (COD_{bio}). The respirogram in Fig. III.2 is already corrected, modifying the method described by Andreottola et al. (2002) and not considering the first DO depletion slope. The respirogram is characteristic of wastewaters with readily biodegradable and slowly biodegradable COD. As can be seen in Fig. III.2, before the addition of the old landfill leachate the endogenous respiration was achieved. After the leachate addition, the OUR values rapidly increased to a peak depending on the amount of biodegradable organic matter initially present in the sample. During the first minutes after leachate addition, the OUR decreased rapidly due to readily biodegradable COD depletion as

well as the slowly biodegradable organic matter. The area delineated by the OUR curve above the endogenous respiration level represents the amount of oxygen consumed at the expense of the total biodegradable COD.

The total biological oxygen demand (TbOD) method, used to verified the total biodegradable COD (COD_{bio}), confirmed the COD_{bio} value measured by using the adapted respirometric method, confirming the assumption of the influence of the dilution effect on the OUR measurements.

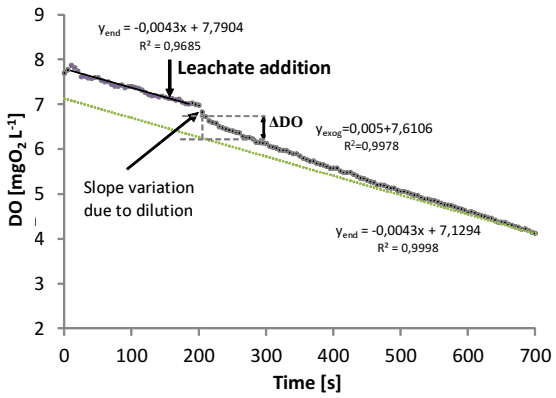

Fig. III.1 Single – OUR test, using activated sludge.
Curve of the oxygen consumption related to the addition of 100 mL of landfill leachate in 1.2 L of the activated sludge of WWTP.

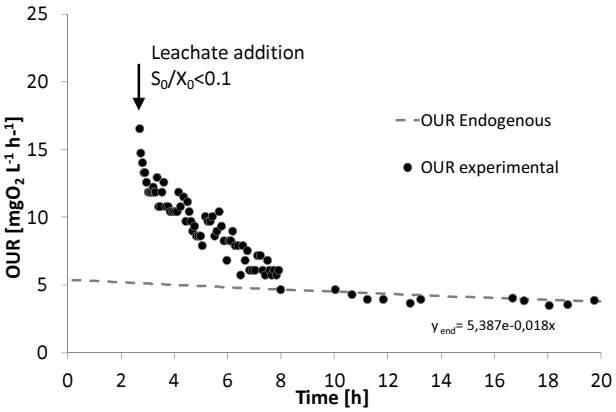

Fig. III.2 Multi-OUR test, using activated sludge.
Respirogram of the activated sludge of WWTP, obtained after the addition of 400 mL of landfill leachate in 1.2 L of the activated sludge of WWTP.

Table III-4 gives the respirometric results. The COD_{bio} (254 mg L^{-1}) was only ~17% of the total COD while the largest part of COD was mainly non-biodegradable (1194 mg L^{-1}), which correspond to the ~83% of the total COD, calculated as the difference between total COD and COD_{bio}. The readily biodegradable COD (21mgL^{-1}), corresponded to ~1.5 % of the total COD and to ~ 1.7 % of the $COD_{soluble}$. The largest part of organic matter was mainly slowly biodegradable and (hydrolyzed) COD. Furthermore, is important to underlined that the RBCOD measured was significantly lower than the BOD_5 value, meaning that BOD_5 values overestimated the readily biodegradable content.

Table III-4. Biodegradable COD fractions.

Component	Concentrations [mg L^{-1}]
TOTAL	1444
BIODEGRADABLE COD– TbOD method	251
BIODEGRADABLE COD– Respirometric method	254
READILY BIODEGRADABLE COD– Respirometric method	21

3.4 Biodegradable COD fractions using the SNAD biomass

When the biodegradable COD fractions of the landfill leachate were evaluated using SNAD bliomass, the COD_{bio} and the RBCOD were lower than the value measured by using the activated sludge of WWTP, 47 mg L^{-1} and 4 mg L^{-1}, respectively. The obtained mean value of Y_{OHO} of the SNAD biomass was 0.71 $gCOD_{cell}$ $gCOD_{oxidized}$, comparable with the value measured for the activated sludge. The lower biodegradable COD fractions detected using the SNAD biomass, were probably due to inhibition effects of chemical compounds present in the leachate on SNAD biomass, which was not acclimated to leachetes, while the activated sludge used in this study was used to treat sporadically landfill leachate as well as municipal wastewater.

Fig. III.3 illustrates the DO depletion during the single-OUR test, performed to measure the RBCOD with SNAD biomass. In Fig. III.3 can be observed a double slope due to the dilution effect as already seen in tests using the activated sludge (Fig. III.1). The OUR data were calculated and the data analysis were performed taking into account the dilution effects.

Fig. III.4 shows an OUR profile determined performing the multi–OUR respirometric experiment with the SNAD biomass and the unfiltered leachate. The OUR profile confirmed the presence of both readily biodegradable and slowly biodegradable COD.

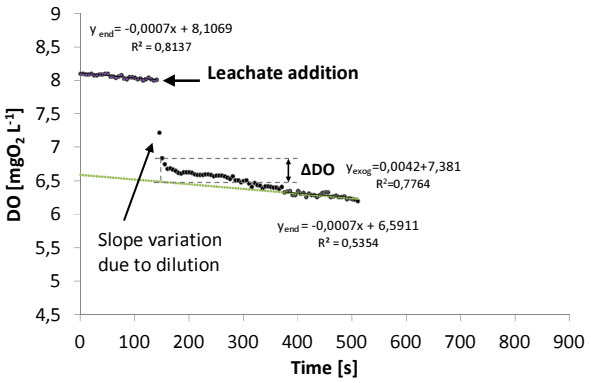

Fig. III.3 Single – OUR test, using SNAD biomass.
Curve of the oxygen consumption related to the addition of 50 mL of landfill leachate in 0.2 L of the SNAD biomass.

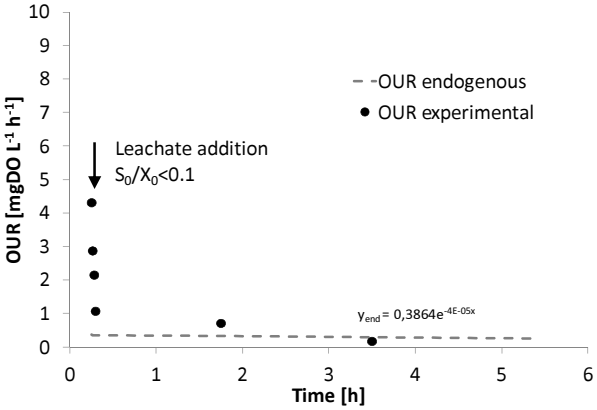

Fig. III.4 Multi-OUR test, using SNAD biomass.
Respirogram of the SNAD biomass, obtained after the addition of 100mL of landfill leachate in 0.2 L of the SNAD biomass.

3.5 Landfill leachate COD characterization

Combining the results of the chemical analysis, used to measure total, soluble and particulate COD, and the results of the respirometric analysis, performed to evaluate the biodegradable fractions, a complete landfill leachate COD characterization was obtained both using the activated sludge and the SNAD biomass. The schemes of the COD characterization for the activated sludge of WWTP and for the SNAD biomass are reported in the Fig. III.5 A and Fig. III.5 B, respectively.

The COD fractions were divided based on solubility (S and X) and biodegradability (B and U). The readily biodegradable COD (S_B), the slowly biodegradable COD (X_B), the soluble inert organic matter (S_U) and the particulate organic matter (X_U) were measured. The lower biodegradable COD fractions detected using the SNAD biomass were probably due to inhibition effects of chemical compounds present in the leachate on the SNAD biomass. The obtained characterization could be useful for biological process simulation models, which require a complete influent wastewater characterization.

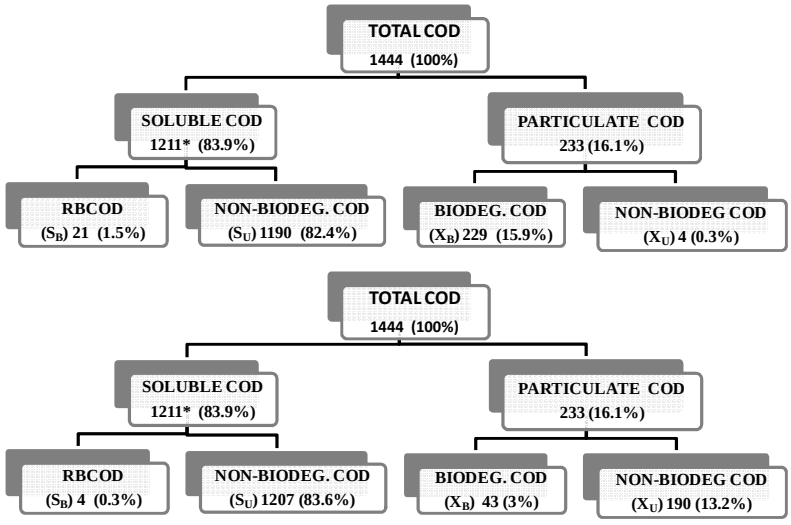

Fig. III.5. COD fractionation of landfill leachate
COD fractionation using (A) an activated sludge of WWTP and (B) a SNAD biomass (mean [mg/L])
* mean of CODsoluble measured by sequential filtration and $ZnSO_4$ coagulation methods.

3.6 Landfill leachate C/N ratio

The low $BOD_5/COD_{filt\_0.45\mu m}$ ratio (0.04) measured with traditional approach indicated that the leachate is characterized by a low biodegradability. The biodegradable fractions measured using the respirometric tests indicated a biodegradability even lower, as confirmed by the $RBCOD/COD_{soluble}$ ratio calculated in this study, around at 0.017.

Further, the results showed that the old landfill leachate is suitable for the applicability of a SNAD process, due to the low biodegradable content compared to the high nitrogen concentration, as confirmed by the low $COD_{soluble}/N$, COD_{bio}/N and RBCOD/N ratios.

A summary of the characteristics of the leachate used in this study together with the characteristics reported in literature of old landfill leachates treated with SNAD processes is given in Table III-5. As can be seen from the Table III-5, the BOD_5/N and $COD_{filt\_0.45\mu m}/N$ ratios measured in this study are comparable to the values previously reported in study on SNAD process (Wang et al. 2010; Xu et al. 2010). However, the truly biodegradable COD/N ratio and the truly RBCOD/N are even lower than the corresponding $COD_{filt\_0.45\mu m}/N$ and BOD_5/N, indicating the possible co-existence of anammox and denitrifiers bacteria (Kumar and Lin 2010) and the applicability of the SNAD process to treat the old landfill leachate.

Table III-5. Characterization of old landfill leachates used in SNAD process and described in literature.

	Traditional				**This study**			
Reference	**$BOD_5/COD_{filt\ 0.45\mu m}$**	**BOD_5/N**	**$COD_{filt\ 0.45\mu m}/N$**	**$Inert/COD_{tot}$**	**$RBBOD/COD_{sol}$**	**COD_{sol}/N**	**COD_{bio}/N**	**RBCOD/N**
Wang (2010)	0.1	0.09	0.87	-	-	-	-	-
Xu (2010)	0.14	0.38	2.67	-	-	-	-	-
This study	**0.04**	**0.06**	**1.1 - 1.5**	**0.8**	**0.017**	**1**	**0.2**	**0.017**

4. Conclusions

A complete characterization of leachate is fundamental both in choosing the most appropriate biological treatment technologies and in modeling the biological processes.

In this study we showed that the combination of physical-chemical and respirometric methods is an appropriate tool for the wastewater characterization. Nevertheless, when an old landfill leachate is analyzed, it is important to (i) perform a careful sampling, (ii) chose the optimal technique to evaluate the soluble and particulate organic and nitrogen matter and (iii) adopt the appropriate procedures for OUR data processing in order to properly evaluate the biodegradable fractions. Concerning the soluble organic matter and the total ammonia concentration we suggest the use of sequential filtration over the filtration at 0.45 µm and the coagulation with $ZnSO_4$ at pH 10.5. Regarding the biodegradable organic COD, the respirometric methods allow to achieve good results. A suitable data processing procedure, especially at the beginning of respirometric tests, is necessary in order to correct the dilution effect, due to the fact that a high volume of leachate over the volume of sludge should be added to the respirometric tests in order to appreciate the biodegradable fractions. The results of the respirometric tests showed that the biodegradability depends on the biomass type (activated sludge or SNAD biomass). In fact the two biodegradable fractions measured, both the S_B and the $X_{B,}$ were lower using SNAD biomass compared to the values measured using the activated sludge. This implies that a proper characterization is essential in modeling biological process which use different biomass type.

Further, as the choice of a biological treatment process is strictly related to the presence of ammonia and biodegradable COD, a correct evaluation of COD components and of the COD_{bio}/N ratio is essential to chose the correct biological process and the technology to apply for treating leachates. In this study, the overall leachate

characterization puts in evidence the presence of both slowly biodegradable COD fractions (X_B) (16% of the total COD) and inhibiting compounds for biological process, such as heavy metals. In order to increase the capacity to degrade substrates usually not biodegraded in continuous conventional wastewater treatment plants and to limit the inhibition effects of some compounds, the use of Sequential Batch Reactors (SBRs) and granular biomass systems could be useful to treat old landfill leachates. Moreover, the analysis of the old leachate showed a high total ammonium concentration and a low COD_{bio}/N ratio which indicate the conventional nitrification denitrification processes are not sustainable biological process. Due to the low readily biodegradable organic matter, a co-existence of anammox and denitrifying bacteria would be possible, avoiding the heterotrophic biomass proliferation, while the high ammonium concentration may allow the development, under oxygen limiting condition, of ammonia oxidizing bacteria over the nitrite oxidizing bacteria, making the SNAD process appropriate to treat old landfill leachates.

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IV. Chapter

Application of the Simultaneous, partial Nitritation, Anammox and Denitrification (SNAD) process to Municipal Solid Waste landfill leachate

This chapter was based on:

Langone M., Miotto V., Andreottola G., 2011. *Innovative biological treatment of wastewater with high nitrogen and low biodegradable matter concentration*. in XIII International Waste Management and Landfill Symposium, Padova: CISA-Environmental Sanitary Engineering Centre, 2011, p. [1-18]. - ISBN: 9788862650007. Proceedings of: Sardinia 2011,

Langone M., Andreottola G. (2013). *Application of the Simultaneous, partial Nitritation, Anammox and Denitrification (SNAD) process to Municipal Solid Waste landfill leachate*. Submitted.

Application of the SNAD process to Municipal Solid Waste landfill leachate

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ABSTRACT

The Simultaneous partial Nitrification, Anammox and Denitrification (SNAD) process for municipal landfill leachate treatment is a new, innovative and significant approach that requires more research to identify and solve critical issues. In this work, the feasibility of the application of the SNAD process in a Sequencing Batch Reactor (SBR) to treat an old landfill leachate has been tested.

First, batch studies were conducted on a synthetic wastewater in order to evaluate a suitable SBR cycle configuration and the SNAD-SBR performances. With an initial total ammonium nitrogen concentration of 68 mg TAN L^{-1} and an initial carbon – nitrogen ratio of 1.5, a total ammonium nitrogen and a total nitrogen removal efficiencies of 100% and 92% were observed, respectively. A high carbon (COD) removal efficiency was achieved (~ 45%). Batch studies on the old landfill leachate were conducted, achieving a total ammonium nitrogen and a total nitrogen removal in the range of 76-87% and 64-79%, respectively. The lower N removal efficiencies were mainly due to the high ammonia-oxidizing bacteria sensitivity to the inhibitors compounds present in the leachate. A lower COD removal was also observed (~ 34%) due to the presence of non-biodegradable or slowly biodegradable organic matter in the leachate.

Influent characteristics, biomass activities and the operative conditions (i.e. pH, DO, T) influenced the SBR cycle configuration set up in the SNAD process. In this study, treating leachate, a good relationship between the SBR cycle configuration and the nitrogen loading rate was determined, in order to achieve a suitable partial nitrification during the first reaction phase of the SBR cycle under oxygen limiting conditions followed by a proper anammox process during the second reaction phase under anoxic conditions.

Keywords: Anammox, Autotrophic Nitrogen Removal, Denitrification, Partial Nitritation, old landfill leachate, Sequencing Batch Reactor, SNAD.

1. Introduction

1.1. Old Landfill Leachate Pollution and treatments

Although the general concept of the sustainable waste management is being increasingly supported by regulators and by the general public, landfilling of waste continues to be a significant waste management method (Wagner 2011). One of the main problems in landfill disposal of Municipal Solid Wastes (MSW) is the generation of leachate, which is a hazardous wastewater. The leachate generally contains a complex mix of inorganic and organic pollutants in dissolved, colloidal or dispersed form (Paxéus 2000): high ammonium nitrogen, variable biodegradable organic (volatile fatty acids) content, organic refractory compounds (humic and fulvic acids), heavy metals, alkalinity, hardness, salinity, chlorinated organic xenobiotic compounds such as benzene and toluene, and numerous other substances that can be considered as pollutants (Kurniawan et al. 2006b). Among them, the most potentially hazardous pollutant is ammonium nitrogen, due to the long-term effects and to its acute and chronic toxicity in aquatic environments (Kurniawan 2009).

Landfill leachate treatment remains one of the greatest problems associated with environmental management of sanitary landfills. A great variety of pre-treatments and combined treatments have been applied to landfill leachates, in order to *i)* improve the biodegradability of refractory and non- biodegradable compounds, such as *advanced oxidation techniques* (Zhang et al. 2006); *ii)* remove heavy metal ions performing *physic-chemical methods* (chemical precipitation, ion-exchange, adsorption, membrane filtration, evaporation, reverse osmosis,

coagulation-flocculation, and electrochemical methods) as recently reviewed by Fu and Wang (2011); iii) achieve the nitrogen and carbon limit standards, by using *physic-chemical methods* (i.e. ammonia stripping, chemical precipitation) (Kurniawan et al. 2006a) and *biological treatment methods*, such as the conventional activated-sludge (Robinson and Maris 1983; Laitinen et al. 2006) and combinations of new innovative nitrogen pathways (Ruscalleda et al. 2008; Xu et al. 2010; Lan et al. 2011).

In particular, many efforts have been made towards developing nitrogen removal processes. Due to their simplicity and high cost-effectiveness, biological treatments are widely applied over physic-chemical methods for nitrogen removal from ammonium rich wastewaters (Mulder 2003). However, the choice of the optimal biological treatment depends on the leachate composition, which is, in turn, a function of several landfill parameters (Deng and Englehardt 2006), such as the type of waste, local weather and climate conditions, age and design of landfill and operation processes. Among them, the age of landfills mainly influences chemical properties, in particular the content of total ammonium nitrogen (TAN), biodegradable chemical oxygen demand (COD_{bio}) and biological oxygen demand (BOD), and provides an important criterium to choose the most adequate biological treatment (Renou et al. 2008). During landfill aging, the concentration of biodegradable carbon and nitrogen organic compounds in leachates normally decreases, while the inorganic ammonium concentration increases. Generally, a high ammonium nitrogen (400-5.000 mgTANL^{-1}), a moderate total organic carbon (5.000-20.000 mg COD L^{-1}), and a low biodegradable organic carbon (20-1.000 mg BOD_5 L^{-1}) characterize the old leachates, which are generated from 10 more years operating landfills (Neczaj et al. 2005; Kurniawan et al. 2006b; Yan and Hu 2009). The resulting BOD_5/COD (< 0.2) and BOD_5/N (< 2.5) ratios are rather low. In particular, the low biodegradable COD/N ratio makes old leachates difficult to be treated by conventional biological process (thus, nitrification and denitrification), as a high oxygen supply should be available for biological nitrification process and supplemental external biodegradable organics have to be added for denitrification, causing a considerable increase in operational costs (Price et al. 2003; Kulikowska and Klimiuk 2008). On the contrary, conventional biological treatments have shown to be very effective in removing nitrogen and organic matter from young landfill leachates, which are generated from 5 less years operating landfills (Laitinen et al. 2006), where the BOD_5/COD and biodegradable COD/N ratios have high values, not requiring an external carbon source to complete the nitrogen removal process. In this context, improved nitrogen biological removal processes have been now implemented to treat old leachates. One of the most challenging process is the Simultaneous partial Nitritation, Anammox and denitrification (SNAD) process in view of its advantages in terms of costs saving (Sri Shalini and Joseph 2012).

The main aim of this study was to investigate the feasibility of the application of the SNAD process in a Sequencing Batch Reactor (SBR) to treat an old landfill leachate, characterized by high ammonia concentration, a low biodegradable COD/N ratio and inhibitory compounds. Simple batch tests were conducted simulating a SBR cycle, evaluating the COD, TAN and TN removal efficiencies. First, batch studies were conducted on a synthetic wastewater in order to evaluate a suitable SBR cycle configuration and the SNAD-SBR performances. Then, the inhibition effects due to the landfill leachate composition on the SNAD biomass were investigated and the optimal SBR configuration was evaluated.

Abbreviations

Parameters		**Reactor Configurations**	
BOD	Biological Oxygen Demand	AUSB	Aerated Upflow Sludge Bed
COD	Chemical Oxygen Demand	CSTR	Continuous Stirred Tank Reactor
CODtot	Total Chemical Oxygen Demand	FBBR	Fixed Bed BioFilm Reactor
CODfiltr	Filtrate Chemical Oxygen Demand	GSBR	Granular Sequencing Batch Reactor
CODsoluble	Soluble Chemical Oxygen Demand	HBR	Hybrid Biofilm Reactor
CODbio	Biodegradable Chemical Oxygen Demand	MBR	Membrane Bioreactor
FA	Free Ammonia (NH_3 -N)	RBC	Rotating Biological Contactors
FNA	Free Nitrous Acid (HNO_2-N)	SBBR	Sequencing Batch Biofilm reactors
N	Nitrogen	SBR	Sequencing Batch Reactors
NO_3^--N	Total Nitrate Nitrogen	UASB	Upflow Anaerobic Sludge Blanket
OUR	Oxygen Uptake Rate		
RBCOD	Readily biodegradable substrate	**Bacteria**	
SBCOD	Slowly biodegradable substrate	AOB	Ammonia-Oxidizing Bacteria
TAN	Total Ammonia Nitrogen	NOB	Nitrite-Oxidizing Bacteria
TNN	Total Nitrite Nitrogen	Anammox	ANaerobic AMMonia -OXidizing Bacteria
TKN	Total Kjeldahl Nitrogen		
TSS	Total Suspend Solid	**General**	
VSS	Volatile Suspend Solid	MSW	Municipal Solid Wastes
		WWTP	Wastewater Treatment Plant
Operative		**Conversion rates**	
DO	Dissolved Oxygen	ν TAN aerobic	aerobic total ammonia removal rate
HRT	Hydraulic Retention Time	ν TNN aerobic	aerobic total nitrite removal rate
KLa	Mass transfer coefficient for oxygen	ν N-NO_3^- aerobic	aerobic nitrate production rate
NLR	Nitrogen Loading Rate	ν TAN anoxic	anoxic total ammonia removal rate
OLR	Organic Loading Rate	ν TNN anoxic	anoxic total nitrite removal rate
ORP	Oxidation-Reduction Potential		
VER	exchange volume ratio	ν N-NO_3^- anoxic	anoxic nitrate conversion rate

Process	
CANON	Completely Autotrophic Nitrogen removal Over Nitrite (CANON)
OLAND	Oxygen-Limited Autotrophic Nitrification/Denitrification
SHARON	Single reactor High activity Ammonia Removal Over Nitrite
SNAD	Simultaneous partial Nitritation, Anammox, Denitrification

1.2. General overview: Innovative biological treatments for old landfill leachate

Since the discovery (Mulder et al. 1995) and the identification (Strous et al. 1997) of anammox bacteria, the traditional theory of biological nitrogen removal has been extended to new nitrogen metabolic pathways. Anaerobic ammonium oxidation (anammox) is an autotrophic process in which bacteria of the phylum Planctomycetes combine, under anoxic conditions, ammonium and nitrite to yield di-nitrogen gas, producing small amounts of nitrate (Strous et al. 1999b). As the anammox process requires nitrite, it should be coupled with the first step of nitrification process (nitritation) in order to oxidize ammonia to nitrite (Hellinga et al. 1998). Nitritation and anammox processes built a shortcut in the nitrogen cycle, allowing time and economic savings. Consequently, several nitrogen removal processes have been developed in wastewater treatment and applied to old leachates, as recently reviewed by Sri Shalini and Joseph, (2012).

Different reactor configurations have been investigated to perform the anammox process treating old landfill leachates: Rotating Biological Contactors (RBC) (Siegrist et al. 1998; Egli et al. 2001; Lv et al. 2011); Upflow Anaerobic Sludge Blanket (UASB) reactor (Zhang and Zhou 2006); up-flow Fixed Bed Biofilm reactor (Liang and Liu 2008) and Sequencing Batch Reactors (SBR) (Ruscalleda et al. 2008).

Treating leachates with anammox process, due to the presence of biodegradable organic matter in the influent, heterotrophic denitrification process could occur. However, as old leachates are characterized by a low biodegradable COD/N ratio, denitrifiers will not outcompete anammox bacteria (Kumar and Lin 2010). The co-existence between anammox and heterotrophic denitrifiers has been performed in different anaerobic reactor configurations, such as the UASB reactor (Zhang and Zhou 2006) and the SBR (Ruscalleda et al. 2008, 2010).

The nitritation process was studied as well as implemented in leachate full scale applications. Different strategies have been adopted to inhibit the second step of nitrification process (nitratation) in order to have the nitrite accumulation: *i)* working at high ammonia concentrations (Anthonisen et al. 1976), *ii)* working under oxygen-limited conditions (Wyffels et al. 2004) and *iii)* operating at temperatures above 25°C and without retention sludge (Hellinga et al. 1998), which is the base of the Single reactor High activity Ammonia Removal Over Nitrite (SHARON) process (Van Hulle et al., 2007). Several reactor configurations have been found to be suitable to perform the nitritation process treating old leachates, among them the continuous stirred tank reactor (CSTR) (Vilar et al. 2010), the Fixed Bed BioFilm Reactor (FBBR) (Liang and Liu 2008), the membrane bioreactor (MBR) (Canziani et al. 2006) and the SBR (Ganigué et al. 2009, 2012; Gabarró et al. 2012; Li et al. 2012).

Moreover, innovative combinations of the biological processes were implemented for treating leachates, by either using separate reactors in series or accomplish two or more processes in the same reactor (single reactor).

Separate reactors in series (Nitritation-Anammox/Denitrification). Xu et al., (2007a) treated a leachate with the SHARON process carried out in a hybrid biofilm reactor (HBR) followed by the anammox process in an anaerobic baffled reactor (ABR). Ruscalleda et al., (2008) studied an urban landfill leachate treatment by the SHARON and anammox processes in two SBR systems. Liu et al., (2010) showed that organic matter in the leachate can be degraded in an UASB reactor, then the effluent from this was passed into an aerated upflow sludge bed (AUSB) reactor for the nitritation process and further into an ammonium oxidation upflow anaerobic sludge reactor for anammox process. Liang and Liu, (2008) treated a leachate by setting two up-flow fixed bed biofilm reactors to perform partial nitritation and anammox process followed by two underground soil infiltration systems for the purification of the remaining pollutants. Later, the same authors showed that in the anammox reactor a decomposition and mineralization of aquatic humic substances was obtained (Liang et al. 2009). Mangimbulude et al., (2012) investigated and proposed a complete removal of ammonium from a leachate, through a two-stage treatment system at landfilling site, where the first aerated leachate pond could be used for the nitritation process, while in the second pond the remaining ammonium and produced nitrate can be converted by a combination of nitrate reduction to nitrite (denitrification) and anammox.

Single reactor (Nitritation/Denitrification/Anammox). Nitrification and denitrification *via* nitrite in a one-single system was implemented for the leachate treatment in an SBR (Spagni et al. 2008; Spagni and Marsili-Libelli 2009) and in a Granular Sequencing Batch Reactor (GSBR) (Wei et al. 2012). In a complete absence of organics in leachates, Sun and Austin, (2007), demonstrated that the nitritation and anammox process took simultaneously place in a subsurface vertical flow wetlands. The occurrence of Simultaneous partial Nitrification, Anammox and Denitrification (SNAD) process in one single reactor, under oxygen limitation, was observed in a single partially aerated full-scale bioreactor treating an old landfill leachate (Wang et al., 2010). SNAD process is one of the most interesting performing nitrogen removal process. It is carried out by bacteria with opposed environmental requirements and it comprises three simultaneous, interacting and sequential processes: *i*) an oxygen-limited (micro-aerobic) process, *partial nitritation* Eq. V-1(Eq. IV-1), where 50% of

ammonia is oxidized to nitrite; *ii*) an anoxic process, a*nammox* (Eq. IV-2), where ammonia and nitrite are converted to nitrogen gas producing a small amount of nitrate (Strous et al. 1999b) and where a small amount of organics could be oxidized to CO_2 reducing nitrate or nitrite to di-nitrogen gas (Kartal et al. 2007b); *iii*) another one anoxic process, *denitrification* (Eq. IV-3) - (Eq. IV-4), where the biodegradable COD (COD_{bio}), as electron donor, could deoxidize nitrate and nitrite to N_2 gas.

$$NH_4^+ + 0.69O_2 + 0.99HCO_3^- \xrightarrow{\text{AOB autotrophic bacteria}} 0.49NO_2^- + 0.01C_5H_7O_2N + 0.5NH_4^+ + 0.52H_2O + 0.94H_2CO_3 \quad \text{(Eq. IV-1)}$$

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \xrightarrow{\text{anammox autotrophic bacteria}} 1.02N_2 + 0.26NO_3^- + 2.03H_2O + 0.066CH_2O_{1.5}N_{0.15} \quad \text{(Eq. IV-2)}$$

$$5C + 4NO_3^- + 2H_2O \xrightarrow{\text{heterotrophic bacteria}} CO_2 + 4HCO_3^- + 2N_2 \quad \text{(Eq. IV-3)}$$

$$3C + 3.5NO_2^- + H_2O \xrightarrow{\text{heterotrophic bacteria}} CO_2 + 2HCO_3^- + 1.75N_2 \quad \text{(Eq. IV-4)}$$

In addition, in the SNAD process, secondary biological processes may take place, i.e. aerobic nitrite oxidation (Wett et al. 2010), nitrite – dependent anaerobic methane oxidation (Luesken et al. 2011c), some of them compromising the total nitrogen removal efficiency. As consequence, a well balance co-existence of different microbial communities in the SNAD reactor is a necessary requirement to stable perform the biogical nitrogen removal process. In that aspect, the reactor configuration and the operative conditions play an important role. Only few studies have been carried out the SNAD process for landfill leachate treatments (Table IV-1). Xu et al., (2007b) showed a cooperative denitrification mechanism with partial nitrification and anammox processes in a Sequencing Batch Biofilm reactors (SBBR), treating landfill leachates, under an intermittently aeration, at low oxygen dissolved concentration (DO < 1.2-1.4 mgL^{-1}) and high temperatures (T = 32°C). Xiao et al., (2009) confirmed, through molecular analysis, that the ammonia removal from the leachate in a sequencing batch biofilm (SBBR) reactor was achieved thanks to the co-existence of nitrifiers, denitrifiers and anammox bacteria. Wang et al., (2010) demonstrated the occurrence of simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) process in a leachate full-scale CSTR under oxygen-limited conditions (DO = 0.3 mgL^{-1}) and high temperatures (T = 30-33°C). The anammox process accompanied with autotrophic nitrification and heterotrophic denitrification in one rotating biological contactor (RBC) was investigated at low temperatures (T < 20°C) (Cema et al. 2007).

To perform the SNAD process, the Sequencing Batch Reactors (SBR) and the Granular Sequencing Batch Reactors (GSBR) have some advantages over the other reactor configurations, as they require a lower space and capital costs, and they are characterized by higher nitrogen removal efficiencies and shock resistances (Artan et al. 2003; Mohan et al. 2005). The great process flexibility of the SBR is very important when considering wastewaters as leachates, which have a high degree of variability (Kennedy and Lentz 2000) and a low COD/N (Yan and Hu 2009). The SBR flexibility, optimizing the reaction phases over the total length of the cycle, allows to regulate the biological processes, in order to achieve an acceptable nitrogen removal. Moreover, the granular biomass, in the GSBR technology, has some advantages with respect to the suspended biomass, especially for anammox-like systems which are characterized by extremely low growth rate (doubling time 11 days) and inhibited by several compounds (oxygen dissolved, nitrite, inorganic compounds, *etc*). The granular biomass allows both the accumulation of large biomass concentrations in reactors without carrier materials and the existence of substrate gradients, preventing inhibitions and causing aerobic and anaerobic conditions favorable for the coexistence of different microorganisms in one single system (Vázquez-Padín et al. 2010). Nevertheless, experiences with SNAD process for the landfill leachate treatment carried out either in an SBR or in a GSBR are

very few (Table IV-1). Lan et al., (2011) studied the simultaneous partial nitrification, anammox and denitrification (SNAD) process for ammonia removal from a synthetic wastewater, characterized by a low COD/N ratio, using a SNAD seed sludge collected from a biological treatment unit (aeration tank) of a full-scale landfill-leachate. The authors investigated the influence of hydraulic retention times (HRT = 3-9 d) on the performance of a GSBR, operating under high temperatures (T=35°C) and a low oxygen level (DO =0.5-1.0 mgL^{-1}).

Xu et al., (2010) showed that an integration of partial-nitrification process, anammox process and heterotrophic denitrification process may be applied to the SBR system treating leachate with periodical air supply (DO = 1.0-1.5 mgL^{-1}) and high temperatures (T = 30°C).

Table IV-1 Overview of typical SNAD process applied to landfill leachate and synthetic wastewater

Reference	Chen et al. 2009	Lan et al. 2011	Xu el al. 2010	Xu el al. 2010	Xiao et al 2009	Wang et al. 2010
Wastewater	Synthetic wastewater	Synthetic wastewater	Synthetic wastewater	Landfill Leachate	Landfill Leachate	Landfill Leachate
CODbio/N	1:2	1:2	0	1:2.6	nd	1:10
Reactor type	NRBC	SBR	SBR	SBR	SBBR	Aeration tank
Volume [L]	small	18 L	3 L	3 L	3 L	384 m^3
SBR configuration	nd	24h cycle	12h cycle	12h cycle	24 h cycle	
Fill	nd	continuous	intermittent	intermittent	intermittent	continuous
Aeration mode	continuous	continuous	intermittent	intermittent	intermittent	continuous
DO [mg L^{-1}]	0.4 – 0.6	0.5 – 1.0	1.0 – 1.5	1.0 – 1.5	0.6	0.3
Inoculum	nitrifying biomass+ anammox sludge	SNAD sludge	active sludge+ anammox sludge	active sludge+ anammox sludge	active sludge	active sludge
HRT	5-6 h	3 d	1.5 d	1.5 d	2-4 h	1.26 d
SRT [d]	nd	very high	-	-	nd	12-18
pH	nd	7-8	7.2-8	7.2-8	7.8-8.54	7.4
T [°C]	35	35	30	30	nd	30
VSS [g L^{-1}]	nd	-	1.2	1.5	nd	1.5
OLR [g COD L^{-1} d^{-1}]	0.69	0.033	0	2.6	-	0.44
NLR [g TN L^{-1} d^{-1}]	0.34	0.066	0.187	0.961	-	0.5
Removal rate [g TN L^{-1} d^{-1}]	nd	0.066*	0.140*	0.896*	-	0.38*
TN tot removal [%]	70%	92,6% (85% PN+ Amx; 7,3% Den)	65%	92%	-	75% (%68 PN+ Amx; 8% Den)
TAN removal [%]	79%	93%	70%	92%	97%	80%
COD removal [%]	94%	72%	0	95% BOD (6,7% COD)	86%	28% (denitrify)

PN = Partial Pitritation, Amx = Anammox, Den = Denitrification
*value calculated

2. Materials and Methods

2.1. Feeding media and old landfill leachate

The synthetic wastewater was prepared according to Third et al., (2001) and optimized for the SNAD process, as no nitrite was added while a carbon source and alkalinity were introduced in the medium. Its composition was similar to that of old landfill leachates, in terms of total ammonia nitrogen (TAN = 900 mgL^{-1}) and total soluble organic matter ($COD_{soluble}$ = 1000 mgL^{-1}) content. Ammonium, as NH_4Cl, was added to the medium in the required amounts and alkalinity, as $KHCO_3$, was added according to the nitritation stoichiometry. COD, as CH_3COONa, was added in a low soluble COD to N ratio (~1). The composition of the synthetic wastewater is reported in the Supplementary Table IV-1.

The raw old landfill leachate was collected from a municipal landfill (Trento, Italy) and stored at 4°C in order to prevent the COD and nitrogen degradation. The chemical characterization of the old landfill leachate was obtained over a period of 1 year. The leachate was characterized by a moderately high total and soluble COD content (1444 $mgCOD_{total}\ L^{-1}$ and 1273±194 $mgCOD_{soluble}\ L^{-1}$), by a high total ammonia concentration (1183 mg TAN L^{-1}), and by a low biodegradable and readily biodegradable COD content, 250 mg $COD_{bio}\ L^{-1}$ and 21 mg RBCOD L^{-1}, respectively. The leachate was characterized by very low C/N ratios: $COD_{soluble}/N \sim 1$; biodegradable $COD_{bio}/N \sim 0.2$, $RBCOD/COD_{total}$ = 0.02. The average values of the principal compounds concentrations are summarized in the Supplementary Table IV-2.

2.2. Seed sludge

The SNAD seed sludge was collected from a biological treatment unit (SBR) of the full-scale Municipal Wastewater Treatment Plant, Zürich (Switzerland). The SBR treated the digester supernatant, at high temperature (T=30°C). The operating conditions of the SBR (1400 m^3) were: DO < 0.8 mgL^{-1}, pH ~ 7.1±0.2, T ~ 30°C, MLSS ~ 3.6 kg $TSSm^{-3}$, N- load ~ 0.625 kg Nm^{-3} d, HRT ~ 1.45 d (Joss et al. 2009). In the Zürich SBR, partial nitrification, anammox and denitrification process was full achieved (Joss et al. 2009). The seed sludge contained granules surrounded by a matrix of brownish flocs. Two kinds of granules (red and brown) could be distinguished. The granules had diameters between 0.5 and 2.0 mm. Molecular analysis of the original inoculum showed the presence of ammonia-oxidizing bacteria of the N*itrosomonas europaea/eutropha* group, anaerobic ammonium-oxidizing bacteria of the "*Candidatus* Brocadia fulgida" type and denitrifying bacteria related to the *Thauera* and *Pseudomonas*. Nitrite-oxidizing bacteria from the genus *Nitrobacter* were also detected (Langone et al. 2013b). The authors showed long filamentous - shaped microorganisms were dominant in the outer layer of the granules, while single cells as well as aggregates were trapped in a net of filamentous bacteria.

2.3. Batch tests

Batch tests were carried out in a lab-bioreactor, equipped as a Sequence Batch Reactor (SBR) and inoculated with the SNAD sludge harvested from the Zürich full-scale SBR. The experimental reactor with its instrumentation and control system is schematized in Fig. IV.1. The cylindrical reactor (working height 0.22 m, inner diameter 0.152 m) made of acrylic plastic had a total working volume of 4 L. The system was fitted with a thermostatic jacket to maintained a fixed temperature of 30°C±1°C. The reactor was equipped with a mixer, acid/base pumps and a set of two peristaltic pumps to introduce the feeding solution and to discharge the effluent. Air was supplied by a Schego M2K3 350 air pump and the gas-flow was maintained constant at 20 nL h^{-1} using a mass-flow meters.

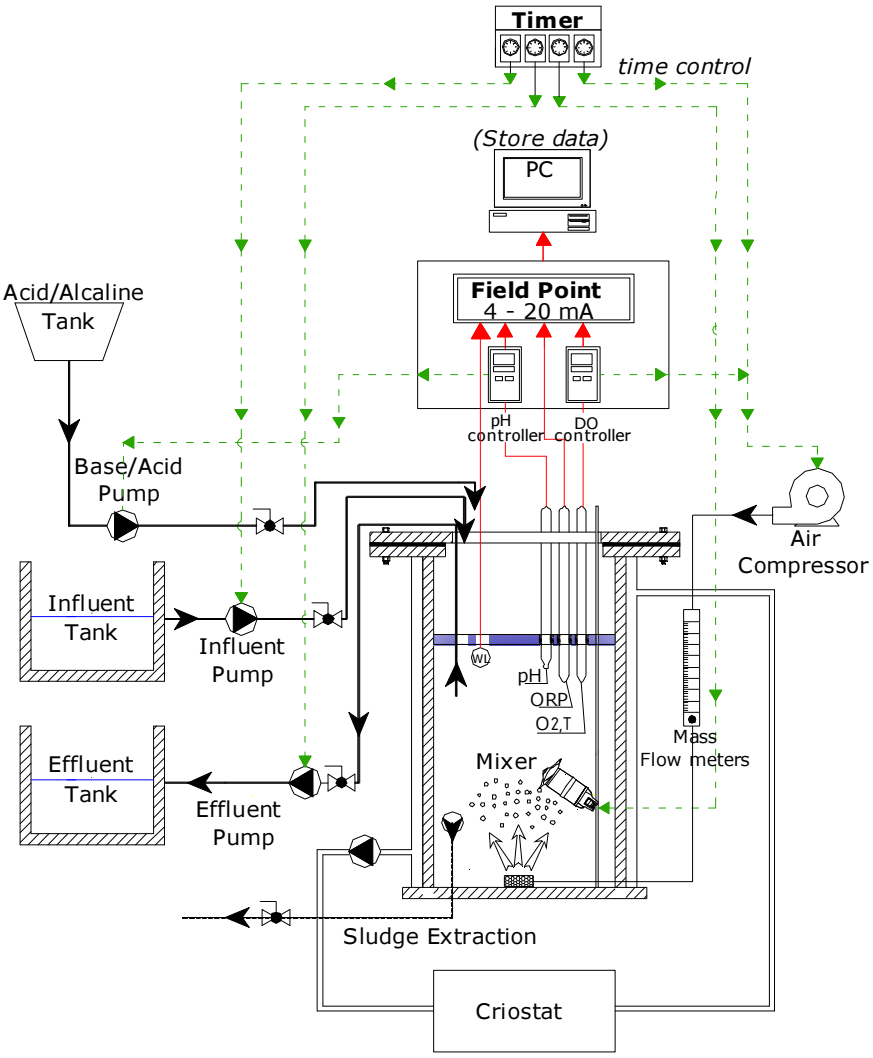


Fig. IV.1 Schematic representation of the 4L lab-scale reactor used for batch tests..

Air was bubbled through n. 4 diffusers at the bottom of the reactor to promote the oxygen transfer into the bulk liquid and to support a suitable mixing. The mass transfer coefficient for oxygen (KLa) was calculated and fixed at a sufficiently high value (0.22 min^{-1} at 20°C), in order to ensure the dissolved oxygen set-point can be attempted with the on/off control (Sin et al. 2004). The initial total suspended solids (TSS) concentration of the whole sludge was 3-4 g TSS L^{-1}. The volatile suspended solids (VSS) were about 70% of total suspended solids (TSS). In the SBR, a high biomass retention was obtained by choosing a suitable settling time in order to have a minimum biomass settling velocity of 0.7 m h^{-1}, which is the ratio between the vertical distance from the water

surface to the effluent discharge point and the settling time (Vlaeminck et al. 2009a). This value means that bio-particles having a settling velocity less than 0.7 m h^{-1} could be withdrawn through the discharge effluent. The exchange volume ratio (VER) and the hydraulic retention time (HRT) were variable depending on the nitrogen loading rate (NLR) applied.

Each batch test lasted for 4 or 6 hours distributed according to the scheme described in Fig. IV.2, simulating an SBR cycle time. Each cycle comprised an unaerated and short filling phase (t_{fill}=3-5min), a micro-aeration phase ($t_{microaer}$ = 1–4 h), an anoxic phase (t_{anoxic} = 1.8–3.0 h), a settling phase (t_{settle} = 5–7 min) and a draw phase (t_{draw} = 10-15 min). The filling phase was mixed and short, creating conditions under which the growth rate is higher for floc-formers than for filaments (Artan and Orhon 2005). The short filling phase ensured that the biomass was exposed initially to a high nitrogen load, and consequently to a high concentration of Free Ammonia (FA), inhibiting the nitrite- oxidizing bacteria (NOB) (Anthonisen et al. 1976). Further, during the mixed fill, heterotrophic bacteria may degrade the organics using either residual oxygen or alternative electron acceptors, such as nitrite, nitrate and sulphate. The reaction phase of the SBR took place after the filling period. A first reaction phase under oxygen-limited conditions (micro-aerobic phase) preceded a second anoxic reaction phase (anoxic phase), as a partial nitritation step is necessary to supplies both NH_4^+-N and NO_2^--N and attain a suitable influent for anammox process. The micro-aeration phase took place at the end of the short filling phase (when in the reactor it was supposed that the maximum ammonia concentration had been achieved). Further micro-aerobic phase was conducted under oxygen limitation (DO < 0.3 mg L^{-1}), in order to further inhibit the NOB activity by low oxygen concentration, due to the lower oxygen affinity of NOB over the AOB (Wiesmann 1994). During the anoxic phase, anammox and denitrification processes occurred. After the micro-aerobic and the anoxic phases (reaction phase), the stirrer was stopped and the biomass aggregates and granules were allowed to settle for 5-7 minutes. In the remaining minutes of the cycle, the supernatant was removed. The length of the SBR cycle and the length of micro-aerobic and anoxic phases depended on both the nitrogen loading rate and the performances of the SNAD process. In this study the phases length of the single batch tests was manually fixed and controlled by programmable timers.

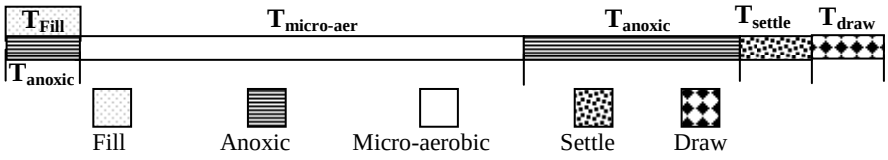
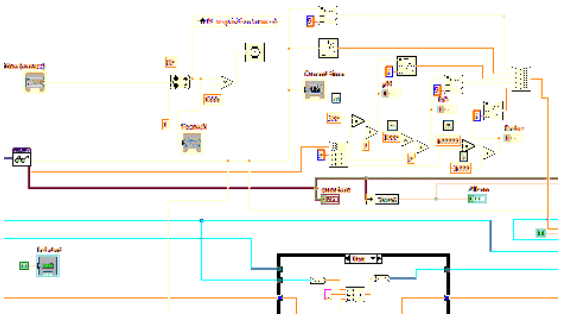


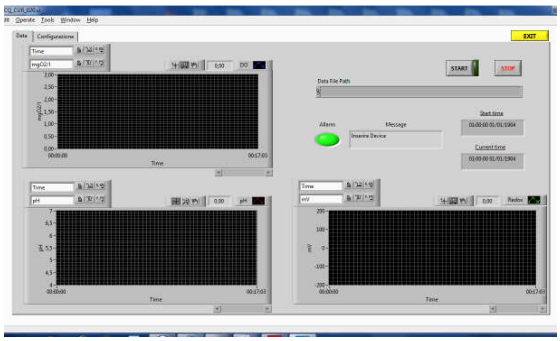
Fig. IV.2 Schematic representation of the SBR cycle configuration

The monitoring system of the batch tests consisted in a data acquisition system (Field Point FP 1000, National Instruments, Austin, TX, USA) and our own software developed using LabVIEW 2010 software (National Instruments, Austin, TX, USA) (Fig. IV.3). The acquisition software developed was able to acquire the on-line parameters, presenting them in a graphical interface. The FP-1000 FieldPoint network module connected an industrial RS-232 network to a FieldPoint analog input module with eight analog voltage or current input channels, 16-bit resolution (FP-AI-110). The FP-AI-110 channel configuration is summarized in the Supplementary Table IV-3. On-line data provided by pH, ORP, DO and temperature sensors were automatically acquired and stored. The pH and ORP were measured using Hanna Instruments electrodes (Hanna Instruments, Italy). The dissolved oxygen (DO) was measured using a polarographic cell (B&C Electronics, Italy). Alarms

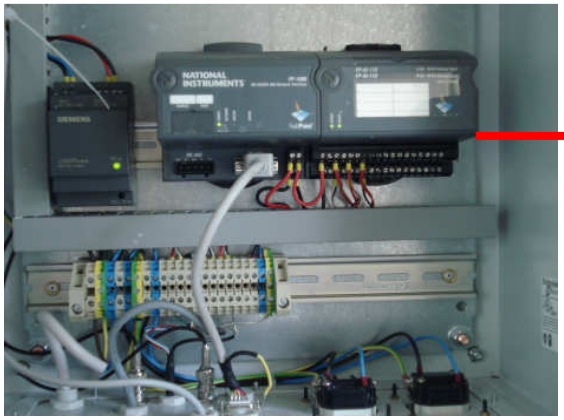
and protective modes were programmed into the system. A liquid detection sensor was installed in the reactor. In case of leachate overflows and touch of the liquid detection sensor, a relay control could automatically stop the influent pump.



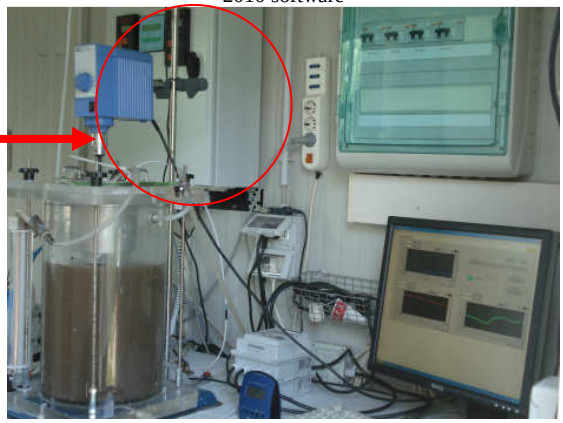
A. Part of monitoring software code developed using LabVIEW 2010 software



B. Software for on-line monitoring developed using LabVIEW 2010 software



C. Acquisition hardware (Field Point FP 1000and FP-AI-110)



D. Installation of data acquisition system

Fig. IV.3 SBR monitoring and control system.

The SBR cycle was managed using a multiple programmable timers combined with a pH and a DO controller. Timers and controllers were connected with the mechanical devices (pumps, mixer and air pump). During the whole cycle, the pH was controlled using a biocontroller (Hanna Instruments, Italy) and it was allowed to vary in the wide range of 7.1 - 8.5, by the addition of acid (1 N HCl) and alkaline (1 N NaOH) solutions. During the micro-aerobic phase, the DO was controlled by a programmable logic controller system (B&C Electronics, Italy), ensuing a low DO concentration around 0.1 – 0.3 mg/L. The ORP was left free to vary.

2.4. Experimental procedure

Subsequent batch tests, simulating one cycle of the operation of SBR, were conducted. The experiments were divided into three periods. In the period I, a first run of assays was carried out to assess the performance of the SNAD process for treating ammonium rich wastewaters with a low COD_{bio}/N ratio. The batch tests were fed with a synthetic medium, where inhibitors were not present, characterized by a COD and TAN concentration of 1000 and 900 mgL^{-1}, respectively. The SBR cycle was modified in order to achieve a partial nitritation process during the micro-aerobic phase and reach, at the beginning of the anoxic phase, a suitable remaining TAN and produced TNN ratio for the later anammox process, assuring the maximum total nitrogen (TN) removal over the SBR cycle. In this run, the biomass was gradually acclimatized to the high total ammonia concentration, by filling the SBR with increasing volumes of synthetic wastewater at the beginning of each cycle. The initial total ammonium nitrogen (TAN) concentration was gradually increased and a low (SW1) and a moderate (SW2) load tests were

operated. At the beginning of the each SBR cycle, TAN concentrations in the reactor were 22.5 mg TAN L^{-1} in SW1 and 66 mg TAN L^{-1} in SW2. The total (TSS) and volatile (VSS) suspended solids content in the reactor were in the range of 3.3-3.5 gTSS L^{-1} and 2.3-2.5 gVSS L^{-1}, respectively. Other details of the batch tests are reported in Table IV-2.

During the period II, the reactor was fed with the raw leachate from a municipal landfill (Trento, Italy), in order to study the applicability of the SNAD–SBR process to the treatment of landfill leachates, which could inhibit the biological activity, as toxic compounds are present. The SNAD biomass, after the inoculation and the start up period using the synthetic wastewater, was gradually acclimated to the old landfill leachate, by filling the SBR with increasing volumes of the leachate at the beginning of each cycle. The raw leachate was characterized by mean COD and TAN concentrations of 1273 mg $COD_{soluble}L^{-1}$ and 1183 mg $TANL^{-1}$, respectively, and a low COD/N ratio. Starting from the results of the first run, three batch experiments (A, B, and C) were performed in order to gradually reach the maximum nitrogen loading rate per cycle applied in the Period I (0.067 kgTAN m^{-3} $cycle^{-1}$). TAN concentrations in the reactor in the three preformed tests, at the beginning of each SBR cycle, were 38, 66, and 81 mgTAN L^{-1}, respectively. The length of the SBR cycle and the duration of the micro-aerobic and anoxic phases were optimized, depending on the initial TAN concentration. The total (TSS) and volatile (VSS) suspended solids content in the reactor were around at 2.7-3.3 gTSS L^{-1} and 1.9-2.3 gVSS L^{-1}, respectively. The details of the batch tests are reported in Table IV-2.

Finally, in a third run, period III, the effects of the high total ammonia concentration on the SNAD performance were investigated. Three high load batch tests (D, E, F) were conducted using increasing the ammonia loading rate and consequently the total ammonia concentrations at the beginning of the 6 h SBR cycles (Table IV-2). The high load tests were then compared with the moderate load tests, looking at the performance of the SNAD process, in terms of nitrogen and carbon removal efficiency.

Table IV-2 Operative strategies during Period I, II and III

		Synthetic wastewater		Old landfill leachate					
		Period I		Period 2			Period 3		
Parameters	**u.m.**	**SW.1**	**SW.2**	**A**	**B**	**C**	**D**	**E**	**F**
NLR per cycle	[kgTAN m^{-3} $cycle^{-1}$]	0.0125	0.0675	0.038	0.065	0.0825	0.105	0.128	0.167
OLR per cycle	[kgCOD m^{-3} $cycle^{-1}$]	0.015	0.075	0.040	0.070	0.085	0.11	0.135	0.175
V fill	[L]	0.06	0.3	0.12	0.21	0.26	0.34	0.41	0.54
Duration of the different SBR cycles									
Total cycle length	[h]	6	6	4	6	6	6	6	6
Filling time	[min]	3-5	3-5	3-5	3-5	3-5	3-5	3-5	3-5
Micro-aeration time	[h]	2.5	2.5	1.3	2.5	3.5	3.5	4.0	3.5
Anoxic time	[h]	2.8-3.0	2.8-3.0	2.2-2.4	2.8-3.0	1.8-2.0	1.8-2.0	1.3-1.5	1.8-2.0
Settling time	[min]	5-7	5-7	5-7	5-7	5-7	5-7	5-7	5-7
Effluent withdrawal	[min]	10-15	10-15	10-15	10-15	10-15	10-15	10-15	10-15

After the addition of both the synthetic wastewater and the leachate, samples were taken, filtered, and analyzed immediately at a certain interval (30-60 min). The carbon and nitrogen performances of the SNAD process were investigated depending on the type of the wastewater and on the configuration of the SBR cycle.

2.5. Analytical methods

Total suspended solid (TSS), volatile suspended solid (VSS), chemical oxygen demand (COD) and total Kjeldahl Nitrogen (TKN) were determined according to the Standard Methods (APHA-AWWA-WPCF 2005).

The COD was analyzed taking into account the chloride interference and further recalculated considering the interference due to the presence of nitrite (nitrite exerts a COD of 1.142 mgO_2/mg TNN).

According to Langone and Andreottola (2013), the raw leachate samples were deeply analyzed for the truly soluble COD ($COD_{soluble}$) and TAN concentrations by using the sequential filtration technique (0.45 and 0.2 µm nominal pore size), for total biodegradable (COD_{bio}) and readily biodegradable organic matter (RBCOD) by using a combination of chemical and respirometric techniques. Ferrous ions (FeII) were determined using a UV/Vis spectrophotometer, while Ferric ions (FeIII) through colorimetric analysis. Metallic ions, among them Manganese, were measured using the inductively coupled plasma mass spectrometry (ICP-MS) (EN ISO 17294-2:2004). Dissolved sulphide (S^{2-}) and hydrogen sulfide (H_2S) were measured according to APAT CNR IRSA, 2003. Alkalinity determinations were conducted using the kit USEPA Buret Titration Method 8221 (Hach Company).

The reactor effluent and the samples of the batch tests were filtered through 0.45 filters, as this procedure required lower amount of sample and was time saving over the sequential filtration. The filtrate was analyzed colorimetrically for total ammonium as nitrogen (TAN) and total nitrite as nitrogen (TNN) (APAT CNR IRSA 2003). Nitrate as nitrogen (NO_{3-}-N) and chemical oxygen demand ($COD_{filtrate}$), were measured according to the Standard Methods (APHA-AWWA-WPCF 2005).

The mass transfer coefficient for oxygen in the reactor (KLa) was estimated following the standard method of measurement of the transfer of oxygen in clean water published by the American Society of Civil Engineering (ASCE 1992).

2.6. Calculations

Concentrations of free ammonia (FA or NH_3-N) and free nitrous acid (FNA or HNO_2-N) were calculated using the equations (Eq. IV-5) - (Eq. IV-7) derived from acid–base equilibrium, as a function of pH, temperature and the sum of unionized and ionized forms, TAN and TNN, respectively (Anthonisen et al. 1976):

$$FA\left[mgNL^{-1}\right] = \frac{TAN}{1+\left(10^{-pH}/K_e^{+NH}\right)} \quad \text{(Eq. IV-5)}$$

$$FNA\left[mgNL^{-1}\right] = \frac{TNN}{1+\left(K_e^{NO}/10^{-pH}\right)} \quad \text{(Eq. IV-6)}$$

$$K_e^{NH} = e^{-6344/(273+T)} \text{ and } K_e^{NO} = e^{-2300/(273+T)} \quad \text{(Eq. IV-7)}$$

The efficiency of total ammonia nitrogen (TAN), total nitrogen (TN) and organic matter (COD) removal were estimated according to the following equation:

$$Efficiency\ [\%] = \left(\frac{C_{ini} - C_{eff}}{C_{ini}}\right) \cdot 100 \quad \text{(Eq. IV-8)}$$

where C_{ini} is the concentration in the liquid after the instant fill phase and C_{eff} is the concentration in the effluent at the end of the SBR cycle.

3. Results

3.1 Period I – feasibility of SNAD process

The batch tests conducted using the synthetic wastewater indicated that a simultaneous carbon and nitrogen removal in one single reactor, under oxygen limitation, from wastewater characterized by a low biodegradable COD and N ratio (C/N = 0.2) was feasible. Fig. IV.4 shows the profiles of nitrogen species over the SBR cycles of the SW1 and SW2 tests, distinguishing the micro-aerobic and the anoxic phases. The nitrogen conversion rate in both the micro-aerobic and the anoxic conditions were evaluated and reported in Table IV-3, together with the nitrogen and carbon removal efficiencies.

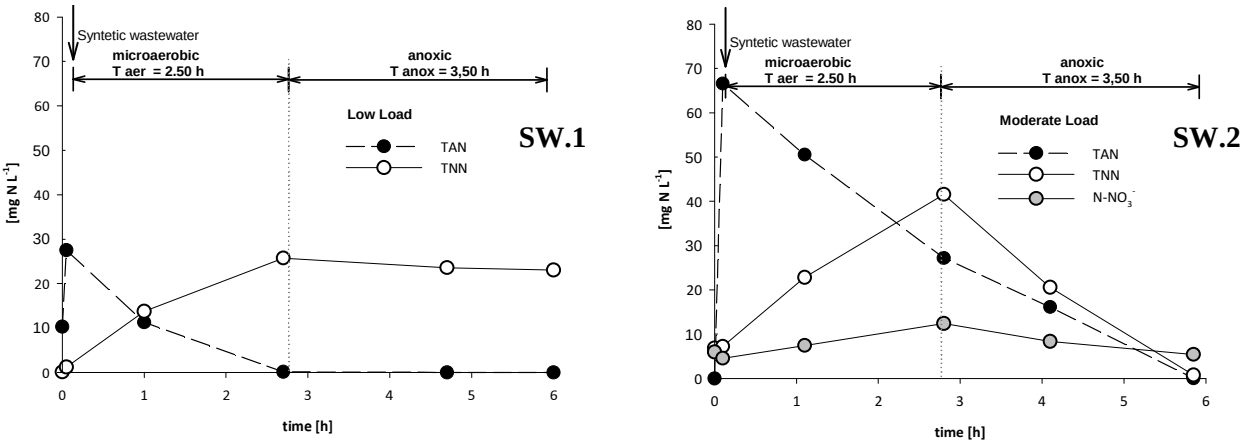


Fig. IV.4 Evolution of nitrogen species over a 6 h SBR cycle using synthetic wastewater: (SW.1): NLR = 0.012 kgTAN $m^{-3}cycle^{-1}$ and (SW.2): NLR = 0.067 kgTAN $m^{-3}cycle^{-1}$

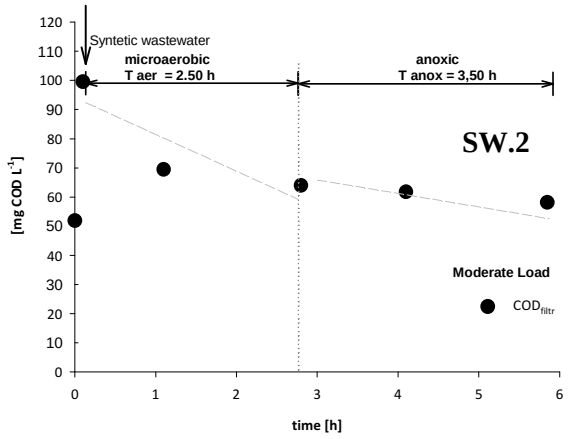


Fig. IV.5 Evolution of COD over a 6 h SBR cycle using synthetic wastewater: (S2):OLR=0.075 kgCOD m^{-3} $cycle^{-1}$

It is clearly shows that during the micro-aerobic phase the nitritation process occurred, as TAN was removed and simultaneously TNN was produced. During the micro-aerobic phase, the nitrate profiles and the low values of the aerobic nitrate production rate (ν NO_3-N aerobic) showed a low activity of the aerobic nitrite oxidizing bacteria (NOBs), which were partially inhibited in the SNAD biomass. The aerobic total ammonia removal rate (ν TAN aerobic), mainly related to the ammonia- oxidizing bacteria (AOBs) activity, was slighter higher during the moderate load test SW2 (-0.163 g TAN g VSS^{-1} d^{-1}) compared with the value measured in test SW1 (-0.136 g TAN g VSS^{-1} d^{-1}), as the AOBs activity are limited by the lower ammonia concentration reached in test SW1. The length of micro-aeration phase was fixed at 2.50 h in both tests. A 100% oxidation of the initial total ammonia (TAN) to nitrite (TNN) occurred in the low load test (SW1), achieving a complete nitritation process

(Fig. IV.4-SW1). On the contrary, a successful partial nitritation process, required for the SNAD process, occurred in the moderate load test (SW2), where only the 50% of the initial TAN was converted into TNN, ensuing an optimal remaining TAN and produced TNN ratio for the subsequent anammox process (Fig. IV.4-SW2). The nitrogen profiles were further analyzed during the anoxic period. The simultaneous depletion of the TAN and TNN was observed in the test SW2 and the anoxic total ammonia removal rate (ν TAN anoxic), which is referred to the anammox activity, was measured (-0.091 gTAN $gVSS^{-1}d^{-1}$). On the contrary, any anammox activity was detected in the test SW1, as there was no ammonia left to react with nitrite during the anoxic phase.

Further, from the COD profile over the SBR cycle, it can be deduced that the aerobic biodegradable organic compounds oxidation was combined with nitritation during the micro-aerobic phase while the denitrification was coupled with anaerobic ammonium oxidation during the anoxic phase (Fig. IV.5–SW2).

Looking at the total performance of the SNAD process, the efficiencies of the TAN removal were high for both the tests (100%). Nevertheless, the total nitrogen (TN) efficiency removal of the low load test (SW1) was extremely low, as during the micro-aerobic phase TAN was completely oxidized to TNN, making impossible to perform the following anammox process during the anoxic phase. High TN (92%) and COD (45%) removal efficiencies were calculated for the moderate load test (SW2).

In addition, the SNAD biomass used in this study showed good settling property, with a settling velocity of 0.9 m h^{-1}, as both flocs and granules were present. Only small particles were discharged with the effluent while the granules entrapped in a crosslinked network formed by flocs successful settled.

Table IV-3 Characteristics of nitrogen and carbon removal in period I

		Synthetic Wastewater	
Parameters	**u.m.**	**SW.1**	**SW.2**
ν TAN aerobic	[g TAN (g VSS d)$^{-1}$]	-0.136	-0.163
ν TNN aerobic	[g TNN (g VSS d)$^{-1}$]	+0.132	+0.141
ν N-NO_3^- aerobic	[g NO_3-N (g VSS d)$^{-1}$]	~0	+0.033
(TNN/TAN)aer	-	-0.97	-0.87
(NO_3-N/TAN)aer	-	~0	-0.18
ν TAN anoxic	[g TAN (g VSS d)$^{-1}$]	-0.0006	-0.091
ν TNN anoxic	[g TNN (g VSS d)$^{-1}$]	-0.0087	-0.137
ν N-NO_3^- anoxic	[g NO_3-N (g VSS d)$^{-1}$]	0	-0.023
(TNN/TAN)anox	-	-	+1.50
(NO_3-N/TAN)anox	-	-	+0.97
Total TAN efficiency removal	%	100	100
Total TN efficiency removal	%	0	92
Total COD efficiency removal	%	nd	45

The ionized (NH_4^+-N-and NO_2^--N) and the unionized (FA/NH_3-N and FNA/HNO_2-N) nitrogen species were calculated during both the batch tests, taking into account the pH on-line monitored in the time-course experiments. In Fig. IV.6 the evolution of total nitrogen ammonia, free ammonia and pH is reported for the test SW2. The batch tests conducted on the synthetic wastewater showed a good relationship between the on-line pH measured and the development of the SNAD process. In SW2 test, the pH value was in the range of 7.6 – 8.5 with no external addition of base or acid, showing a step increase during the feeding event and a linear decrease during the micro-aerobic phase, reaching the minimum value of 7.6 at the end of the micro-aerobic phase, due to the proton production linked to the AOB activity. During the anoxic phase, the pH behavior was influenced by denitrification and anammox process. The overall trend resulted in a pH increase due to the OH^- contribution by the denitrification process and to the H^+ consumption by anammox reaction, while only a little amount of

alkalinity is consumed during the anammox process. Further the FA concentration during the SBR cycle was always lower than 10 mg NH_3-NL^{-1}.

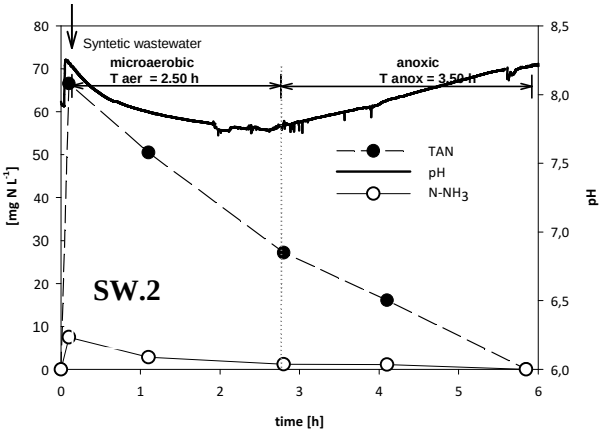


Fig. IV.6 Profiles of pH, TAN and FA in an SBR cycle using synthetic wastewater:
(S2) :NLR=0.27 kgTAN m^{-3} d^{-1}

3.2 Period II–feasibility of SNAD process for treating old leachate and SBR phases optimization

Landfill leachates contained some inhibitory compounds, which may inhibit biological activity, such as heavy metals and high salts concentration. Treating leachates and gradually increasing the nitrogen loading rate, it was possible to achieve a good nitrogen and carbon removal efficiency performing the SNAD process. The profiles of the nitrogen species over the SBR cycles of the batch tests performed are reported in the Fig. IV.7, while the performance details of each tests are reported in Table IV-4.

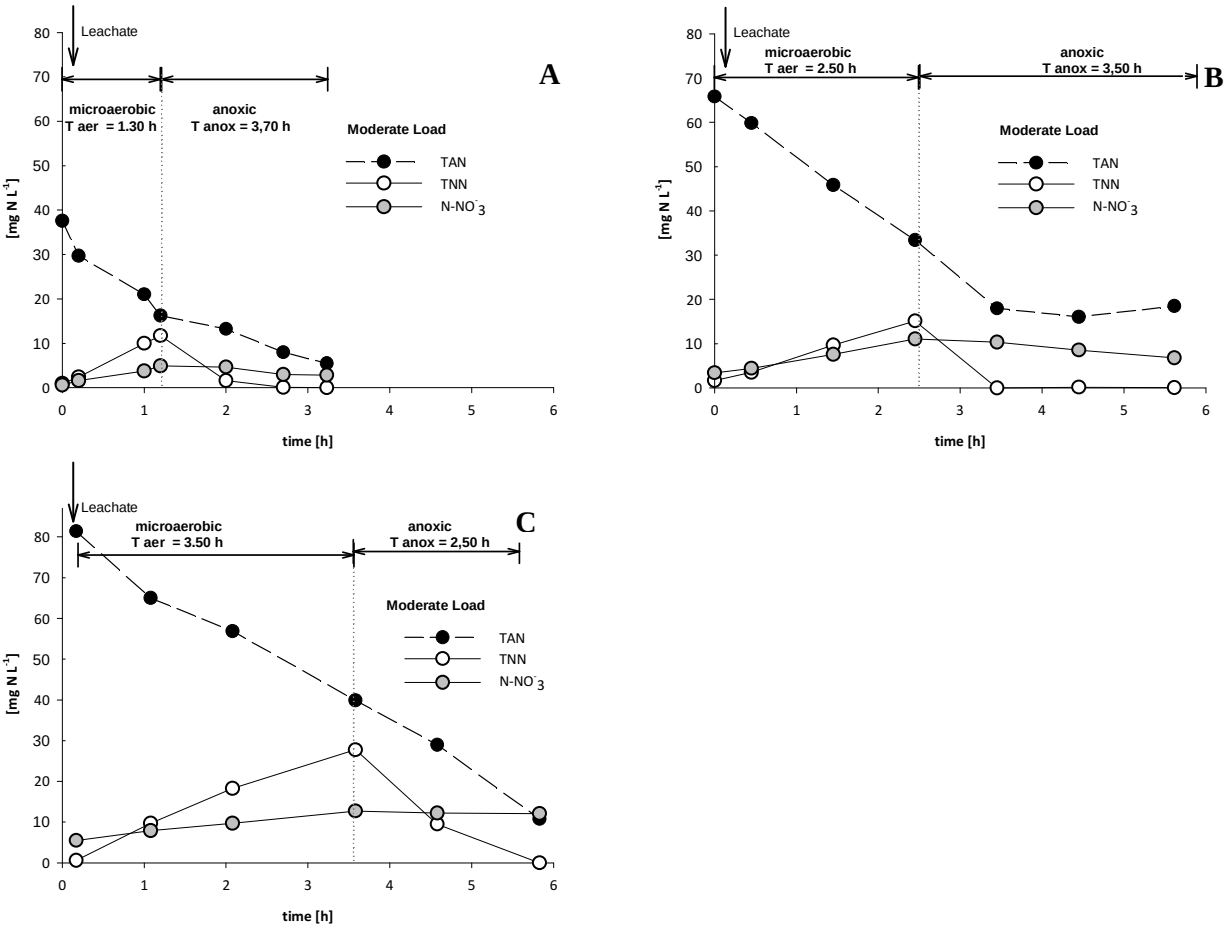


Fig. IV.7 Evolution of nitrogen species over an SBR cycles using old landfill leachate:
(A): NLR=0.038 kgTAN m^{-3} $cycle^{-1}$, (B): NLR=0.065 kgTAN m^{-3} $cycle^{-1}$, (C): NLR=0.082 kgTAN m^{-3} $cycle^{-1}$

The nitrate profiles during the micro-aerobic phase suggested a low NOBs activity (ν NO_3-N aerobic), while the total ammonia removal and nitrite profiles clearly showed the occurrence of the nitritation process during the micro-aerobic phase and the anammox process during the anoxic phase.

High TAN removal efficiencies (76-87%) were observed in the moderate load tests. At the end of the cycles residual TAN, TNN and NO_3^--N were found (lower than 10 mg L^{-1}), resulting in TN (total ammonia) removal efficiencies in the range of 64-79%. A mean COD removal efficiency of ~34% was measured.

Looking at the total efficiency of the SNAD process for the leachate treatment, the SBR cycle configurations were compared: *i*) a 4.0 h SBR cycle and a 1.30 h micro-aeration phase was suitable for a loading rate of 0.038 kgTAN m^{-3} $cycle^{-1}$, corresponding to an initial TAN concentration of 38 mg TAN L^{-1}; *ii*) a 6.0 h SBR with 2.5 h of micro-aeration phase was suitable for a loading rate of 0.065 kgTAN $m^{-3}cycle^{-1}$, corresponding to an initial TAN concentration of 66 mg TAN L^{-1}; *iii*) finally, a micro-aeration phase of 3.5 h in a 6.0h SBR was adequate for a loading rate of 0.0825 kgTAN m^{-3} $cycle^{-1}$, corresponding to an initial TAN concentration of 81 mg TAN L^{-1}

Table IV-4 Characteristics of nitrogen and carbon removal in period II

		Old landfill leachate		
Parameters	u.m.	A	B	C
		6	7	10
ν TAN aerobic	[g TAN (g VSS d) $^{-1}$]	-0.139	-0.142	0.140
ν TNN aerobic	[g TNN (g VSS d) $^{-1}$]	+0.097	+0.090	+0.107
ν NO_3-N aerobic	[g NO_3-N (g VSS d) $^{-1}$]	+0.033	+0.036	+0.022
(TNN/TAN)aer	-	-0.7	-0.64	-0.77
(NO_3-N/TAN)aer	-	-0.24	-0.25	-0.16
ν TAN anoxic	[gTAN (g VSS d) $^{-1}$]	-0.073	-0.093	-0.117
ν TNN anoxic	[gTNN (g VSS d) $^{-1}$]	-0.104	-0.112	-0.140
ν NO_3-N anoxic	[g NO_3-N (g VSS d) $^{-1}$]	-0.003	-0.013	-0.003
(TNN/TAN)anox	-	+1.42	+1.20	+1.20
(NO_3-N/TAN)anox	-	+0.035	+0.14	+0.024
Total TAN efficiency removal	%	86	76	87
Total TN efficiency removal	%	79	64	74
Total COD efficiency removal	%	31	23	34

The SBR configuration plays an important role for the development of a stable SNAD process. In particular, the length of micro-aerobic phase is one of the main factor to achieve high SNAD performances, as it dictates a proper partial nitritation process, which is an essential step for the following anammox process.

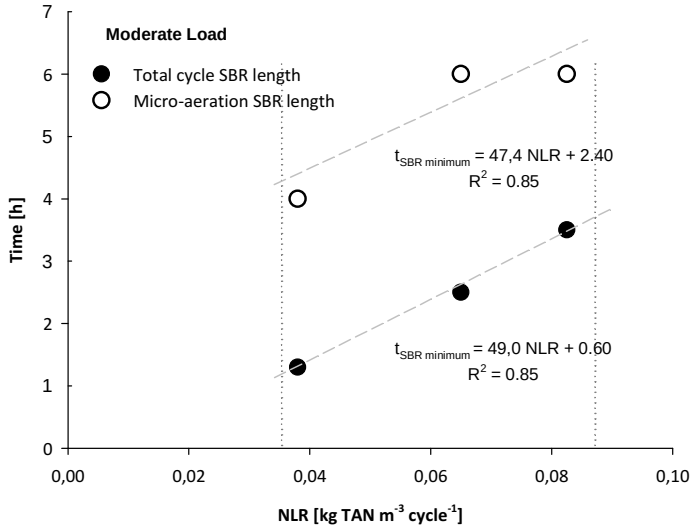


Fig. IV.8 Optimal length of the total cycle length and the micro-aerobic phase in the SBR cycle

Treating leachates, in this study we determined a good relation between the nitrogen loading rate per cycle and both the total SBR cycle and the minimum length of micro-aeration phase, under oxygen limited conditions, to achieve a partial nitritation process and a high TN removal efficiency (Fig. IV.8). The relation was validated in the range of 0.035 – 0.085 kgTAN m^{-3} $cycle^{-1}$. However, this finding were both wastewater and biomass specific as the performance of the SNAD process depended on the activity of the each group of microorganisms involved and on their interactions.

3.3 Period III –Effect of high total ammonia nitrogen on SNAD performance

The SNAD performance, in terms of nitrogen and carbon removal efficiency, significantly decreased when a NLR higher than 0.08 kgTAN m^{-3} $cycle^{-1}$, corresponding to an initial TAN concentration of 81 mg TAN L^{-1}, was applied (Fig. IV.9).

In the high load tests (D,E,F), the total ammonia nitrogen (TAN) removal efficiency decreased from values of 76 -87%, reached in the moderate load tests (A,B,C), to 30 – 50%. Considering the total nitrogen (TN) removal efficiency, a decrease from 64-79% to 20-50% was observed.

We showed that the lower nitrogen efficiencies were mainly linked the lower AOBs activity (Fig. IV.10). In fact, the aerobic ammonia removal rate started to decrease when a concentration of 105 mg TAN L^{-1} was reached in the reactor at the beginning of the SBR cycle. As the pH at the beginning of the SBR was always a value higher than 8, the corresponded FA concentration in the high load tests (D,E and F) was always higher than 10 mg NH_3–NL^{-1}, thus inhibiting the AOBs activity (Anthonisen et al. 1976).

From the analysis of the batch studies, we also concluded that acceptable anoxic ammonia removal rates were measured ensuing, at the beginning of the anoxic phase, a maximum TNN concentration of 20-25 mg L^{-1}. From nitrogen species profiles, we observed that lower nitrite concentrations resulted a in substrate limitation for anammox bacteria, while repeated nitrite concentrations higher than 20-25 mg L^{-1} caused losses of anammox activity due to the nitrite long-term effect.

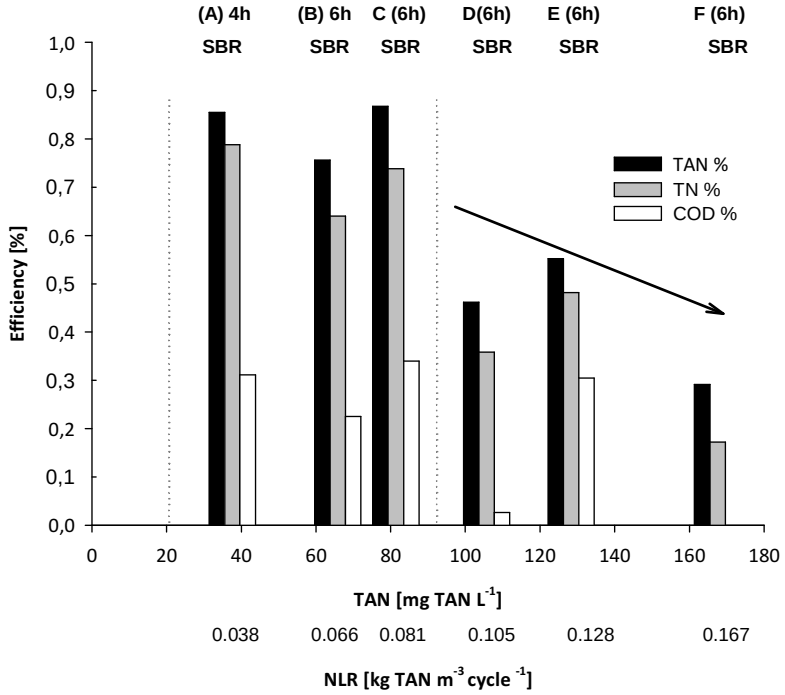


Fig. IV.9 Effect of high total ammonia concentration (TAN) on the SNAD performance.

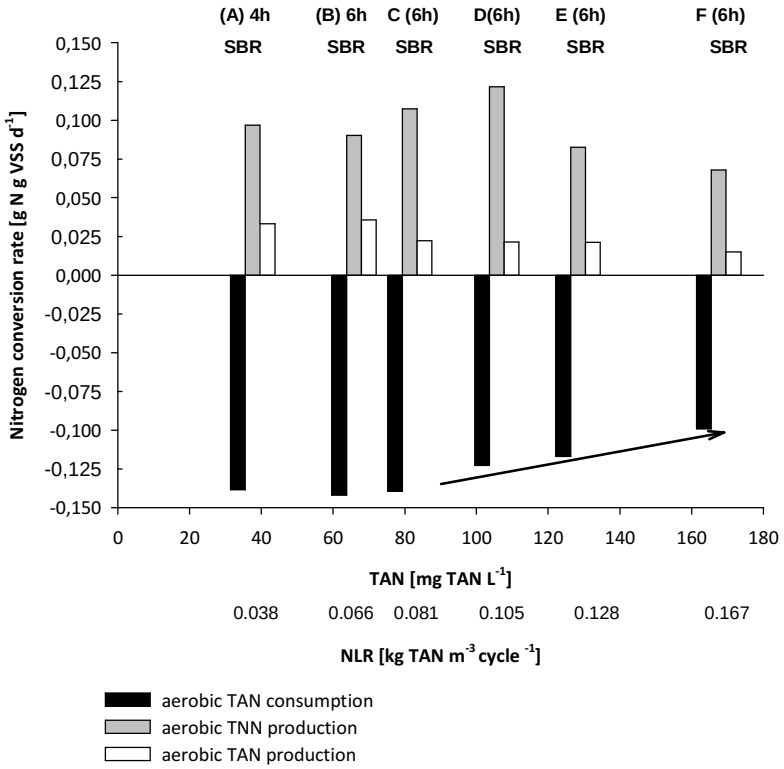


Fig. IV.10 Aerobic conversion rates during micro-aerobic phase related to the initial micro-aerobic TAN concentrations

4. Discussion

4.1 Feasibility of SNAD process

This research showed the possible application of the Simultaneous partial Nitritation, Anammox and Denitrification process for treating both synthetic and industrial wastewaters, such as old landfill leachates, containing COD and ammonium with a COD_{biod}/N ratio up to 1. In the batch tests conducted in this study, the SNAD process successfully occurred and anammox process was combined with nitritation, anaerobic ammonium oxidation, organic compounds oxidation and denitrification process.

Good results were achieved treating the synthetic wastewater, where no inhibitor compounds were present. The chosen cycle of the SBR (t_{cycle}=6h, t_{aer}=2,5h) was optimal to treat ammonium rich wastewaters, with a loading rate of 0.067 kgTAN m^{-3} $cycle^{-1}$, corresponding to an initial TAN concentration of 66 mg TAN L^{-1} (Test SW2). A TAN removal of 100% was achieved, and at the end of the cycle very small quantities of TNN and N-NO_3^- were found, resulting in a consistent TN removal (92%). Also a high COD removal was observed (around at 45%) due to the totally biodegradable organic matter used in the synthetic influent (CH_3COONa). The alkalinity, expressed as $CaCO_3$, and the TAN ratio, in the reactor, at the beginning of the micro-aeration phase was 7.51, sufficiently high to carry on the nitritation reaction (stoichiometric ratio of nitritation: $CaCO_3$/TAN=7.07). The filtered COD and TAN ratio, in the reactor, at the beginning of anoxic phase was around 2, enough low to allow the co-existence of denitrifying and anammox bacteria. The overall total ammonia removal rate (ν TAN aerobic), the total nitrite (ν TNN aerobic) and nitrate (ν NO_3-N aerobic) production rate, in the reactor, during micro-aerobic phase were 0.163gTAN $gVSS^{-1}d^{-1}$, 0.141TNN $gVSS^{-1}d^{-1}$ and 0.0033 gNO_3-$NgTSS^{-1}d^{-1}$ respectively. The resulting consumed TAN and produced TNN ratio was 0.87, instead of 1, characteristic of nitritation process, as NOBs were not completely inhibited. During the anoxic phase, the overall

total ammonia (ν N-NO_3^- anoxic) and nitrite (ν TNN aerobic) removal rate were 0.091 g TAN $gVSS^{-1}d^{-1}$ and 0.137 gTNN $gVSS^{-1}d^{-1}$ respectively, resulting in a consumed TNN to consumed TAN ratio of 1.5. The ratio TNN/TAN was higher than the stoichiometric ratio of the anammox process (1.3). We explained these results considering that denitrification via nitrite occurred during the anoxic phase. Due to the presence of the biodegradable organic matter, denitrifiers competed with anammox bacteria for nitrite consumption, resulting in a higher nitrite removal rate (ν TNN anoxic) compared to the anammox reaction. In addition, also denitrification via nitrate occurred, as showed by both the COD efficiency (Table IV-3) and the NO_3-N and COD profiles in the anoxic phase (Fig. IV.4 - SW2 and Fig. IV.5 - SW2), resulting in a negative anoxic nitrate removal rate (ν NO_3-N $^-$ anoxic).

Applying a NLR maximum of 0.067 kg $TANm^{-3}cycle^{-1}$, at the beginning of the SBR cycle, when the TAN concentration and the pH were the highest over the cycle, the FA concentrations reached its maximum value (8 mg NH_3-NL^{-1}). The FA value reached was lower than the minimum ammonia inhibition threshold reported in literature for AOB species. On the contrary, the FA value of 8 mg NH_3-NL^{-1} may partially contribute to inhibit the NOB species (Anthonisen et al. 1976), even if Kim et al. (2008a) reported different threshold for *Nitrobacter* spp. (30 ~ 50 mg NH_3-N L^{-1}) and *Nitrospira* spp. (0.04 ~ 0.08 mg NH_3-N L^{-1}) and Turk and Mavinic (1989) showed an adaptation of the nitrite oxidizing bacteria to NH_3, confirmed also by other studies (Villaverde 2000). During the experiment, the FNA were always lower than 0.0014 mg HNO_2-N L^{-1}, not inhibiting either AOB or NOB.

4.2 SNAD process for treating old landfill leachate

In this study, we showed that despite feeding the reactor with the old leachate, the SNAD process was feasible, achieving a satisfactory nitrogen and carbon removal when a NLR in the range of 0.038-0.08 kg TAN $m^{-3}cycle^{-1}$ was applied. Nevertheless, an acclimatization period to the leachate was necessary, as landfill leachates are characterized by higher conductivity and dissolved salts values compared to those measured for the SNAD biomass as well as by the presence of heavy metals, which may inhibit the biological activity. Therefore, sudden high filling volumes of leachates to the biological system may cause a drastic variation of the environmental conditions of the biomass, having a negative impact on the microbial activity of nitrifying (Del Borghi et al. 2003) and anammox bacteria (Dapena-Mora et al. 2007). This effect could be due to an increase of the osmotic pressure in the medium surrounding the cells, affecting the transport system through the membrane (Dapena-Mora et al. 2007). Nevertheless, several studies showed that biological systems may be acclimated gradually to leachate characteristics (Ganigué et al. 2007; Ruscalleda et al. 2010; Xu et al. 2010).

The chosen cycle of the SBR (t_{cycle}=6h, t_{aer}=2.5h) was optimal to the old landfill leachate, with a loading rate of 0.065 kgTAN m^{-3} $cycle^{-1}$, corresponding to an initial TAN concentration of 68 mg TAN L^{-1}. However, lower nitrogen removal efficiencies were achieved treating the landfill leachate compared to the efficiency measured treating the synthetic wastewater. These findings were explained by the fact that in the raw leachate inhibitory compounds were present, slightly inhibiting the biomass activities. In particular, the AOBs seems to be more influenced by the leachate composition (Aktas and Çeçen 2001). The aerobic total ammonia removal rates (ν TAN aerobic) were comparable in all the tests performed with leachate (A, B and C), but lower (mean values: -0.140 g TAN $gVSS^{-1}d^{-1}$) than the values measured in the test SW2 using the synthetic wastewater (-0.163 g TAN $gVSS^{-1}d^{-1}$), which had a similar initial TAN concentration in the reactor (65–80mg $TANL^{-1}$). On the contrary,

the anoxic total ammonia removal rates, after the biomass acclimation, were not negatively affected by the leachate composition, probably as anammox bacteria were protected from inhibitors by the layer of nitrifiers and denitrifiers that covers the granule. The nitrate profiles during micro-aerobic phase suggested a low NOBs activity (ν NO_3-N^- aerobic). Furthermore, lower COD removal efficiencies were detected treating leachates. It can be explained taking into account the presence of non-biodegradable or slowly biodegradable organic matter present in the leachate. The alkalinity/TAN ratio at the beginning of the micro-aeration phase was sufficiently high (8.8-12) to carry on the nitritation process. During the micro-aerobic phase, the mean produced TNN and consumed TAN ratio was 0.70 and the produced N-NO_3^- and consumed TAN ratio was 0.2, as NOB were not completely inhibited and a small amount of nitrite was converted into nitrate. The filtered COD/N ratio at the beginning of anoxic phase, when denitrifiers may compete with anammox bacteria, was 3.4. However, we consider the biodegradable COD_{bio}/N ratio lower than 3.4, due to the low amount of biodegradable fraction present in the old landfill leachate, thus not compromising the anammox activity. In fact, due to the lower biodegradable organic matter present in the influent, over the anoxic phase the consumed TNN to consumed TAN ratio was in the range of 1.2 -1.4. This value was close to the stoichiometry one reported for anammox process (1.32), confirming the lower activity of the denitrifiers compared to the results obtained using the synthetic wastewater.

4.3 SBR configuration for SNAD process

The SNAD performance, in terms of nitrogen and carbon removal efficiency, were significantly high applying a loading nitrogen rate (NLR) in the range of 0.038-0.08 kg TAN $m^{-3}cycle^{-1}$, while decreased when a NLR higher than to 0.08 kg TAN m^{-3} $cycle^{-1}$, corresponding to an initial TAN concentration of 81 mg L^{-1}, was applied.

At initial TAN concentrations higher than 80 mg L^{-1}, the lower nitrogen efficiencies were mainly linked the lower AOBs activity, which were partially inhibited by the high FA concentration at the beginning of the SBR cycle, when the pH and the TAN reached their maximum values. Concerning the anammox activity, we observed that at the end of the micro-aerobic period, nitrite concentrations lower than 20 mg $TNNL^{-1}$ resulted a in substrate limitation, while repeated nitrite concentrations higher than 20-25 mg $TNNL^{-1}$ caused losses of anammox activity due to the nitrite long-term effect. This finding were confirmed in other studies. Bettazzi et al. (2010) observed that repeated injections of nitrite higher than 30 mg $TNNL^{-1}$ determined a loss of anammox activity. The authors also found that the inhibition increased after each spike.

The SBR configuration plays an important role to perform a proper SNAD process. Applying a NLR in the range of 0.038-0.08 kg TAN $m^{-3}cycle^{-1}$, the length of both the total SBR cycle and the micro-aerobic and anoxic phase can be expressed as function of the nitrogen loading rate per cycle. The relation between the NLR and the SBR configuration found in this study ensured to achieve a partial nitritation process during the micro-aerobic phase and consequently reach, at the beginning of the anoxic phase, a suitable remaining TAN and produced TNN ratio for the subsequent anammox process. Then, the anoxic period would be sufficiently long to allow the depletion of the TAN and TNN by anammox process.

5. Conclusions

This research evaluates the use of SNAD process to treat old landfill leachates. This study was carried out as an initial step towards development of a continuous SNAD process performed in an SBR under oxygen limited conditions. First of all, batch studies on synthetic wastewater showed that the simultaneous removal of carbon and nitrogen from effluents with low C/N ratio was feasible in one single reactor. An integration of partial nitritation, anammox and heterotrophic denitrification process was successfully achieved at high pH values (7.5 and 8.5) and high temperatures (T = 30°C) under oxygen limited conditions (DO < 0.5 mg L^{-1}). This operative condition were optimal to treat a nitrogen loading rate of 0.067 kg $TANm^{-3}cycle^{-1}$, achieving at the beginning of the micro-aerobic phase a maximum FA concentration of 10 mg NH_3-N L^{-1}, which made sure the nitrification process stopped at the ammonium oxidation step. Further, it was preferable to achieve a maximum nitrite concentration of 20-25 mg $TNNL^{-1}$ at the beginning of the anoxic phase, in order to prevent the anammox bacteria inhibition, due to the nitrite long term effects. Using synthetic wastewaters, where no inhibition compounds were present, a TAN and TN removal efficiencies of 100% and 92% were observed, respectively. A high COD removal efficiency was achieved (45%) probably due to ready biodegradable organic matter used in the synthetic influent (CH_3COONa).

A progressive adaptation of biomass from the synthetic wastewater to the raw leachate was carried out via increasing the filling volumes of the leachate in the SBR. Despite feeding the reactor with the old leachate, which contained trace of inhibitory compounds and high conductivity values, both the aerobic ammonia oxidizing - and anammox bacteria showed high microbial activity values after a biomass acclimatation to the landfill. Acceptable SNAD performance, in terms of nitrogen and carbon removal efficiency, were achieved applying a loading nitrogen rate (NLR) in the range of 0.038 – 0.08 $kgTANm^{-3}cycle^{-1}$. However, the overall nitrogen and carbon removal efficiencies using leachate were lower compared to the efficiencies detected using the synthetic wastewater. This could be explained taking into account both the high AOBs sensitivity to the inhibitory compounds present in the leachates and the lower biodegradable organic matter present in the leachate with respect to the synthetic wastewater, that also reduce the total nitrogen removal efficiency. Anammox activity was found to be not negative affected by leachate treatment probably as anammox bacteria were protected from inhibitors by the layer of nitrifiers and denitrifiers that covers the granule.

To design an optimal nitrogen and carbon removal in a SNAD-SBR systems, the characteristic of wastewaters, the microbial activity and the reactor operative conditions (i.e. pH, T, DO, concentration of TAN, FA and TNN) should be take into account. In this study, concerning the leachate treatment, a relation between the nitrogen loading rate per cycle and the SBR configuration was determined in order to achieve a suitable partial nitrification during the micro-aerobic phase followed by a proper anammox process during the anoxic phase. However, this finding were wastewater and biomass specific as the performance of the SNAD process depends on the activity of the each group of microorganisms involved and on their interactions. Similar tests could be useful during the design and management of the SNAD-SBR, in order to apply the optimal SBR configuration and achieve high nitrogen removal efficiencies in a continuous SNAD process.

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Supplementary Material

Further details concerning the characteristic of the synthetic wastewater and the old landfill leachate as well as the acquisition and control hardware and software.

Supplementary Table

Supplementary Table IV-1 Characteristics of the synthetic wastewater	
Parameter	**This study [g L^{-1}]**
NH_4 Cl	3.41
K**HCO_3**	8.50
CH_3COONa	1.38
Medium Composition*	
CaCl_2 $2H_2O$	0.30
MgSO_4 $7H_2O$	0.20
FeSO_4	0.00625
EDTA	0.00625
K**H_2PO_4**	0.0250
Trace elements solution*	1.25 mL L^{-1}
Trace element composition (1L)*	
Zn$SO_4 \cdot 7H_2O$	0.43
Mn$Cl_2 \cdot 4H_2O$	0.99
Cu$SO_4 \cdot 5H_2O$	0.25
Na**Mo**$O_4 \cdot 2H_2O$	0.22
Ni$Cl_2 \cdot 6H_2O$	0.19
H_3**B**O_4	0.014

* adapted by * Thirds et al. 2001

Supplementary Table IV-2 Characteristics of the raw leachate from the municipal wastes landfill site of Trento city. (Italy)

Parameter	**This study**	**old landfill leachate [mg L^{-1}]** (Kjeldsen et al. 2002; Kurniawan et al. 2006b)
CONDUCTIVITY [mS cm^{-1}]	10.7±1	-
REDOX [mV]	30±10	-
pH	8.1±0.1	7.5 - 9
TSS [mg L^{-1}]	123 ± 129	-
COD_{total} [mg L^{-1}]	1444 ± 86	
$COD_{filt\ 0.45\ \mu m}$ [mg L^{-1}]	1307 ±193*	5.000-20.000
$COD_{toluble}$* [mg L^{-1}]	1237 ± 125+	
COD_{bio}** [mg L^{-1}]	250**	
BOD_5 [mg L^{-1}]	70±17	20-1.000
RBCOB [mg L^{-1}]	21**	
TOTAL AMMONIUM NITROGEN (TAN_{filt}) [mg L^{-1}]	1241±136+	400-5000
TOTAL NITRITE NITROGEN (TNN) [mg L^{-1}]	<0.04*	-
NITRATE (NO_3-N) [mg L^{-1}]	10±2*	-
TOTAL KJELDAHL NITROGEN (TKN) [mg L^{-1}]	1288±169	-
ORGANIC NITROGEN (Norg) [mg L^{-1}]	57±39	-
TOTAL PHOSPHORUS (P) [mg L^{-1}]	14±2	6
SULFATE[mg L^{-1}]	1200*	10-420
ALKALINITY ($CaCO_3$) [mg L^{-1}]	7.000 – 15.000*	-
HYDROGEN SULFIDE (H_2S^-)[mg L^{-1}]	0.04*	-
Fe(II) [mg L^{-1}]	2,1*	-
Fe(III) [mg L^{-1}]	1,8*	
Mn(II) [mg L^{-1}]	0,9*	-
ALUMINIUM [mg L^{-1}]	0.42 0.1*	-
ARSENIC (As) [mg L^{-1}]	<0.08*	0.01-1
BARIUM (Ba) [mg L^{-1}]	0.39±0.03*	-
BORON (Bo) [mg L^{-1}]	5.6±0,79*	-
CADMIUM (Cd) [mg L^{-1}]	<0.005*	0.005
CHROMIUM (Cr) [mg L^{-1}]	0.21±0.04*	0.28
IRON (Fe) [mg L^{-1}]	3.8±0.44*	3 - 280
LEAD (Pb) [mg L^{-1}]	<0.04*	0.09
MANGANESE (Mn) [mg L^{-1}]	0.1±0.007*	0.03-45
MERCURY (Hg) [mg L^{-1}]	<3.0*	0.00005-0.16
NICKEL (Ni) [mg L^{-1}]	0.1±0.015*	0.17
ZINC (Zn) [mg L^{-1}]	0.18±0.15*	0.03-4
COPPER (Cu) [mg L^{-1}]	0.01±0.002*	0.065
SELENIUM (Se) [mg L^{-1}]	<0.003*	-
STANNUM (Sn) [mg L^{-1}]	0.06±0.006*	-

* values after filtration 0.45 µm

** values determined by respirometric analysis

\+ values determined after ultrafiltration 0.45 µm and 0.2 µm

Supplementary Table IV-3 FP-AI-110 channel configuration

FP – AI -110 - FieldPoint analog input module with eight analog voltage or current input channels, 16-bit resolution

Device	**Description**	**Physical channel**	**Channel Name**	**output range**	**Work range**	**Work measure**
pH sensor	on-line reactor pH	AI0	Numeric 1 pHr	±21.5 mA	4 – 20 mA	4 -10
DO sensor	on-line reactor DO	AI2	Numeric 2 DOr	±21.5 mA	4 – 20 mA	0-10 mg L^{-1}
ORP sensor	on-line reactor ORP	AI3	Numeric 3 ORPr	±21.5 mA	4 – 20 mA	-1000 - + 1000 mV
Temperature sensor	on-line reactor Temperature combined with DO sensor	AI4	Numeric 4 TDOr	±21.5 mA	4 – 20 mA	5 - 40 °C
free	-	AI4		±21.5 mA		
free	-	AI5		±21.5 mA		
free	-	AI6		±21.5 mA		
free	-	AI7		±21.5 mA		

V. Chapter

Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process at moderate temperature treating anaerobic digester effluent

This chapter was based on:

Langone M., Andreottola G., Cadonna M. (2013). *Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process at moderate temperature treating anaerobic digester effluent.* Submitted

Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process at moderate temperature treating anaerobic digester effluent

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ABSTRACT

The integration of the nitritation, anammox and heterotrophic denitrification via nitrate and nitrite was investigated at moderate temperature (T=25°C). Treating a synthetic wastewater, a mean nitrogen removal rate of 0.14 g $NL^{-1}d^{-1}$ was registered and high total ammonium (97%), total nitrogen (93%) and organic matter (77%) removal efficiencies were obtained. High removal efficiencies were also achieved treating anaerobic digester effluent, but a higher nitrate accumulation occurred in the system, due to the lower biodegradable COD and pH in the influent. The influent carbon-nitrogen ratio governs the contribution of denitrification over the anammox process and the total nitrogen removal efficiency of the SNAD process. At moderate temperature, a proper control of the free ammonia levels, and thus of pH, seemed to be a key factors of the nitratation inhibition together with the oxygen limiting conditions. A new primer set, targeting the *hao/hzo* gene, was applied for the identification of the anammox bacteria.

Keywords:Anaerobic digester effluent, Anammox, hzo/hao gene; SNAD, Sequencing Batch Reactor, Temperature

Abbreviations

Parameters		**Reactor Configurations and process**		**Operative**	
COD	Chemical Oxygen Demand	CANON	Completely Autotrophic Nitrogen removal Over Nitrite	DO	Dissolved Oxygen
CODtot	Total COD	CAS	Conventional Activated Sludge	HRT	hydraulic retention times
CODfiltr	Filtrate COD	DEMON	Deammonification process	KLa	mass transfer coefficient for oxygen
CODsoluble	Soluble COD	GSBR	Granular Sequencing Batch Reactor	NLR	Nitrogen Loading Rate
CODbio	Biodegradable COD	GSB-ANR	Granular Sludge Bed reactor	NRR	Nitrogen Removal Rate
C_T	cycle number PCR	MAP	Magnesium-Ammonia-Phosphate	OLR	Organic Loading Rate
FA	Free Ammonia (NH_3- N)	OLAND	Oxygen-Limited Autotrophic Nitrification/Denitrification	VER	Volume Exchange Ratio
FNA	Free Nitrous Acid (HNO_2-N)	SHARON	Single reactor High activity Amm. Removal Over Nitrite	ORP	Oxidation-Reduction Potential
MLSS ~	Mixer Liquor Suspend Solid	SNAD	Simultaneous partial Nitritation, Anammox, Denitrification	EC	Conductivity
N	Nitrogen	SND	Simultaneous Nitrification and Denitrification	SAUR	Specific Ammonia Uptake Rate
NO_3^--N	Total Nitrate Nitrogen	RBC	Rotating Biological Contactors	SNUR	Specific Nitrite Uptake Rate
RBCOD	Readily biodegradable COD	SBBR	Sequencing Batch Biofilm reactors	SAA	Specific Anammox Activity
TAN	Total Ammonia Nitrogen	SBR	Sequencing Batch Reactors	SOUR	Specific Oxygen Uptake Rate
TNN	Total Nitrite Nitrogen	WWTP	Wastewater Treatment Plant		
TKN	Total Kjeldahl Nitrogen	PCR real time	Real-time polymerase chain reaction		
TSS	Total Suspend Solid	AOB	Ammonia-Oxidizing Bacteria		
T_M	melting temper	NOB	Nitrite-Oxidizing Bacteria		
VSS	Volatile Suspend Solid	Anammox	ANaerobic AMMonia -OXidizing Bacteria		

1. Introduction

Anaerobic digestion is widely applied throughout the world for the treatment of wastes and wastewaters and it is the most sustainable approach for the sludge stabilization. In the anaerobic digester reactor, the biodegradable organic carbon (COD) in the influent is converted to methane, which may be used for electricity and heat purposes. Ammonium (TAN) is not removed and therefore the anaerobic digestion process generally yields an ammonium-rich effluent (containing about 500-600 mg $TANL^{-1}$), low in biodegradable COD content and characterized by a low COD/N ratio. Those characteristics make anaerobic digester effluents more difficult to apply a conventional way of nitrogen removal via the nitrification–denitrification processes.

Generally, the effluent of the anaerobic digestion plant is recirculated, after a dewatering treatment, to the conventional activated sludge (CAS) tanks in the wastewater treatment plants (WWTPs). Although it is a

consolidated practice, it has significant effects on the cost balance of the WWTPs. Indeed, digester effluent may contain reducing and inhibitory compounds, which could compromise the whole efficiency of the CAS systems. Then, the high nitrogen concentrations of the anaerobic digester effluent require, in the CAS system, a large amount of electricity to provide enough oxygen for nitrification. Further, the amount of COD contained in these anaerobic effluents is usually not enough to carry out the removal of nitrogen by the conventional nitrification-denitrification processes and organic matter source should be added for the denitrification stage. A study of the nitrogen balance of the urban WWTP of Trento, Italy, shows that a significant fraction of the inlet nitrogen load (15-20%) is recycled with the return liquors from sludge dewatering to the main stream, even if it contributes only a small amount (1%) to the influent water flow (Fig. V.1). The results of this study are also comparable to the results of other researches (Mulder et al. 2001; Fux et al. 2002; Fux and Siegrist 2004).

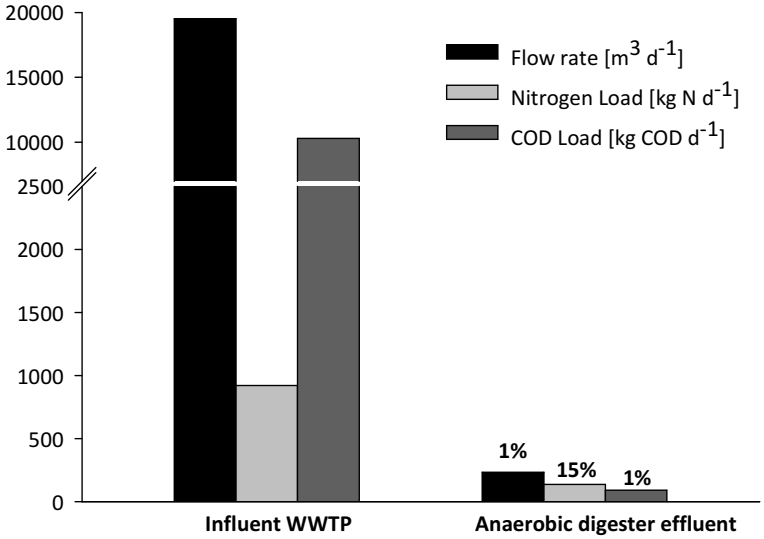


Fig. V.1 N-balance without treatment of anaerobic digester effluent

In the last decades, many efforts have been made to optimize the anaerobic digestion output management. A dewatering post–treatment is however required. Both a liquid and a solid stream, with different final pathway, are obtained. According to national and local regulations, the solid fraction is generally disposed to landfill, when it cannot be used in agriculture. The anaerobic digester supernatant is commonly directed back to the WWTPs. Due to the high concentration of nitrogen, a separate treatment of the digester supernatant would significantly reduce the nitrogen load of the main stream, improving the nitrogen elimination and reducing the operative costs.

Chemical elimination of ammonium with Magnesium-Ammonia-Phosphate (MAP) precipitation, or with air stripping are feasible but more expensive than conventional biological process, by means of nitrification and denitrification with addition of an organic carbon source (Mulder 2003). To this end, several innovative biological nitrogen removal processes and technologies have been developed, such as the simultaneous nitrification and denitrification (SND) via nitrate (Collivignarelli and Bertanza 1999), simultaneous nitrification and denitrification (SND) via nitrite (Zeng et al. 2004), the combined SHARON (single reactor system for high activity ammonium removal over nitrite) and the anammox process in two independent units (Van Dongen et al. 2001b; Furukawa et al. 2009; Vázquez-Padín et al. 2009b). The possibility to combine the nitritation with the anammox process for treating anaerobic digester effluents in one single stage has also been investigated: the completely autotrophic nitrogen removal over nitrite process (CANON) (Vázquez-Padín et al. 2009c), the deammonification process (DEMON) (Wett 2007), the simultaneous nitritation, anammox and denitrification

(SNAD) process (Joss et al. 2009; Zhang et al. 2012). SNAD process consists in a biological innovative treatment based on a partial biological autotrophic oxidation of ammonium to nitrite (nitritation) coupled and followed by an autotrophic anaerobic ammonium oxidation via nitrite (anammox). Further, both the oxidation of the biodegradable COD and the anoxic denitrification of NO_2^- or NO_3^- to gaseous N_2 by heterotrophic microorganisms occur, using the small amount of the organic matter in the influent as carbon and energy source. Recently, significantly high nitrogen removal rate (NRR) were achieved treating synthetic wastewater and using a granular sludge bed reactor (GSB-ANR process) at high temperature (T=30°C) to perform the autotrophic nitrogen removal processes in a single reactor (Wang et al. 2012). The authors reached a NRR of 2.57 kg N $m^{-3}d^{-1}$, which was significantly higher than the level reported for the autotrophic nitrogen removal processes in "1 single reactor" systems. Nevertheless, the organic carbon in the synthetic wastewater was not present. Generally, the presence of organic carbon could affect the anammox bacteria activity, either as organic carbon could be inhibitory (Chamchoi 2008) or heterotrophic denitrifiers can be competitive with anammox bacteria for the utilization of nitrite (Kumar and Lin 2010).

Different studies have been carried out in order to enhance the co-existence of different bacteria, which live in different environmental conditions (Table IV-1). Lan et al., (2011) studied the simultaneous partial nitrification, anammox and denitrification (SNAD) process for ammonia removal from a synthetic wastewater, characterized by a low COD/N ratio, using a SNAD seed sludge collected from a biological treatment unit (aeration tank) of a full-scale landfill-leachate. The authors investigated the influence of hydraulic retention times (HRT = 3 - 9 d) on the performance of a granular sequencing batch reactor (GSBR), operating under high temperatures (T=35°C) and oxygen limitation (DO = 0.5-1.0 mgL^{-1}). De Clippeleir et al. (2009), treating synthetic wastewater in a sequential batch reactor (SBR) showed that a low critical minimum settling velocity (0.7 mh^{-1}) and a low volumetric exchange ratio (25%) were essential to ensure a fast start-up of a simultaneous nitritation, anammox denitrification process, reaching a nitrogen removal rate of 1.1 g N $L^{-1}d^{-1}$.

Vlaeminck et al. (2009b) showed the feasibility to treat digested black waters from vacuum in a lab-scale oxygen-limited autotrophic nitrification/denitrification (OLAND) rotating biological contactor (RBC). Working on the nitrite oxidizing bacteria inhibition at free ammonia levels above 3 mg N L^{-1}, the authors achieved a stable nitrogen removal rate. Zhang et al. (2012) applied a completely autotrophic nitrogen removal over nitrite (CANON) process using a sequencing batch biofilm reactor (SBBR) at 30°C, to remove nitrogen and organic carbon from a swine digester liquor reaching a nitrogen removal rate of about 0.1 kg $Nm^{-3}d^{-1}$, showing the importance of a low C/N ratio (< 1.2).

Nevertheless, the sequencing batch reactor (SBR) is one of the most applied reactor configuration for treating the anaerobic digester effluents. A first full-scale application of the nitritation, anammox and denitrification process treating anaerobic digester effluents was implemented by Wett (2007). The author developed the deammonification technology (DEMON) in an SBR treating the anaerobic digestion effluent at the WWTP Strass (Austria) with a nitrogen load up to 0.68 kgN m^{-3} d^{-1} and high temperature (T=30°C). It consisted in a pH-controlled process on the intermittent aeration phase. Ensuing a low DO concentration in the system, the pH-controlled process simultaneously allowed both to repress the second oxidation step from nitrite to nitrate and to develop the anammox process. The results showed a 40% saving on oxygen consumption with respect to a CAS system and the specific energy demand was reduced from 2.9 kWh kgN^{-1} to 1.16 kWh kgN^{-1}, comparing to about 6.5kWh kgN^{-1} of the mainstream treatment. The presence of anammox bacteria and the high microbial diversity in this system had been later confirmed by Innerebner et al. (2007). In Zürich, Switzerland, the partial nitritation,

anammox and denitrification process in an SBR, which relied on online measurements of ammonium, conductivity, and oxygen and employed one feeding per cycle under oxygen limitation and high temperature (T=30° C), was developed by Joss et al. (2009). The co-existence of nitrifying, anammox and denitrifying bacteria had been then demonstrated (Langone et al. 2013b).

However, few studies are present at moderate temperature (Table V-1). Vázquez-Padín et al. (2009a) treated the supernatant of an anaerobic digester at 20 °C in an one single stage granular SBR, with a mean granules diameter of 3 mm. In an SBR working with an hydraulic retention time (HRT) of 0.25 d, operating at a dissolved oxygen concentration of ~3 mg L^{-1} and a continuous feeding, a mean nitrogen removal rate of 1 kg $Nm^{-3}d^{-1}$ was reached, achieving a total nitrogen removal efficiency of 60%. Vázquez-Padín et al. (2009b) treated an anaerobic digester effluent by a CANON system, operating at room temperature (20–24°C) in an air pulsing reactor. The biomass was composed by flocs and granules with an average diameter of 1.6 mm. The authors, working at limiting dissolved oxygen conditions (DO=0.5 mg L^{-1}) and HRT of 0.5 d, reached a maximum nitrogen removal rate of 0.45 $kgNm^{-3}d^{-1}$ and a total nitrogen removal between 35 and 64%.

Table V-1 Overview of typical innovative nitrogen removal processes in SBR applied to anaerobic digester effluent and synthetic wastewater

Reference	De Clippeleir et al. 2009	Lan et al. 2011	Wett 2007	Joss et al 2009	Vázquez-Padín et al 2009b	Vázquez-Padín et al 2009a
Wastewater	Synthetic wastewater	Synthetic wastewater	Digester effluent	Digester effluent	Digester effluent	Digester effluent
CODbio/N	0	1:2	1:3	1:2	1:1.2	nd
Reactor type	SBR	GSBR	SBR	SBR	SBR	GSBR
Volume	2.5 L	18 L	500 m^3	1400 m^3	1.5L	1.5L
SBR configuration	1h cycle	24h cycle	8h cycle	6-8h cycle	5.75h cycle	3 h cycle
Fill	instantaneous	continuous	continuous	instantaneous	continuous	continuous
Aeration mode	continuous	continuous	intermittent	continuous	Air pulsing	Air pulsing
DO [mg L^{-1}]	0.3-0.7	0.5 – 1.0	0.2-0.3	<0.8	0.1 -2	2.7
Inoculum	OLAND biomass	SNAD sludge	activated sludge	activated sludge + anammox	granular nitrifying + anammox	granular heterotrophic
HRT [d]	0.2 d	3 d	4*	1.45*	0.5	0.25
pH	>7.4	7-8	7.0 - 7.1	7.1 ±0.1	7.5-7.9	-
T [°C]	33±1	35	27.8 ±1	30±3	20-24	20
VSS [g L^{-1}]	2.3	-	3	3.6	1.5 – 4.5	5
OLR [g COD L^{-1} d^{-1}]	0	0.033	0.14*	0.2*	-	-
NLR [g TN L^{-1} d^{-1}]	1.5*	0.066	0.43*	0.45*	0.7	1.6*
Removal rate [g TN L^{-1} d^{-1}]	1.1	0.061*	0.37*	0.42*	0.25-0.45	1.0
TN tot removal [%]	73%*	92,6% (85% PN+ Amx; 7,3% Den)	85.8%	94.6*	35 - 64%	60%
TAN removal [%]	-	93%	90.3%	95%*	nd	nd
COD removal [%]	0	72%	56%*	37%*	nd	nd

PN= Partial Nitritation, Amx= Anammox, Den= Denitrification

The present study aimed to determine the operational key factors so as to investigate the effect of the high and moderate temperature on an SBR system performing a simultaneous nitritation, anammox and denitrification process. Further, the feasibility of the application of the SNAD-SBR to the anaerobic digested liquor treatments at moderate temperature (T=25°C) was investigated. A new primer set targeting the *hao/hzo* was used for the identification of the anammox bacteria. During the experiment the biomass activities were monitored and the contribution of each biological process involved in the SNAD-SBR was investigated.

2. Materials and Methods

2.1. Feeding media and supernatant from anaerobic digester

In this study, the synthetic wastewater was prepared according to Third et al., (2001) and adapted for the SNAD process, thus no nitrite was added while a carbon source and alkalinity were introduced in the medium. The synthetic wastewater composition was similar to that of digester supernatants, in terms of total ammonia nitrogen (TAN = 425±50 mgL^{-1}) and total soluble organic matter ($COD_{soluble}$ = 293±112 mgL^{-1}) content. Ammonium, as NH_4Cl, was added to the medium in the required amounts and alkalinity, as $KHCO_3$, was introduced according to the nitritation stoichiometry. COD, as CH_3COONa, was added in a low soluble COD to N ratio (0.5). The total soluble COD was entirely biodegradable, as we used acetate as a carbon source. The composition of the synthetic wastewater is reported in the Supplementary Table V-1.

The real wastewater used in this study was collected from the dewatering system treating the anaerobic digesters sludge of the municipal Wastewater Treatment Plant (WWTP) in Trento, Italy, in the spring and summer season of 2012. The supernatant was stored at 4°C in order to prevent the COD and nitrogen degradation. The supernatant composition was characterized by a seasonal pattern with a higher biodegradability during the spring period compared to the summer period. The total COD measured during the spring and the summer period was 364 mg $CODL^{-1}$ and 320 mg $CODL^{-1}$, respectively, while the total ammonia concentration was 384 mg $TANL^{-1}$ and 480 mg $TANL^{-1}$, respectively. Concerning the biodegradability, it was measured that the biodegradable COD (COD_{bio}) during the spring and the summer period was 175 mg L^{-1} and 145 mg L^{-1}, respectively, while the readily biodegradable COD (RBCOD) was 89 mg L^{-1} and 7 mg L^{-1}, respectively. The COD/N ratio was 0.5 – 0.6 during the summer time, when environmental temperatures of 30 degrees Celsius were achieved, with peaks of 0.8 - 0.9 during the spring time, when the environmental temperature was about 25 degrees Celsius. The main characteristics of the supernatant used for the present study are given in the Supplementary Table V-2.

2.2. Seed sludge

The SNAD seed sludge was collected from a biological treatment unit (SBR) of the full-scale Municipal Wastewater Treatment Plant, Zürich (Switzerland). The SBR treated the digester supernatant, at high temperature (T = 30°C). The operating conditions of the full scale SBR (V = 1400 m^3) were: DO <0.8 mgL^{-1}, pH 7.1±0.2, T ~ 30°C, MLSS ~ 3.6 kg/m^3, N- load ~ 0.625 kg Nm^{-3} d, HRT ~ 1.45 d. In the Zürich SBR, the partial nitrification, anammox and denitrification process was full achieved (Joss et al. 2009). The seed sludge contained granules surrounded by a matrix of brownish flocs. Two kinds of granules (red and brown) could be distinguished. The granules had diameters between 0.5 and 2.0 mm. Molecular analysis of the original inoculum showed the presence of ammonia-oxidizing bacteria of the N*itrosomonas europaea/eutropha* group, anaerobic ammonium-oxidizing bacteria of the "*Candidatus* Brocadia fulgida" type and denitrifying bacteria related to the *Thauera* and *Pseudomonas*. Nitrite-oxidizing bacteria from the genus *Nitrobacter* were also detected (Langone et al. 2013b). The authors showed long filamentous - shaped microorganisms were dominant in the outer layer of the granules, while single cells as well as aggregates were trapped in a net of filamentous bacteria.

2.3. Experimental lab scale reactor

SBR reactor. A lab scale (8 L) Sequence Batch Reactor (SBR) was inoculated with the SNAD sludge from the SBR in Zürich that removed organic matter and nitrogen from an anaerobic digested plant at T = 30°C. In this study, the 8 L SNAD-SBR was operated with first the synthetic wastewater and then the anaerobic digester supernatant from the municipal WWTP of Trento, Italy. Fig. V.2 shows the reactor setup. The cylindrical reactor (working height 0.295 m, inner diameter 0.189 m) made of acrylic plastic had a minimum and a maximum operating volume of 7.0 and 8 L, respectively. It was water-jacketed, allowing the mixed liquor temperatures to be controlled by a thermostated circulating water bath. The reactor was equipped with a mechanical stirrer to ensure a complete but mild mixing, at low rpm (150), to minimize the size granule reductions. The reactor was also equipped with acid/base pumps and a set of three peristaltic pumps to introduce the feeding solution and to discharge the effluent and eventually the sludge. Aeration was carried out by n. 6 air diffusers placed at the bottom part of the reactor. Air was supplied by a Schego M2K3 350 air pump and the gas-flow was maintained constant at 50 nL h^{-1} using a mass-flow meters.

The mass transfer coefficient for oxygen (KLa) was calculated and fixed at a sufficiently high value (0.15 min^{-1} at 20°C), in order to ensure the dissolved oxygen set-point can be attempted with the on/off control (Sin et al. 2004). The DO set-point was fixed at a set point value of 0.2 ±0.1 mgO_2 L^{-1}, inhibiting the activity of the nitrite oxidizing bacteria (NOBs) over the ammonia oxidizing bacteria (AOBs), due to the lower oxygen affinity of NOBs (Wiesmann 1994). The pH was controlled, allowing to vary in a wide range (7.3 - 8.5) by the addition of acid (1 N HCl) and alkaline (1 N NaOH) solutions.

During the experiment, the total suspended solids (TSS) concentration of the whole sludge varied between 3 - 4 g $TSSL^{-1}$. The volatile suspended solids (VSS) were about 70% of the total suspended solids (TSS). In the SBR, a high biomass retention was obtained by choosing a suitable settling time in order to have a minimum biomass settling velocity of 0.7 m h^{-1}, which is the ratio between the vertical distance from the water surface to the effluent discharge point and the settling time (Vlaeminck et al. 2009a). This value means that bio-particles having a settling velocity lower than 0.7 m h^{-1} could be withdrawn through the discharge effluent.

The pH and the ORP were measured using Crison Instruments electrodes, (mod. 53.35 and 53.55, respectively, Crison Instruments Italy). The dissolved oxygen (DO) was measured using a polarographic cell (mod. 60.50 Crison Instruments, Italy) while the conductivity was measured using a conductivity (EC) meters (mod. 53.88 Crison Instruments, Italy). Finally, the temperature was measured both by the PT 100 included in the DO sensor $T_{(DO)}$, and by the PT 1000 included in the conductivity meters $T_{(CE)}$.

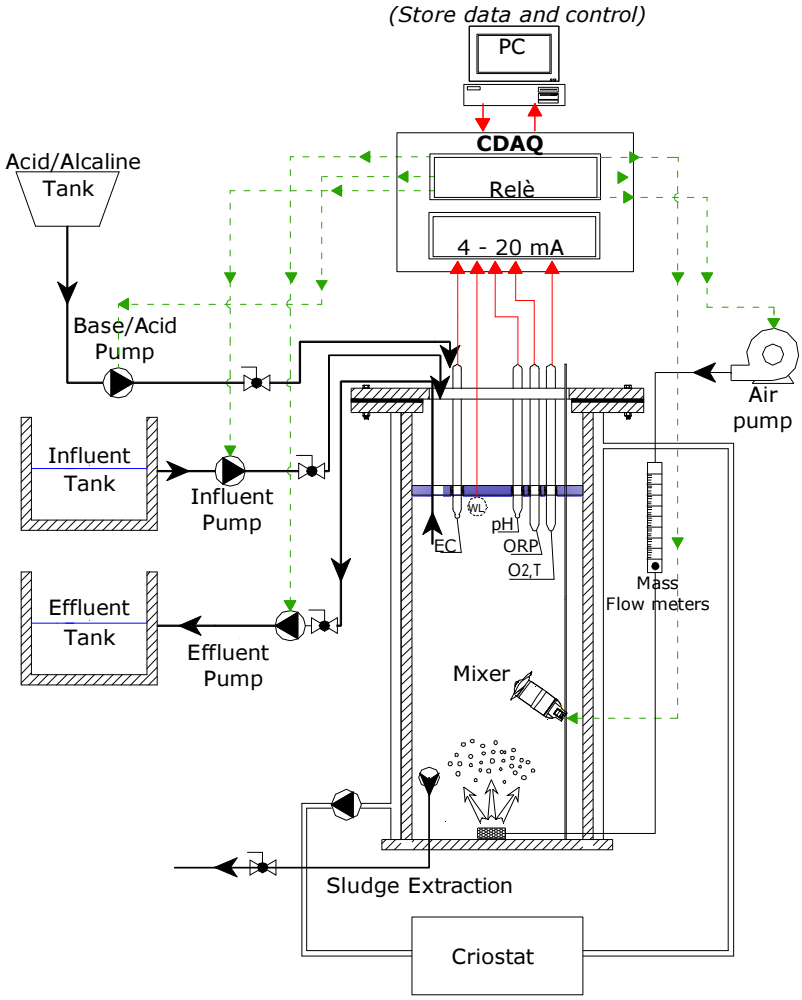


Fig. V.2 Schematic representation of the 8L lab-scale SBR.

SBR cycle. The SBR was operated with three 8 h operational cycles per day, according to the scheme described in Fig. V.3. The reactor was operated with a fixed residual volume of ~7.0 L, which resulted in a volumetric exchange ratio of ~12% per cycle and a hydraulic residence time of 2.7 - 3 d.

Each cycle consisted of 480 min and of six different phases: filling, micro-aerobic reaction, anoxic reaction, settling, drawing and idle. The cycle started with a short feeding (6.6 min) during a mixing period of 10-15 min followed first by a 120-210 min micro-aeration and mixing phase and then by a 234-325 min anoxic and mixing phase. After the completion of these 3 steps, the biomass was allowed to settle for 7 min. The cycle ended with a 12 min window for the effluent discharge and eventually for the sludge wastage. The sludge wastage phase could be set by choosing how often, the volume and the mode of the sludge discharge: either at the end of the settling phase, extracting a concentrated sludge or at the end of the last mixing phase, discharging a mixed sludge. A final idle interval was optional. Both the filling and the drawing phases were considered to occur in an instant.

The filling phase was mixed and short, creating conditions under which the growth rate was higher for floc-formers than for filaments (Artan and Orhon 2005), in order to improve the granulation process. The short filling phase also ensured that the biomass was exposed initially to a high nitrogen load (NLR), and consequently to a high concentration of Free Ammonia (FA), inhibiting the Oxidizing Bacteria (NOB) (Anthonisen et al. 1976), which perform the aerobic oxidation of nitrite to nitrate. The filling phase was mixed and non-aerated and inserted into a short idle period where no oxygen was added, in order to allow the consumption of i) residual

oxygen or alternative electron acceptors, such as nitrite, nitrate and sulphate thanks to heterotrophic bacteria which may use the organics present in the influent (ii) residual nitrite thanks to autotrophic anammox bacteria which may use the ammonium present in the influent. The following micro-aerobic phase, under oxygen limitation, allowed the development of nitritation process, especially due to the lower oxygen NOB affinity. Then, the mixing phase allowed the development of both anammox and denitrification process. The settling phase was short to achieve a high biomass retention, improving the granulation phenomena (Liu et al. 2005). The phase lengths of the SBR cycles tests were fixed and controlled by a control systems based on a simple timer- and logic control.

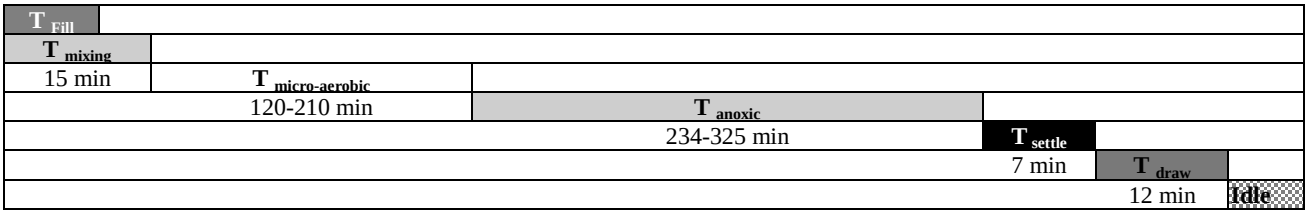


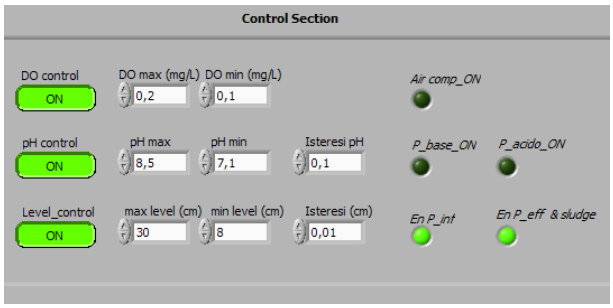
Fig. V.3 Schematic representation of the SBR cycle configuration

2.4. Monitoring and control system

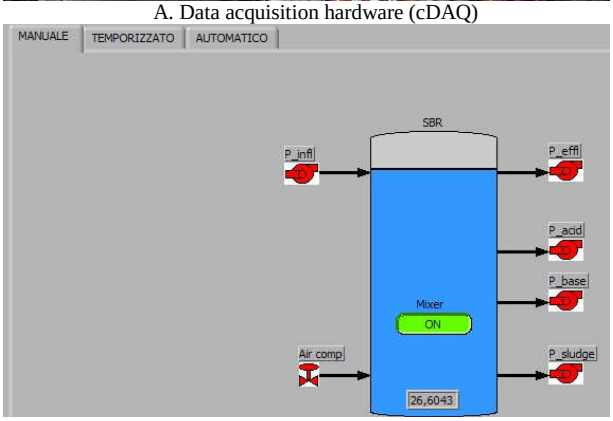
The monitoring and control system of the SBR consisted in a data acquisition system (cDAQ-9174, National Instruments, Austin, TX, USA) and our own software developed using LabVIEW 2010 software (National Instruments, NI, Austin, TX, USA) (Fig. V.4). The cDAQ-9174 (four slots) USB chassis was used to measure analog I/O signals over a Hi-Speed USB 2.0 interface, through two NI C-Series modules: a 16 analog voltage or current input channels, 16-bit resolution (NI9208) module and a 8-Channel C-Series Relay module, 60 VDC/30 Vrms, 750 mA (NI 9485). The cDAQ channel configuration is summarized in the Supplementary Table V-3. Ten analog input (AI) channels were used to obtain the analog input signals, i.e., level, pH, DO, ORP, CE and $T_{(DO)}$, $T_{(CE)}$ in the reactor and pH, CE ,$T_{(CE)}$ in the influent tank. The acquisition and control software developed was able to acquire the on-line parameters, presenting them in a graphical interface (Supplementary Fig. V-1). Signals were sampled and stored in a simple *.xls* file for further processing. Control actions were transmitted by the cDAQ to the relay output board (AO), which controlled the on/off switch of electrical devices.

The software established a simple control strategy based on the time and on the measurements of DO, pH and liquid level of the bulk liquid. During the whole cycle, the pH was controlled, allowing to vary in a pH range by the on/off control of the acid/base pumps. During the micro-aerobic phase, the DO was controlled, ensuing a low DO concentration, by the on/off control of the air pump. The ORP and conductivity were monitored and left free to vary. The SBR had a level control which stopped either the influent pump when a maximum water level was reached (8.0 L maximal reactor volume) before the 6.6 min were up or the effluent and sludge pumps when a minimum water level was reached (7.0 L minimal reactor volume) before the 12 min were up. The software was able to repeat over the time a previously defined cycle operation by controlling the switching on/off devices.

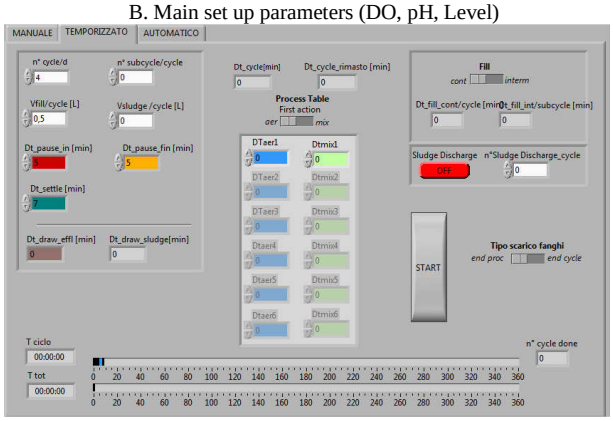
A. Data acquisition hardware (cDAQ)



B. Main set up parameters (DO, pH, Level)



C. Manual control



D. Time control

Fig. V.4 SBR monitoring and control system.

(A) Data acquisition and control hardware (cDAQ). From (B) to (D) Screenshots of the data acquisition and control software developed using LabVIEW 2010 software: (B) main control section: pH, DO and Level; (C) manual control; (D) time control.

2.5. Pilot-plant operation experimental set-up and operation

The experiment conducted on the SNAD-SBR was divided into two periods. In Period I the reactor was fed with the synthetic wastewater and the effect of the temperature on the SNAD process was investigated at 30°C (Step 1) and 25°C (Step 2). In Period II, the liquid temperature inside the SBR was maintained at 25°C and the reactor was fed with a mixture of the synthetic wastewater (the concentration of total nitrogen ammonia and biodegradable organic matter was maintained at around 425±50 mgL^{-1} = 293±112 mgL^{-1}, respectively) and the anaerobic digestion effluent from the urban WWTP in Trento, Italy. The proportion of the anaerobic digestion effluent in the feed progressively increased during the whole period from 20%v/v until the 100%v/v of raw digested effluent.

The nitrogen loading rate (NLR) was set at a value of 0.12 – 0.15 $kgN\ m^{-3}\ d^{-1}$ during the whole experiment. Lower NLRs were applied for a short period of time (< 5 days) at the beginning of the different reactor operating conditions. During Period I, the reactor suspended solids concentration was about 3-3.5 g TSS L^{-1}, while the volatile suspended solids (VSS) were about 70% of total suspended solids (TSS). During Period II a low amount of SNAD biomass was introduced in the reactor and the reactor suspended solids were 4.10 g TSS L^{-1}. In each period, concentrations of COD, TAN, total nitrite nitrogen (TNN) and nitrate (NO_3-N) were measured per half hour over some specific 8h cycles, to study in more detail the processes and the conversion of the C and N species.

The maximum activity of aerobic and anaerobic ammonium oxidizing-, aerobic nitrite oxidizing- and denitrifying bacteria were measured in the Period I. Moreover, in each period, concentrations of COD,

ammonium, nitrite and nitrate were synchronously measured per half hour over some specific 8h cycles, to study in more detail the processes and the conversion of the C and N species.

2.6. Potential specific activity assays

Activity assays were conducted in order to determine the potential activities of aerobic ammonia- and nitrite-oxidizing bacteria (AOBs and NOBs), anaerobic ammonium oxidizing bacteria (anammox) and denitrifying bacteria, considering both denitrification via nitrate and via nitrite. The activity assays were performed during the reactor operations in Period I. Prior to the activity tests, the biomass, taken from the reactor, was washed 3-5 times with tap water, devoid of oxygen, to remove residual dissolved reactor compounds. The last sludge wash was performed using HEPES 20 mM as a buffer solution at the desiderate pH. Activity was determined performing specific batch tests for each biomass and by measurement of the rate of substrates consumption. To this end, samples (5 mL) were taken at regular intervals from the batch tests, filtered through a 0.45 µm-pore-size filter, and analyzed for TAN, TNN, NO_3^--N and COD. Measurements were performed as described below over a time span of 3-4 h with a sampling interval of 30 min.

2.6.1. Aerobic activity assays

Aerobic experiments were conducted to evaluate the potential specific activity of AOBs and NOBs at 20°C. The tests were performed with a respirometric assay in order to compare the experimental results in terms of ammonia uptake rate (AUR), nitrite uptake rate (NUR) and oxygen uptake rate (OUR). A detailed description of the procedure for OUR calculation using a respirometer can be found elsewhere (Spanjers et al. 1998). However, the OUR reported in this work correspond to the exogenous OUR obtained subtracting the endogenous OUR value to the total measured OUR. The dissolved oxygen (DO) consumption was recorded on-line. For each test, the VSS concentration was evaluated and the specific AUR (SAUR), NUR (SNUR) and OUR (SOUR) were obtained dividing the AUR, NUR and OUR by the VSS concentration in the assays. The batch assays were performed in 250 mL glass flasks with a 200 mL working volume. The reactors were water-jacketed at 20°C using a heated circulating water bath. All incubations were performed under a continuous mixing using a mechanical device (150 rpm), rather than a magnetic stirrer, to avoid the granules abrasion. Dissolved oxygen (DO) and temperature were measured in the liquid through the DO probe. The DO probe was connected through a RS-232 port to a computer, which was used for storing and monitoring all data. The flasks were incubated aerobically, aerobic conditions were maintained under active mixing and oxygen furniture thought an air pump. The concentration of total ammonia, nitrite and nitrate was measured by taking samples.

AOB activity assays. This experiment was designed to evaluate the potential specific activity of AOBs at 20°C, minimizing the interference of NOBs during the respirometry in order to compare AUR and OUR results, adapting the protocol proposed by Ciudad et al. (2006). Both high free ammonia and non-limiting DO concentrations were used in order to inhibit NOB activity. As the real substrates of AOBs is the non-ionic form of ammonium (NH_3 or FA), which inhibits NOBs at concentration range of 1-10 mgN-NH_3-NL^{-1}, the AOBs assays were conducted under pH 8 and sufficiently high FA levels, inhibiting NOBs and non limiting AOBs activity. Substrate was added, from a sterile 10 g $TANL^{-1}$ stock solution, to initial concentrations of 130 mg TAN L^{-1}, corresponding at about 5 mg NH_3-NL^{-1}. The DO was monitored and controlled to be in the range between 2.5 – 3.5 mg L^{-1}, in order to limit NOBs activity. The potential aerobic ammonia oxidation activity was calculated from the slope of the total ammonia concentration over the time (AUR). The OUR_{AOB} was compared

to the AUR, taking into account that the aerobic oxidation of ammonia to nitrite require 3.16 mg O_2 per mg NH_4^+-N^{-1} oxidized.

NOB activity assays. Non-limiting concentrations of nitrite and DO were used in order to measure the potential aerobic nitrite activity at 20°C, according to the protocol proposed by Ciudad et al. (2006). As free nitrous acid (HNO_2 or FNA) inhibits NOBs at concentration higher than 0.01-0.07 mg HNO_2-N L^{-1} , the assays were conducted under pH 7.5 and non-limiting nitrite concentration and non-inhibiting free nitrous acid concentration. Substrate was added, from a sterile 10 g TNN L^{-1} stock solution, to initial concentrations of 70 mg TNN L^{-1}, corresponding at about at 0.0056 mgN-HNO_2 L^{-1}. The dissolved oxygen concentrations were monitored and controlled to be in the range between 4 – 6 mg L^{-1}. Further, for all the experiments, thiourea (20 mg L^{-1}) was added to inhibit the AOB activity consuming the possible residual ammonia. The potential aerobic nitrite oxidation activity was calculated from the slope of the total nitrite concentration over the time (NUR). The OUR_{NOB} was compared to the NUR, taking into account that the aerobic oxidation of nitrite to nitrate require 1.1 mg O_2 per mg NO_2^--N oxidized.

2.6.2. Anaerobic activity assays

Anaerobic experiments were conducted to evaluate the potential specific activity of anammox and denitrifying bacteria at 23 ± 1°C. For the anaerobic batch tests, 100 mL glass flasks were used, containing 80 mL of mixed liquor. Once the buffering solution (20 mM HEPES, pH 8) and substrates were added, the flasks were closed with a silicone membrane cap. Directly hereafter, the bottles were made anaerobic by alternatingly applying under-pressure and flushing with N_2 gas, as no growing effect was investigated. An overpressure of 1 bar was maintained in the bottles. The flasks were incubated on a shaker at 23 ± 1°C. Liquid samples were taken for ammonium, nitrite, nitrate and COD analyses.

Anammox activity assays. This experiment was designed to determined the potential specific activity of anammox bacteria (SAA), minimizing the interference of denitrifiers. No COD was used. Non-limiting concentrations of either total nitrogen ammonia or total nitrogen nitrite were used. Further non- inhibitory concentration of total nitrogen nitrite were reached. Flushed substrate solutions of TAN and TNN, from sterile 10 mg L^{-1} stock solutions (NH_4Cl and $NaNO_2$), were supplemented by means of needled syringes. Initial concentrations were 70 mg $TANL^{-1}$ and 25 mg $TNNL^{-1}$. For all the experiments, thiourea (20 mg L^{-1}) was added to inhibit the nitritation process if traces of DO were present. The potential specific anammox activity was estimated from the slope of the curve of the ammonium concentration along the time, dividing by the biomass (VSS) concentrations.

Denitrifiers activity assays. Potential denitrification activities via nitrite and nitrate of the biomass were measured, according to the protocol proposed by Constantin and Fick (1997). Into each bottle, KNO_3, from a sterile 10 mg L^{-1} stock solutions, corresponding to 200 mg NO_3^--N L^{-1} in 80 mL solution, was added by means of needled syringes. Then a flushed substrate solutions of CH_3COONa (sterile 100 g L^{-1} stock solutions) was supplemented to the bottles. The initial amount of the carbon substrate in the 80 mL solution corresponded to approx. 2000 mg $CODL^{-1}$. The same operation was repeated using nitrite. $NaNO_2$, from a sterile 10 mg L^{-1} stock solutions, corresponding to 200 mg TNN L^{-1} in 80 mL solution, was added followed by the addition of the carbon substrates as done in the tests with nitrate. The potential denitrifiers activities via nitrate and nitrite were estimated from the slope of the curve of the nitrate and nitrite concentration over the time, respectively. The values measured were then referred to the biomass (VSS) concentrations.

2.7. Molecular techniques

2.7.1 Sampling and genomic DNA extraction

To maximize the DNA extraction efficiency, representative samples (0.2 mL) with red and brown granules and brownish flocs were taken from the SNAD-SBR. Granules and flocs were aseptically broken to separate the single cells, by passing through a series of syringe needles (0.60 - 0.30 mm). Further, in order to investigate the anammox distribution in the SNAD biomass, both the suspended biomass (0.2 mL) and total granular biomass (0.02 g) were analyzed. In addition, brownish granules (0.02 g) were separated and further analyzed. Total bacterial DNA was extracted from the SNAD biomass samples using QiAamp$^{(R)}$ DNA Stool (QIAGEN) Kit, according to manufacturer's instructions. The final DNA samples (0.2 mL) were diluted 100 times with DNA, and RNase-free water and stored at - 20°C until further analysis.

2.7.2 SYBR Green Real-Time PCR

Although real-time polymerase chain reaction (PCR) amplification with 16S rRNA gene is widely used in anammox bacteria detection, functional genes could also be used as biomarkers. In this study oligonucleotide primers targeting the *hao*/*hzo* gene have been used as functional markers to detect anammox bacteria in the SNAD sludge. Up to now, *hao*/*hzo* gene has been successfully used to detect anammox bacteria from various environmental samples, including wastewater treatment plants, as recently reviewed by Li and Gu (2011).

It was already known that anammox bacteria converts ammonium and nitrite into dinitrogen gas. Hydrazine (N_2H_4) is an intermediate. Anammox bacteria oxidize hydrazine to dinitrogen gas with a hydroxylamine-oxidoreductase- like protein (*hao*). Thus, the pathway for anammox catabolism includes both hydrazine and hydroxylamine (NH_2OH) and more than 200 genes are involved (Strous et al. 2006). A key enzymes of anammox process is the octahaem cytochrome c protein, which is responsible of the bright red colour of the anammox biomass. It is related to the *hao*/*hzo* gene, which is capable of oxidizing hydrazine to N_2 and of producing, under anoxic conditions, NO and N_2O from hydroxylamine.

In order to amplify a part of the *hao*/*hzo* gene of the anammox bacteria a new primer set has been designed. The PCR oligonucleotide primers were designed by using the alignement (CLUSTAL W) of the published DNA sequence of the *hao*/*hzo* gene of *Ca*. Brocadia sp. (GenBank accession number FM163628.1) and the *hao*/*hzo* gene of *Ca*. Kuenenia sp. (GenBank accession number FM163630.1), using the Primer Express Software (Applied Biosystems). The specificity of the primers was checked and confirmed using NCBI's Advanced Blast program. In addition, to test the specificity of the primer set, real time PCR amplifications with the newly designed primers were performed with genomic DNAs that carried target and no target sequences which were obtained in a previous study (Langone et al. 2013b).

The real-time PCR was performed in the 7500 Thermocycler real–time PCR system (Applied Biosystems) using 1 x SYBER Green PCR Master Mix (Applied Biosystems). Amplification was carried out with the mixture solution shown in Table V-2. The new primer set for anammox bacteria consisted of the forward primer (5' GGTCTTCATGGTACGCTATATCAGG 3') and the reverse primer (5' GATCAGCCATCGACATACCCA 3'). A DNA template of 5 µL was added to 20 µL of the mixture solution. The final concentration of each primer in the final PCR solution (25 µL) was 300 nM. The PCR temperature program was initiated with 2 min at 50°C and 10 min at 95°C followed by 40 cycles of 15 s at 95°C and 1 min at 63°C. A melting curve analysis was done after amplification to distinguish the targeted PCR product from the non- targeted PCR product. During the PCR

experiments, negative controls were used to demonstrate any background contamination. Environmental samples from conventional activated sludge of WWTPs and a template without DNA were used as negative controls.

Table V-2 Compositions of mixture solutions for real-time PCR for anammox bacteria

	[µL]
SYBR® Green PCR Master Mix (Applied Biosystems) including: SYBR® Green I dye, AmpliTaq Gold® DNA Polymerase, dNTPs with dUTP, dye molecule and optimized buffer	12.5
Primer Forward (10 µM)	0.75
Primer Reverse (10 µM)	0.75
DNA, and RNase-free water	6
Total	20

2.7.3 Real-Time PCR data analysis

The 7500 Thermocycler real–time PCR system and software (Applied Biosystems) were used for data analysis. The thermocycler real–time PCR system monitors the fluorescence of the reaction mixture just before the denaturation step of each amplification cycle, and records the cycle number at which the fluorescence crosses a specific threshold value (classified as the threshold cycle, C_T). Thus, the C_T is a measure of the quantity of the transcript of interest, and target the DNA copy number. The C_T values are inversely related to the DNA copy number. Further, the real-time machine determines the melting point (T_M) of the product at the end of the amplification reactions, which depends on its base composition. The identity of the PCR product from a sample can be confirmed performing a melting curve analysis comparing its melting temperature (T_M) with the T_M of the product from the positive control. The T_M peaks of the products were calculated based on initial fluorescence (F) curves by plotting the negative derivative of fluorescence over temperature versus temperature (-dF/dT versus T). As no a standard curve was constructed in this study, the real time PCR results should be considered qualitative and can be used for the detection of anammox bacteria. A qualitative comparison of different samples through the C_T values can be only performed when a same quantity of the initial samples is analyzed.

2.8. Analytical methods

Total ammonium as nitrogen (TAN) and total nitrite as nitrogen (TNN) were measured colorimetrically (APHA-AWWA-WPCF 2005). Total suspended solid (TSS), volatile suspended solid (VSS), nitrate as nitrogen (NO_3^--N) and chemical oxygen demand (COD) were determined according to the Standard Methods (APHA-AWWA-WPCF 2005). The COD was always analyzed taking into account the chloride interference and further recalculated considering the interference due to the presence of nitrite (nitrite exerts a COD of 1.142 mgO_2/mg TNN). The truly soluble COD ($COD_{soluble}$) and TAN concentrations of the raw influent were analyzed by using the sequential filtration technique (0.45 and 0.2 µm nominal pore size). The total biodegradable (COD_{bio}) and readily biodegradable organic matter (RBCOD) of the influent were measured by using a combination of physical-chemical (sequential filtration (Roeleveld and Van Loosdrecht 2002) and coagulation using $ZnSO_4$ at pH 10.5 (Mamais et al. 1993; Hu et al. 2002)) and respirometric techniques (Ziglio et al. 2001; Andreottola et al. 2002).

Alkalinity determinations were conducted using the kit USEPA Buret Titration Method 8221 (Hach Company). The KLa of the system was estimated following the standard method of measurement of the transfer of oxygen in clean water published by the American Society of Civil Engineering (ASCE 1992).

2.9. Calculations

Concentrations of free nitrous acid (FNA or HNO_2-N) and free ammonia (FA or NH_3-N) were calculated using the equations Eq. V-1 - Eq. V-3, derived from acid –base equilibrium, as a function of pH, temperature and the sum of unionized and ionized forms, TNN and TAN, respectively (Anthonisen et al. 1976):

$$FNA\left[mgNL^{-1}\right] = \frac{TNN}{1+\left(K_e^{NO}/10^{-pH}\right)} \quad \text{Eq. V-1}$$

$$FA\left[mgNL^{-1}\right] = \frac{TAN}{1+\left(10^{-pH}/K_e^{+NH}\right)} \quad \text{Eq. V-2}$$

$$K_e^{NO} = e^{-2300/(273+T)} \text{ and } K_e^{NH} = e^{-6344/(273+T)} \quad \text{Eq. V-3}$$

The efficiency of total ammonia nitrogen (TAN), total nitrogen (TN) and organic matter (COD) removal were estimated according to the Eq. V-4:

$$Efficiency\ [\%] = \left(\frac{C_{\text{inf}} - C_{eff}}{C_{\text{inf}}}\right)\cdot 100 \quad \text{Eq. V-4}$$

where C_{inf} is the concentration in the influent and C_{eff} is the concentration in the effluent of the SBR.

The nitrogen loading rate (NLR) and organic loading rate (OLR) in each period were calculated by using the Eq. V-5 and Eq. V-6, respectively:

$$NLR\ [gN\ m^{-3}d^{-1}] = \frac{TAN_{\text{inf}}}{HRT} \quad \text{Eq. V-5}$$

$$OLR\ [gCOD\ m^{-3}d^{-1}] = \frac{COD_{\text{inf, filtr}}}{HRT} \quad \text{Eq. V-6}$$

3. Results and discussion

3.1 Period I: temperature effect on the reactor performance

In Period I, the 8 L SNAD-SBR, inoculated with SNAD biomass from the SBR of Zürich, was operated using synthetic wastewater at two different temperature, 30°C (Step 1) and 25°C (Step 2). In the influent, the carbon to ammonium ratio was about 0.5 mg COD/mg TAN in period I at 30°C and 0.9 in period II at 25° in order to simulate the seasonal pattern of the composition of the anaerobic digestion effluent. The alkalinity to ammonium ratio was in the range 8-10 mg$CaCO_3$/mg TAN, similar to the digester effluent characteristics. The $CaCO_3$/TAN was higher than the stoichiometry value for the complete nitritation reaction (7.8 mg$CaCO_3$/mg TAN), and thus, it was not possible to stop the nitritation exactly at 50% ammonia conversion using the alkalinity limitation, as reported in other studies (Bagchi et al. 2010).

As Fig. V.5 shows, in Period I, the NLR was set within 0.13-0.15 kgN m^{-3} d^{-1}. The HRT was ~3 d. A lower NLR had been applied for a short period of time at the beginning of the two stages at different temperature. The applied OLR was between 0.08 - 0.14 kgCOD m^{-3} d^{-1}.

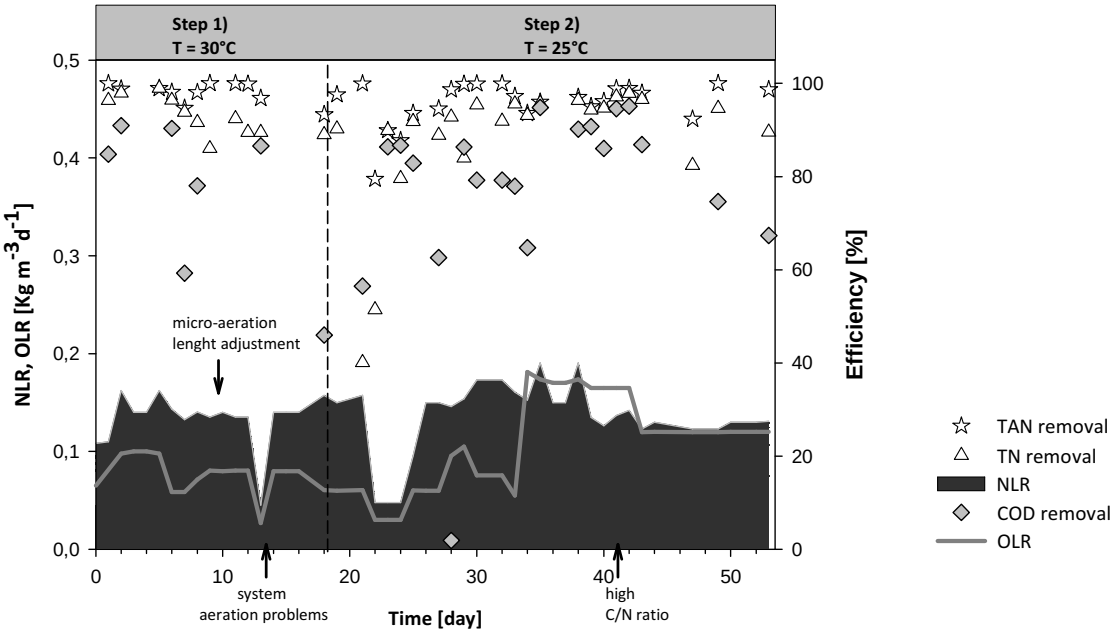


Fig. V.5 Evolution of NLR, OLR and SNAD-SBR performance during period I (step 1 at 30°C and step 2 at 25°C).

Fig. V.5 also shows the operational performance of the SNAD system, including the total ammonia (TAN), the total nitrogen (TN) and the organic carbon (COD) removal efficiencies in the reactor during the whole Period I. As can be seen, in the step 1, at 30°C, high nitrogen removals (%) were achieved almost immediately in the system. Initially, operating the reactor with a duration of the micro-aerobic phase of 230 min , the total ammonia removal and the total nitrogen efficiencies were higher than 98% and 93%, respectively. The COD removal was also high (81%). The simultaneous nitrogen and carbon removal was successful achieved. Nevertheless, as Fig. V.6 shows, after 7 days from the start of the experiment at 30°C, a nitrite (TNN) accumulation in the reactor was detected, suggesting that nitritation process ($NH_3 \rightarrow NO_2^-$) occurred for more than the 50%, due to the gradually AOB acclimatization to the SBR operative conditions (high Free Ammonia, high temperature and low dissolved oxygen). As consequence the system was characterized by high TAN removal efficiency but the TN removal started to decrease. Thus, the length of the micro-aerobic phase was

progressively and manually adapted and reduced in order to achieve a partial nitritation (50%) and a suitable influent for the following anammox process. The duration of the micro-aerobic phase had been decreased from 230 min of the first days to 210 min and then to 170 min (Fig. V.6). The concentrations of TNN gradually decreased and the TN removal efficiency increased again. However, the NO_3^--N concentration in the effluent suggested both the presence of NOB in the reactor and the not complete inhibition of their activity (nitratation process, $NO_2^- \rightarrow NO_3^-$).

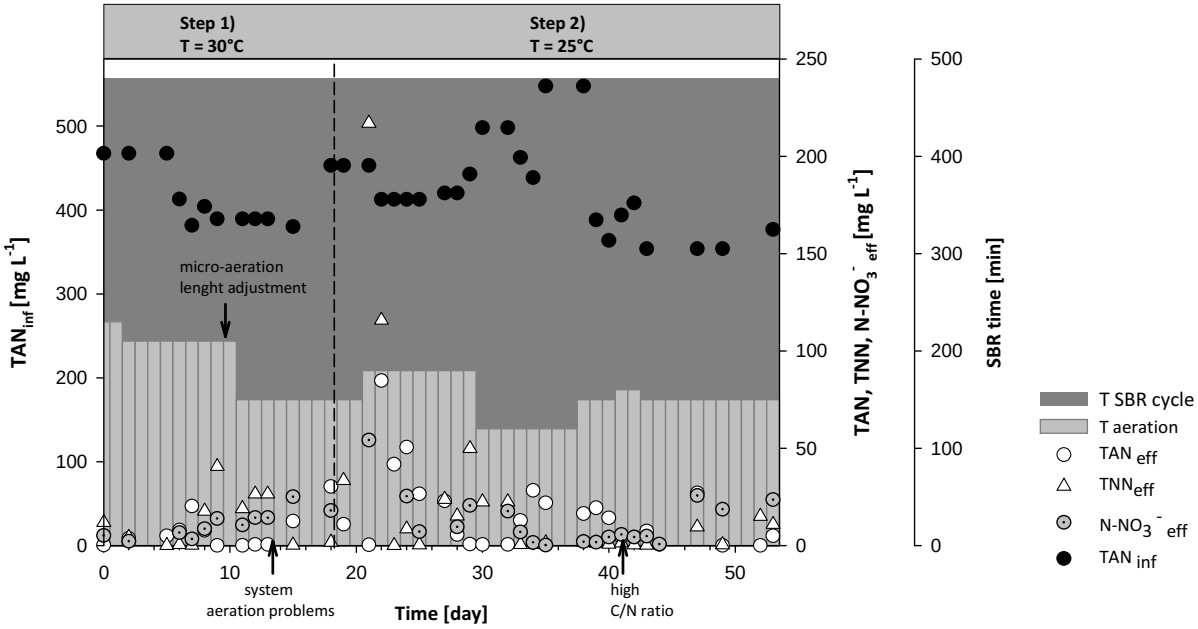


Fig. V.6 Evolution of nitrogen species during period I (step 1 at 30°C and step 2 at 25°C).

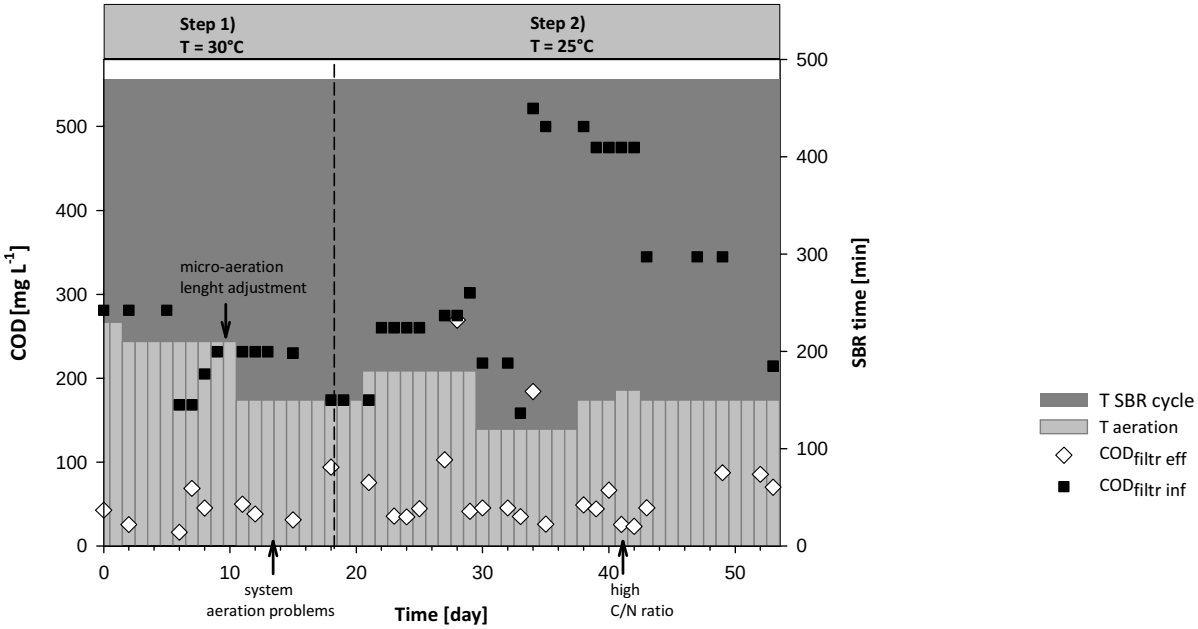


Fig. V.7 Evolution of COD during period I (step 1 at 30°C and step 2 at 25°C).

When the reactor temperature was decreased to 25°C (step 2), the NLR was decreased to 0.05 kgN $m^{-3}d^{-1}$ for few days and then reported to the previous value (NLR=0.13 – 0.15 kgN $m^{-3}d^{-1}$, HRT~3d).

The aeration duration was initially prolonged (180 min) due to lower rates of ammonia oxidation with the decrease in temperature. After the biomass was acclimatized to the moderate temperature (T=25°C), the length of the micro-aerobic phase was decreased again and finally optimized and fixed at 150 min. The system was

stabilized to the new temperature after ~10 days (3-4 times of the HRT). At 25°C, the average of TAN and TN removal efficiencies were 97% and 93%, respectively (Fig. V.5). The COD removal efficiency was 77%. The SNAD sludge in the reactor was gradually adapted to the lower operating temperature, which seems did not deteriorate the stable SNAD performance. In Period I, after a necessary acclimatization period, the reactor was operated in a stable manner at both temperatures. Total ammonium nitrogen and total nitrogen were successful removed at both 30° and 25°C. A high COD removal (%) was achieved in this experiment both at 30° and 25°C, due to the COD oxidation and to the denitrification process via nitrite and nitrate (Fig. V.5 - Fig. V.7).

The Table V-3 summarizes the most important features of the reactor during Period I, after that a stable SNAD process was achieved. Effluent characteristics were similar for both step 1) and step 2) (TAN < 10 mg NL^{-1}, TNN < 13 mg NL^{-1}, NO_3^--N< 9 mg NL^{-1}), and seem to be mainly governed, regardless of the temperature, by the SBR configuration, and in particular by the length of the micro-aeration phase.

Table V-3 Characteristic of the nitrogen removal at step 1 (30°C) and step 2(25°C) (mean values)

Parameter	**Period I - step 1)**	**Period I - step 2)**
Wastewater	Synthetic wastewater	Synthetic wastewater
Temperature [°C]	30	25
Days [d]	1 - 16	28 – 53
VER [%]	12	12
HRT [d^{-1}]	3	3
NLR [kgN m^{-3} d^{-1}]	0.13	0.15
OLR [kgCOD m^{-3} d^{-1}]	0.08	0.14
NRR[kgN m^{-3} d^{-1}]	0.12	0.14
TAN influent [mgN L^{-1}]	415	428
CODfiltr influent [mgCOD L^{-1}]	231	319
TAN out [mgN L^{-1}]	5	10
TNN out [mgN L^{-1}]	13	9
NO_3-N out [mgN L^{-1}]	9	9
CODfiltr out [mgCOD L^{-1}]	40	70
VSS [g L^{-1}]	2.1±0.2	1.9±0.4
TAN removal rate [%]	98	97
TN removal rate [%]	93	93
COD removal rate [%]	81	77
Operative day –Period I	9	35
AOB activity assay (NH_3 →NO_2^-)		
SAUR [kgTANrem $kgSSV^{-1}$ d^{-1}] T=20°C	-0.133*	-0.178**
TNN prod/TAN rem	0.8*	0.9*
NOB activity assay (NO_2^-→NO_3^-)		
SNUR [kgTNNrem $kgSSV^{-1}$ d^{-1}] T=20°C	-0.024*	-0.015**
$SOUR_{NOB\_max}$ [kgDO rem $kgSSV^{-1}$ d^{-1}] T=20°C	-0.028*	0.017**
Anammox activity assay(NH_3 +1.32NO_2^-→0.26 NO_3^- + N_2)		
SAA [kgTANrem $kgSSV^{-1}$ d^{-1}] T=23°C	-0.035*	-0.025**
TNN/TAN	1.3	1.2
Denitrifiers activity assay via nitrate(NO_3^-→NO_2^-)		
Potential specific denitrifiers activity via nitrate [kgN-NO_3^- rem $kgSSV^{-1}$ d^{-1}] T=23°C	-0.214*	-0.429**
Potential nitrite production [kgN-NO_2^- prod $kgSSV^{-1}$ d^{-1}] T=23°C	+0.183*	+0.400**
TNN prod/NO_3^--N rem	0.85*	0.93
Denitrifiers activity assay via nitrite (NO_2^-→N_2)		
Potential specific denitrifiers activity via nitrite [kgTNNrem $kgSSV^{-1}$ d^{-1}] T=23°C	-0.119*	-0.112**

* activity assay performed after 9 days of operative reactor at 30°C

** activity assay performed after 35 days of operative reactor at 25°C

3.2 Period I: C/N effect on the reactor performance

In period I, treating synthetic wastewater at 25°C (Step 2), first a C/N ratio of 0.5 (18 – 32 d) and then a C/N ratio of 0.9 (34 – 52 d) were applied. When the C/N ratio of 0.9 was used in the synthetic wastewater, a higher COD removal efficiency was measured together with a high TN removal efficiency, comparable to the TAN removal efficiency (Fig.V 5). This means that at a C/N ratio of about 1, a higher contribution of denitrification was measured, which contribute to improve the total nitrogen removal efficiency of the system. Nevertheless, C/N ratios higher than 2 are considerate critical for anammox and denitrifying bacteria co-existence (Kumar and Lin 2010).

3.3 Period I: DO shocking effect on the reactor performance

At the 13th day, a dissolved oxygen shock was induced, achieving a high DO concentration DO in the reactor (2–3 mg L^{-1}). The oxygen shock led to an increase of the NOB activity over the AOB activity. This was confirmed by the complete depletion of nitrite and by a nitrate accumulation in the system from the 13th day. Both the TAN and the TN removal efficiencies decreased. A DO increase may cause indeed both a higher nitrate production over the nitrite production and an inhibition of the anammox process, affecting the overall SNAD performance. After 5 days from the aeration system problems, the reactor recovered its performances.

3.4 Period I: microbial activities

The microbial activity of each bacteria involved in the SNAD process was measured performing the activity assays and it is reported in Table V-3. The potential AOB activity at 20°C (SAUR, expressed in kg of TAN removed per kg of reactor VSS per day) of the biomass samples taken from the reactor working at 25°C (step 2) was higher compared with that measured when the reactor worked at 30°C (step 1), probably due to the fact that the nitrifying microorganism community in the SBR got optimized by oxygen limited conditions for long-term operation. Under oxygen limitation, the maximum specific growth rate of ammonia-oxidizing bacteria (AOB) was higher than that of nitrite-oxidizing bacteria (NOB), determining an apparent higher SAUR in the batch assays at 20°C, due to the higher dominance of the AOB over the NOB in the SNAD biomass compared to the start of the experiment. According to the findings reported above, the potential NOB activity of the biomass at 20°C (SNUR, expressed in kg of TNN removed per kg of reactor VSS per day) taken from the reactor working at 25°C (step 2) was lower compared to the SNUR measured when the reactor worked at 30°C (step 1). This could be due to (i) a slight wash-out of the NOB in the reactor, as NOB grow slowly than AOB under oxygen limiting condition and at temperature higher than 25°C and (ii) an inhibition effect of Free Ammonia on NOB activity. However, the NOB were present during the whole Period I and were active as confirmed by the NOB activity assays. In addition, even if the AOB activity assays were specifically designed to measure the AOB activity, the NOB influence in the AOB activity assay was detected. The produced TNN and the removed TAN ratio, was indeed lower than the stoichiometric value of the complete nitritation process (1). As reported in the Table V-3, a lower interference of the NOB activity was detected in the step 2 (TNN_{prod}/TAN_{rem}=0.9), which indicated the gradually disappearance and/or inhibition of the NOB. The interference of the NOB in the AOB activity assays was also confirmed by the $SOUR_{AOB}$ value, which was, both in the step 1 and step 2, higher than the corresponded SAUR value, as it was influenced by the oxygen consumption of both AOB and NOB (data not showed). This last result indicated that the AOB activity in a SNAD process is preferable measured by the AUR rather than respiromentric data analysis (OUR). On the contrary, the $SOUR_{NOB}$ measured by respirometric technique developed in this experiment were perfectly comparable to the corresponding SNUR, obtained

considering the stoichiometry of nitratation process (1.11 g O_2/gNO_2^--N). This implies that the NOB activity assay was optimized for the NOB activity measurement. Thus, respirometric data analysis (OUR) can be performed rather than the direct measurement of nitrite uptake rate (NUR), in order to evaluate the NOB activity, saving time and reagents costs.

Concerning the anammox bacteria, as can be observed from the Table V-3, the potential anammox activity at 23°C (expressed in kg of TAN removed per kg of reactor VSS per day) of the biomass taken from the reactor working at 30°C (step 1) was slightly higher than the value reported for the sludge sampled in step 2, at 25°C. The lower activity detected in the step 2, could be due either to a lower abundance of anammox bacteria in SNAD biomass, due to a wash-out of anammox bacteria present in the flocs or to a partial inhibition of anammox bacteria, due to the lower reactor operative temperature (25°C) or to DO shocks. However, the TNN_{rem}/TAN_{rem} ratios in both the anammox activity assays were close to the 1.32 required by the anammox process, meaning that the assays was optimized for anammox bacteria and there were any interferences by other microorganisms.

Finally, as expected, denitrifiers were active in the SNAD biomass and both nitrate and nitrite removal processes were observed in the activity tests for heterotrophic denitrification. While the denitrifiers which perform the reduction of nitrite to dinitrogen gas seemed to be not influenced by the different working reactor temperatures, the apparent denitrification activity via nitrate (expressed in kg of NO_3^--N removed per kg of reactor VSS per day) were higher in the step 2, when the reactor worked at 25°C. This could be due to a higher abundance of the denitrifiers which perform denitrification via nitrate in the second step over the first, due to the higher COD/N ratio (0.9) in the influent. Finally, we also observed that the $TNN_{prod}/NO_3^-N_{rem}$ ratios in the denitrification via nitrate activity assays, were lower than 1, which is the stoichiometric value of the denitrification via nitrite using acetate. This means that mainly nitrate were reduced to nitrite, but a low amount of nitrate was directly converted to dinitrogen gas.

3.5 Period II: acclimatizing to the digester effluent at moderate temperature

In Period II, the SNAD-SBR was fed with a mixture of the raw digester effluent and the synthetic wastewater. The temperature of the SBR was always controlled at 25°C. During the Period II, the carbon to nitrogen ratio was about 0.5 while the alkalinity to ammonium ratio was variable but high, in the range of 8 - 15 $mgCaCO_3/mg$ TAN. Fig. V.8 presents the evolution of the SNAD-SBR performance over the Period II. As observed, the NLR was set between 0.12 – 0.15 kgN m^{-3} d^{-1}, with the exception of a shock loading induced at 17th day, when the digester effluent was adding in the proportion 20% v/v, followed by a lower NLR for a short period of time. Moreover, a lower NLR had been applied for a short period of time at the beginning of the Period II. The mean HRT was ~ 2.5 - 3 d. The OLR applied was about an half of the NLR, which is typical of the digester effluent composition.

A low TAN and TN removal efficiencies were initially observed when the digester supernatant was started to introduce into the system. Each time that a stable nitrogen conversion was observed, the digester effluent to synthetic wastewater proportion in the influent was increased. After a period of 10 days (3-4 times of the HRT) the system achieved high TAN and TN removal efficiencies, treating 50%v/v of digester supernatant in the influent. After 14 days of operation, the SBR was operated with 100% of raw digester effluent and TAN and TN removal efficiencies higher than 99% and 91% were respectively observed for few days. The COD removal was in the range of 66-80%, lower compared to the Period I, where synthetic wastewater had been treated at 25°C. The lower COD efficiency has been attributed to the lower biodegradable organic content of the anaerobic digester effluent compared to the synthetic wastewater. Even if the total COD concentrations in anaerobic

digester effluent were comparable with the total COD content in the synthetic wastewater, the biodegradable COD of the digester liquor was only 50% of the total organic carbon content.

However, high mean values of TAN (96%), TN (88%) and COD (58%) removal efficiencies were obtained, gradually treating the digester effluent.

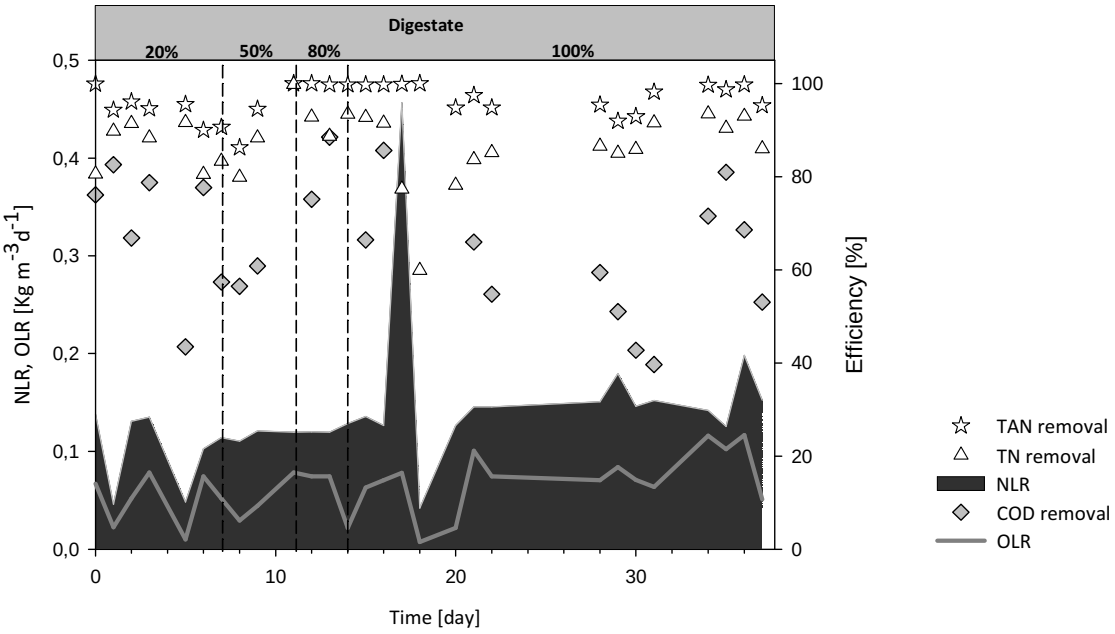


Fig. V.8 Evolution of NLR, OLR and SNAD performance during period IIat 25°C.

Table V-4 summarizes the most important features of the reactor over the Period II, after the stable process was achieved treating 100% v/v anaerobic digester effluent at moderate temperature (T=25°C) and at a mean NLR of 0.15 kg N m^{-3} d^{-1}. High nitrogen removal efficiencies were measured. Nevertheless, a higher nitrate concentration in the SBR effluent was observed compared to the synthetic wastewater, due to the lower biodegradable COD in the digester effluent, reducing the denitrification contribution to the SNAD process. Further, treating the digester effluent, a decrease in the pH of the system was detected, as detailed showed in the next section, which entail that SBR, in Period II, worked at lower Free Ammonia concentration, allowing a NOB reactivation and compromising the SNAD performances.

Table V-4 Characteristic of the nitrogen removal at 25°C , treating anaerobic digester effluent

Parameter	**Period II**
Wastewater	Anaerobic digester effluent
Temperature [°C]	25
Days [d]	20 - 37
VER [%]	12
HRT [d^{-1}]	3
NLR [kgN m^{-3} d^{-1}]	0.15
OLR [kgCOD m^{-3} d^{-1}]	0.08
NRR[kgN m^{-3} d^{-1}]	0.14
TAN influent [mgN L^{-1}]	417
CODfiltr influent [mgCOD L^{-1}]	217
TAN out [mgN L^{-1}]	15
TNN out [mgN L^{-1}]	2
NO_3-N out [mgN L^{-1}]	35
CODfiltr out [mgCOD L^{-1}]	92
VSS [g L^{-1}]	3.6±0.2
TAN removal rate [%]	96
TN removal rate [%]	88
COD removal rate [%]	58

Fig. V.9 and Fig. V.10 show the evolution of influent and effluent versus time, nitrite, nitrate and carbon concentrations and the duration of the micro-aerobic phase, which ranged within 120 – 150 min. After a period of 10 day, the complete TAN depletion can be observed with no nitrite accumulation. Nevertheless, compared to the SNAD performance in the Period I, a higher nitrate accumulation in the reactor was detected, according to the lower COD and TN removal efficiency measured.

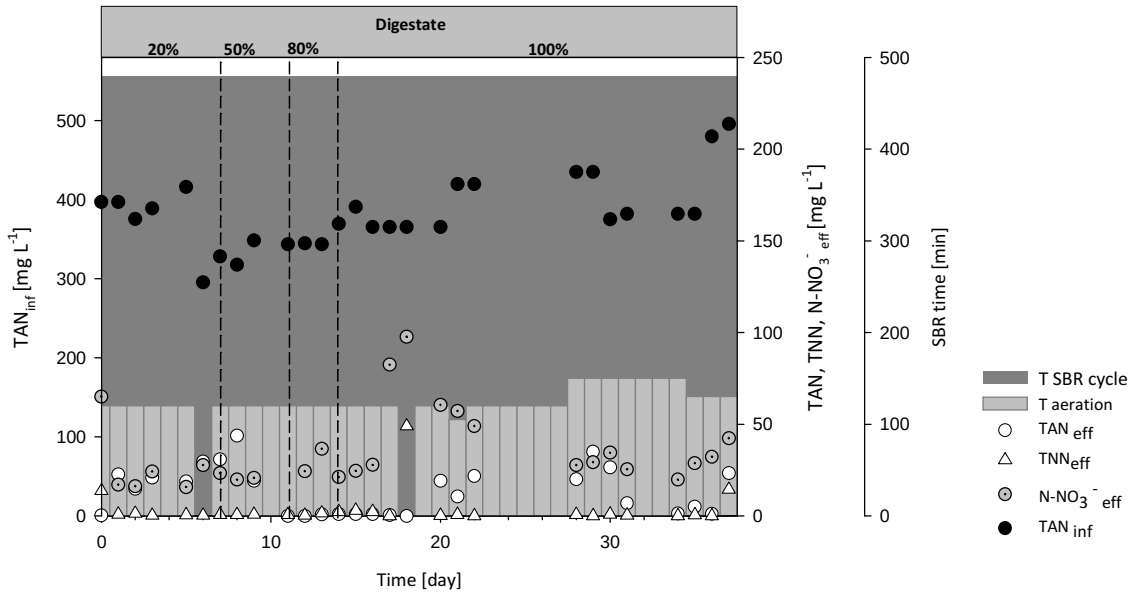


Fig. V.9 SBR reactions phases and evolution of nitrogen species during period II at 25°C.

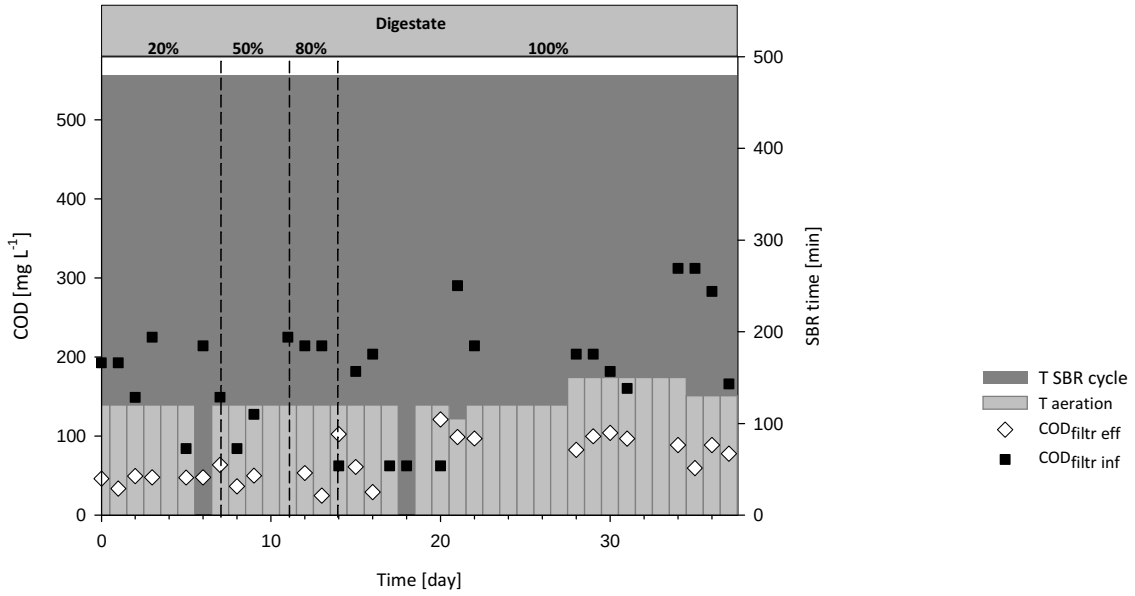


Fig. V.10 SBR reactions phases and evolution of COD during period II at 25°C.

3.6 Period II: Load shocking effect on the reactor performance

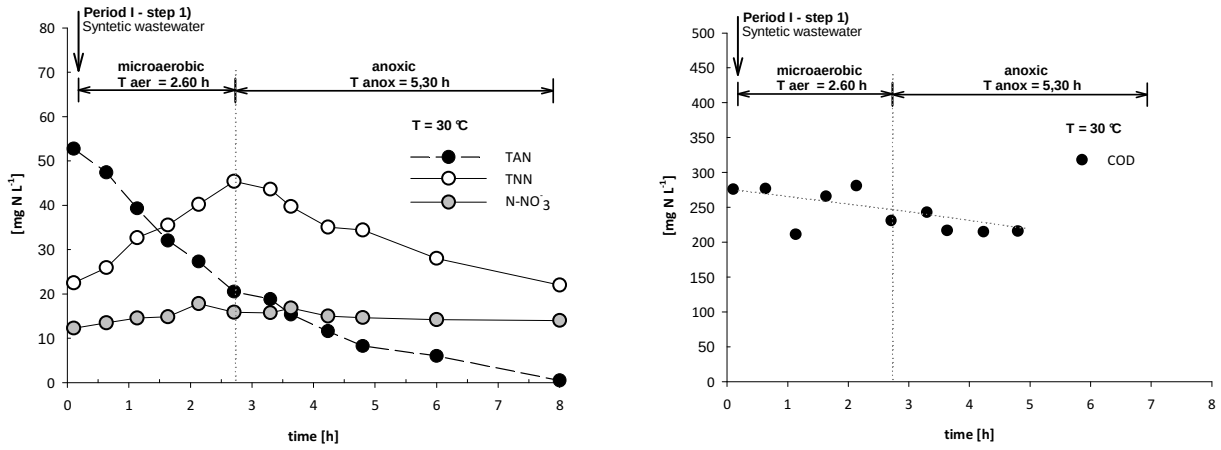
At 17th day, the system lost its stability, due to an induced shock loading (0.44 kg N m^{-3} d^{-1}) and dissolved oxygen shock. After an immediate decrease of the applied NLR followed by 9-10 days (3-4 times of the HRT) of stable influent ammonium concentration, the system recovered its performance, and the TAN and TN removal efficiencies evolved towards 91–99% and 85-93%, respectively.

The shock loading affected the SNAD system, mainly due to a possible slow response of AOB to the increased loading rate together with an unsuitable SBR configuration, which did not appeared to be appropriate for the higher NLR applied. Further, the aeration failure caused a DO increase in the system, which also led to an increase of the NOB activity and consequently to a high nitrate concentration in the reactor effluent. In addition, the high DO levels could partially inhibit anammox bacteria. However, the SNAD-SBR quickly recovered its efficiency, showing the high flexibility of the system under shock loading operations and shock intrusions of oxygen.

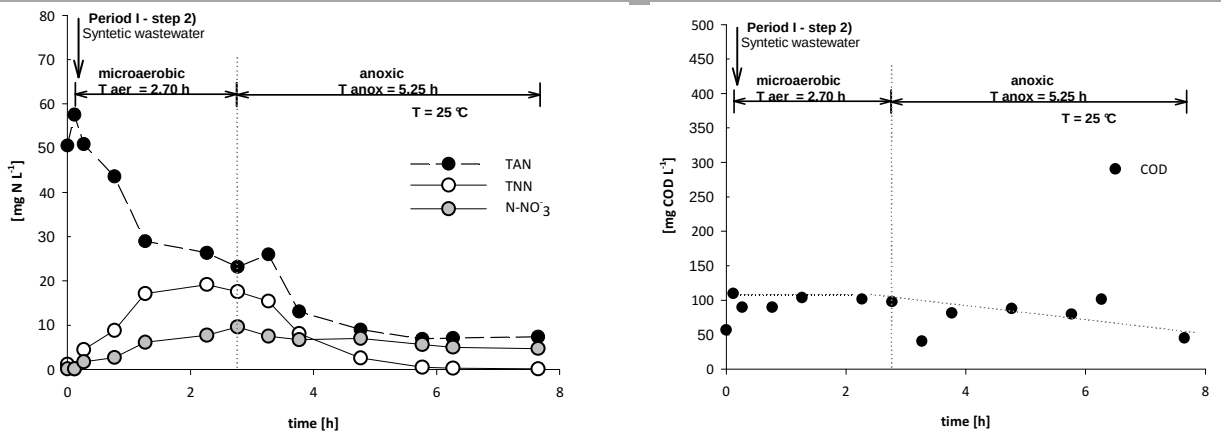
3.7 Evolution of the nitrogen over a 6h SBR cycle

In order to study in more detail the SNAD process and the conversion of the nitrogen and carbon species, a 8 h cycle profile was analyzed at day 12 of Period I – step 1) at 30°C, at day 43 of period I – step 2) at 25°C, treating synthetic wastewater (Fig. V.11 a - b) and at day 22 of Period II at 25°C, treating anaerobic digester effluent (Fig. V.11 c).

a) Period I – step 1)



b) Period I – step 2)



c) Period II

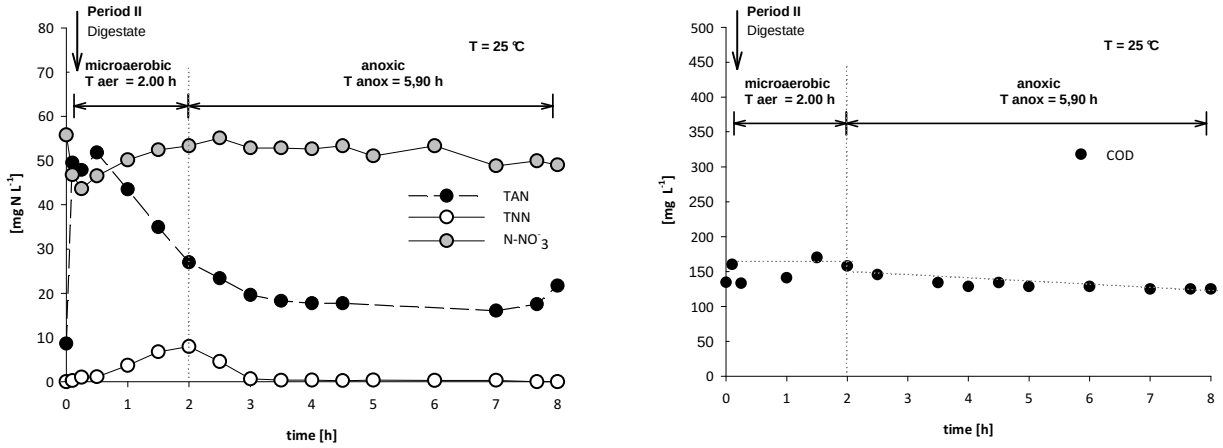


Fig. V.11 Evolution of carbon and nitrogen species over a 8 h SBR cycle: (a) Period I- step 1); (b) Period I- step 2), c) Period II

The Fig. V.11 presents both the evolution of carbon and ammonium, nitrite and nitrate inside the reactor on different days, characterized by different temperatures and different wastewaters. In general, in all nitrogen profiles it was observed that TAN concentration gradually decreased with elapsed time under micro-aerobic condition, from 50-60 to 20-30 mg TAN L^{-1}, depending on the aerobic ammonia oxidation rate and on the duration of the micro-aerobic phase. The TAN removal in the micro-aerobic stage could be also caused by other processes. First, the aeration may resulted in an effect of air ammonium stripping. Second, the cell synthesis may resulted in nitrogen requirements. Third, anammox process might be carried out with the simultaneous consumption of ammonium and nitrite, as anaerobic micro-ecological environments would exist as well as anammox process may be active under oxygen limitation, even if it would be inhibited by low concentration of dissolved oxygen (Yan et al. 2012). On the contrary, an increase of TNN concentration due to nitritation process was observed, reaching a maximum TNN concentration of 40 mg $TNNL^{-1}$, depending both on the NOB washed out or inhibition and on the nitrite accumulation of the previous cycles. Moreover, during the whole experiment, a slightly increase of the NO^-_3–N in the micro-aerobic phase was detected, confirming the not completely wash-out or inhibition of NOB and a possible interaction of anammox bacteria in micro-aerobic conditions.

The residual TAN and TNN at the micro-aerobic phase were consumed in the following anoxic phase, due to the anammox process. TNN and NO^-_3–N were also utilized by heterotrophic bacteria. The depletion of the TAN by anammox process depended on the concentration of TNN at the end of the micro-aerobic phase. However, TNN could be also produced in the anoxic phase during the first step of the heterotrophic denitrification (NO_3^- to NO_2^-), if biodegradable carbon source is available. The decreasing COD profiles in the anoxic phase showed that heterotrophic denitrifiers are active during the anoxic phase, especially using synthetic wastewater where a higher readily biodegradable organic matter was present.

In Fig. V.11, it is clearly shown the effect of the micro-aerobic phase length on the SNAD efficiency. In Period I, both in the step 1 and 2, the duration of micro-aerobic phase (2.5 -2.7 h) led to reach high nitrite concentration at the end of the aerobic phase. Thus, in the following anoxic phase, the nitrite produced by AOB were sufficiently high to deplete the main part of the residual ammonia by anammox process. In Period II, the shorter duration of the micro-aerobic phase (2 h), imposed to avoid further nitrate accumulation in the system thanks to the long oxygen exposure, allowed to reach lower nitrite concentration, which were not enough to carry

on the complete depletion of TAN by anammox process (Fig. V.11 c), which clearly stopped after 3 hours, when the nitrite were completely depleted. Further, in the Period II, even if there was a nitrate accumulation in the system, significant reduction of NO_3^- to NO_2^- did not occurred by denitrification via nitrite process, as can be also seen from the correspondent almost constant COD profile, due to the low amount of readily biodegradable COD in the anaerobic digestion effluent. Thus, during the anoxic phase, no nitrite were produced to further perform the anammox process. At the end of the anoxic phase, when TNN were completely depleted and nitrate were present, a TAN production was also observed, probably as a result of sulfur-oxidizing DNRA (dissimilatory nitrate reduction to ammonia) activity, reducing NO_3^- to NH_4^+, in contrast to the conversion of nitrate to N_2 by denitrification (Sher et al. 2008).

To study the results in more depth and quantify the nitrogen conversion process during the SNAD process, experimental data were analyzed and the consumption or production of ammonium, nitrite and nitrate at each reaction stage were calculated, as shown in Table V-5. The ratios of the main consumed and produced substrates at micro-aerobic and anoxic phases were calculated and also listed in Table V-5. The ratio values were compared with the stoichiometry values of the corresponded reactions and could be used as an useful criteria to confirm the extent of the different biological processes.

Under oxygen limited condition, when the reactor temperature decreased from 30 to 25°C, the aerobic specific ammonia consumption rate measured in the SBR, mainly related to the AOB activity, decreased by 1.15 times from 0.130 to 0.113 g TAN $gVSS^{-1}$ d^{-1}, which was satisfactorily described using the Arrhenius expression (∂ =1.035). This temperature coefficient is similar to that proposed by Guo et al. (2010) under temperature from 20 to 35°C (∂ = 1.051), according to the fact that the temperature coefficient should be considered separately in different temperature intervals for the aerobic ammonium oxidation process. Further, the aerobic specific ammonia consumption rate did not decreased treating digester effluent, most likely due to the gradually adaption of the sludge to the real wastewater. However, the nitrate profiles during the micro-aerobic phase indicated that NOB were active. This was also confirmed by the TNN_{prod}/TAN_{rem} ratio values, which were always lower than the stoichiometry value of the nitritation process (1). The lower TNN_{prod}/TAN_{rem} ratio values indicated that a part of nitrite were converted into nitrate by NOB as well as that anammox process could occurred under oxygen limited conditions. Moreover, looking at the $NO_3^-N_{prod}/TAN_{rem}$ ratio, it can be observed a gradually increase of the NOB contribution over the time, expecially treating the anaerobic digester effluent. The nitrate production rate increased during the course of the experiment, as NOB were gradually reactivated, indicating that, working at moderate temperature, in addition to the oxygen limiting conditions, other selective pressures (i.e. high FA and FNA) should be provided to inhibit NOB bacteria. Finally, it is interesting to note that the $(TNN+N\text{-}NO_3)_{prod}/TAN_{rem}$ ratio is about 0.9, indicating that other process, as reported above, may contribute to the ammonia removal in during the micro-aerobic phase.

Concerning the anoxic period, as can be seen from the Table V-5, the anoxic specific ammonia consumption rate was higher at 30°C than at 25°, while no difference were detected at 25 °C treating either synthetic wastewater or digester effluent. When the temperature decreased from 30 to 25°C, the anoxic specific ammonia consumption rate decreased by 1.2 times from 0.072 to 0.060 g TAN $gVSS^{-1}$ d^{-1}, due to the temperature negatives effect on the anammox activity (Dosta et al. 2008). However, the anoxic specific ammonia consumption rates were slightly lower than the values calculated by Dosta et al. (2008) using biofilm biomass or granular anammox in a complete anoxic reactor. In the SNAD reactor the DO concentration inhibits anammox bacteria, reducieng the the anoxic specific ammonia consumption rate. The temperature effect on anammox

bacteria can also be detected looking at the potential SAA measured in the anammox activity assays at 23°C, which is lower than the values detected during the working period of the SBR at 30° and 25°C.

Despite the anammox activity detected in the anoxic period, no nitrate production was measured during the anoxic phase, but rather a decrease in nitrate concentration was always observed. This result was linked to the heterotrophic denitrifiers activities which reduce the nitrate produced by NOB in the micro-aerobic phase and the nitrate produced by anammox process into nitrite and N_2. An apparent denitrification activity via nitrate ($NO_3^- \rightarrow NO_2^-$), about 0.006 - 0.011 kg NO_3^--N kg VSS^{-1} d^{-1}, was detected during both the Period I and II. Similar results were reported by Xu et al. (2010) in their study on the SNAD process, using an influent with a low C/N ratio. However the NO_3^--N_{rem}/COD_{rem} ratio was lower compared to the stoichiometric value reported for denitrification via nitrate, as nitrate were simultaneously produced by anammox process.

Table V-5 Characteristic of the nitrogen removal at 30°C and 25°C

Parameter	Period I step 1)	Period I step 2)	Period II	Stoichiometry
Wastewater	Synthetic wastewater	Synthetic wastewater	Digester effluent	-
T [°C]	30	25	25	-
Operative day	9	43	22	
Micro-aerobic phase				
ammonia consumption rate [kgTANrem $kgSSV^{-1}$ d^{-1}]	-0.134	-0.113	-0.111	-
nitrite production rate [kgTNNprod $kgSSV^{-1}$ d^{-1}]	+0.091	+0.076	+0.047	-
nitrate production rate [kgNO_3^-N prod $kgSSV^{-1}$ d^{-1}]	+0.025	+0.027	+0.045	-
TNNprod/TAN rem	-0.68	-0.67	-0.42	-1 gTNN/gTAN nitritation
NO_3^-N prod /TAN rem	-0.19	-0.24	-0.40	- 1 g NO_3^-N /gTAN nitratation
NO_x^-N prod /TAN rem	-0.87	0.91	-0.83	- 1 g NO_x^-N /gTAN nitrification
Anoxic phase				
ammonia consumption rate [kgTANrem $kgSSV^{-1}$ d^{-1}]	-0.072	-0.060	-0.061	-
nitrite consumption rate [kgTNNrem $kgSSV^{-1}$ d^{-1}]	-0.095	-0.055	-0.060	-
nitrate conversion rate [kgN-NO_3^-prod $kgSSV^{-1}$ d^{-1}]	-0.011	-0.009	-0.006	-
COD consumption rate [kgCOD prod $kgSSV^{-1}$ d^{-1}]	-0.080	-0.088	-0.060	-
TNNrem/TAN rem	+1.32	+0.92	+0.98	+1.32 gTNN/gTAN anammox process
NO_3^-N /TAN rem	+0.15	+0.15	+0.10	-0.26 g NO_3^-N gTAN anammox process
NO_3^-N rem/CODrem	+0.13	+0.102	+0.10	+0.87 g NO_3^-N /g COD denitrification $NO_3^- \rightarrow NO_2^-$
TNN rem /COD rem	+1.18	+0.62	+1.0	+0.58 gTNN /gCOD denitrification $NO_2^- \rightarrow N_2$
NO_x^-N rem /COD rem	+1.32	+0.72	+1.1	0.35 g NO_x^-N /gCOD denitrification $NO_3^- \rightarrow NO_2^- \rightarrow N_2$

In the period I, step 1), the TNN_{rem}/TAN_{rem} ratio in the anoxic phase maintained in a value of 1.32, commonly assumed in literature for anammox reaction (Strous et al. 1999b). Nevertheless, this value is only apparent as other simultaneous processes occurred, such as the reduction of NO_3^- to NO_2^- and the reduction of NO_2^- to N_2 by heterotrophic denitrifying bacteria. Probably, in the period I, step 1), anammox process occurred and the overall contribution of NO_2^- production and NO_2^-consumption by denitrification was zero. In the other two SBR cycles analyzed, the TNN_{rem}/TAN_{rem} ratio was lower than 1.32, probably due to either a higher activity of heterotrophic

bacteria which reduce NO_3^- to NO_2^- over the heterotrophic bacteria which reduce NO_2^- to N_2^-, or a contribution of DNRA which produce ammonium reducing NO_3^-.

In the anoxic phase, the co-existence of anammox bacteria and denitrifiers was also confirmed by the $(TNN+NO_3\text{-}N)_{rem}/COD_{rem}$ ratio, which was higher than the stoichiometric value of the overall denitrification (0.35), as anammox process together with denitrification contribute to the NO_2 removal.

3.8 Stoichiometry model based evaluation of the SNAD system

The consumption of nitrogen compounds in partial nitrification and anammox processes and the consumption of carbon and nitrogen in denitrification were modeled using the stoichiometric equations and the experimental data (Wang et al. 2010; Lan et al. 2011). Unlike previous stoichiometric models reported in literature, which consider only the influent and the effluent characteristics for modeling, in this study the nitrogen and carbon profiles in the SBR were considered. The model was developed considering that simultaneous bioprocesses could occur during the SBR cycle, in both the micro-aerobic and the anoxic phase. In particular, during micro-aerobic phase the co-existence of the nitritation, nitratation and anammox process was considerate, while in the anoxic phase anammox process, denitrification via nitrate and denitrification via nitrite were taken simultaneously into account. Aerobic denitrification, DNRA and synthesis processes were not considered. The following stoichiometric relationships were used for modeling the biological activities:

(i) in nitritation, the molar ratio $TNN_{removed}$:$TAN_{removed}$ of 1:1 (1 g TNN/g TAN) was utilized. During the micro-aerobic phase, the nitrite produced by AOB could be utilized both in the nitratation and anammox processes. The residual TAN could be used in anammox process during the following anoxic phase while TNN could be used both in anammox and denitrification via nitrite processes.

(ii) in nitratation, the molar ratio $NO_3^-\text{-}N_{produced}$:$TNN_{removed}$ of 1:1 (1 g NO_3^--N/g TNN) was adopted. The nitrate produced in micro-aerobic phase could be utilized in the denitrification via nitrate process during the anoxic phase.

(iii) in anammox process, the $TNN_{removed}$:$TAN_{removed}$ molar ratio and the molar ratio of $NO_3^-\text{-}N_{produced}$:$TAN_{removed}$ were 1.32:1 (1.32 g TNN/g TAN) and 0.26:1 (0.26 g TNN/g TAN), respectively. The nitrate produced could be further utilized in denitrification via nitrate in the anoxic phase.

(iv) in denitrification via nitrate ($NO_3^- \rightarrow NO_2^-$) and in denitrification via nitrite ($NO_2^- \rightarrow N_2$), CH_3COOH was considered as the biodegradable organic matter. Theoretically, denitrification via nitrate utilize 4 mol of NO_3^- per mole of CH_3COOH consumed (0.87 g NO_3^--N/g COD) producing 4 mol of NO_2^- (0.87 gNO_2^--N/gCOD). Further, in the SNAD system, it was assumed that the produced TNN could be used both in anammox and denitrification via nitrite processes during the anoxic phase. Denitrification via nitrite can utilize 8 mol of NO_2^- per 3 mole of CH_3COOH consumed (0.58 g NO_2^--N /g COD) producing N_2.

Starting by the nitrogen and carbon concentrations in the reactor at the beginning of the SBR cycle, the percentages in which nitritation and nitratation processes occurred in the micro-aerobic phase were fixed according to the concentration of the nitrogen species measured at the end of the micro-aerobic phase. In the anoxic phase it was supposed that anammox reaction occurred only if both TNN and TAN were present in the suitable ratio (TNN:TAN=1.32:1), achieving the final TAN concentration measured at the end of the SBR cycle. In the anoxic phase, if the nitrite concentration was not high enough to carry on the anammox process, first denitrification via nitrate occurred, in order to produce nitrite. Finally, several steps of denitrification via nitrate and nitrite occurred until the final concentrations of oxidized nitrogen compounds were achieved. The final concentration of COD was verified. The contribution of each process in the SNAD system, based on the

stoichiometric modeling, is shown in Table V-6 for each SBR cycle investigated in the different periods. The detailed modeling concept and the outcomes at 30°C using synthetic wastewater (period I, step 1), based on the evolution of carbon and nitrogen species over the 8 h SBR cycle, is shown in Fig. V.12.

Table V-6 Stoichiometry model results of the SNAD process in period I and II

Parameter	**T=30°C**	**T=25°C**	**T=25°C**
Wastewater	Synthetic wastewater	Synthetic wastewater	Digestate
Period	I – step 1	I– step 2	II
day	12	36	
T [°C]	30	25	25
Influent CODtot/N	0.5	0.9	0.9
Influent CODbio/N	0.5	0.9	0.5
Micro-aerobic phase			
Nitritation (aerobic ammonia-oxidizing bacteria) %TANin removed	56	45	38
Nitratation (aerobic nitrite-oxidizing bacteria) %TNNin removed and converted in NO_3^--N	6	20	29
Anammox (anaerobic ammonia-oxidizing bacteria) %TANin removed	5.2	4.2	7.5
Anoxic phase			
Anammox %TANin removed	23.3	35	18.7
Denitrification via nitrate (heterotrophic bacteria) %CODin removed	3	14	2.9
Denitrification via nitrite (heterotrophic bacteria) %CODin removed	3	0	0

The results of the stoichiometric modeling indicated that, during the micro-aerobic phase, nitritation was the prevalent TAN removal process (40 – 56%). The anammox process was showed to contribute to the TAN removal efficiency in very low percentage (5 – 7%) in micro-aerobic phase. On the contrary, during the anoxic phase anammox process was the prevalent process, contributing 23 – 35 % to the total TAN removal. NO_3^- produced in nitratation and in anammox process were utilized in denitrification along with COD further producing NO_2^-. A low NO_2^- consumption due to denitrification via nitrite was estimated. This reveals that the SNAD system has the capability of acting as shortcut nitrification–denitrification (SND), however the removal efficiency of the SND process in this system is far less than the SNAD efficiency. In our SNAD-SBR, denitrification contributed lower than anammox process to the total nitrogen removal process.

Comparing the different periods, it can be observed that nitratation was active during the whole experiment and its contribution was growing at 25°C compared to the 30°C, due to the long term effect of the moderate temperature under oxygen limiting conditions. The role of nitratation process was even higher treating the anaerobic digester effluent, as the pH of the system was lower and thus lower free ammonia levels were achieved in the system. Regarding the denitrification process, its contribution was almost high at 25°C (14%) treating synthetic wastewater characterized by a COD_{bio}/TAN ratio of 0.9. Treating wastewaters with a lower CODbio/TAN (< 0.5), the denitrification contribution was lower than 5% both at 30 and 25°C.

The stoichiometric modeling could be an useful tool to better understand the evolving of the SNAD process and the contribution of each process involved under different shocking conditions, such as ammonia load, DO, pH and temperature.

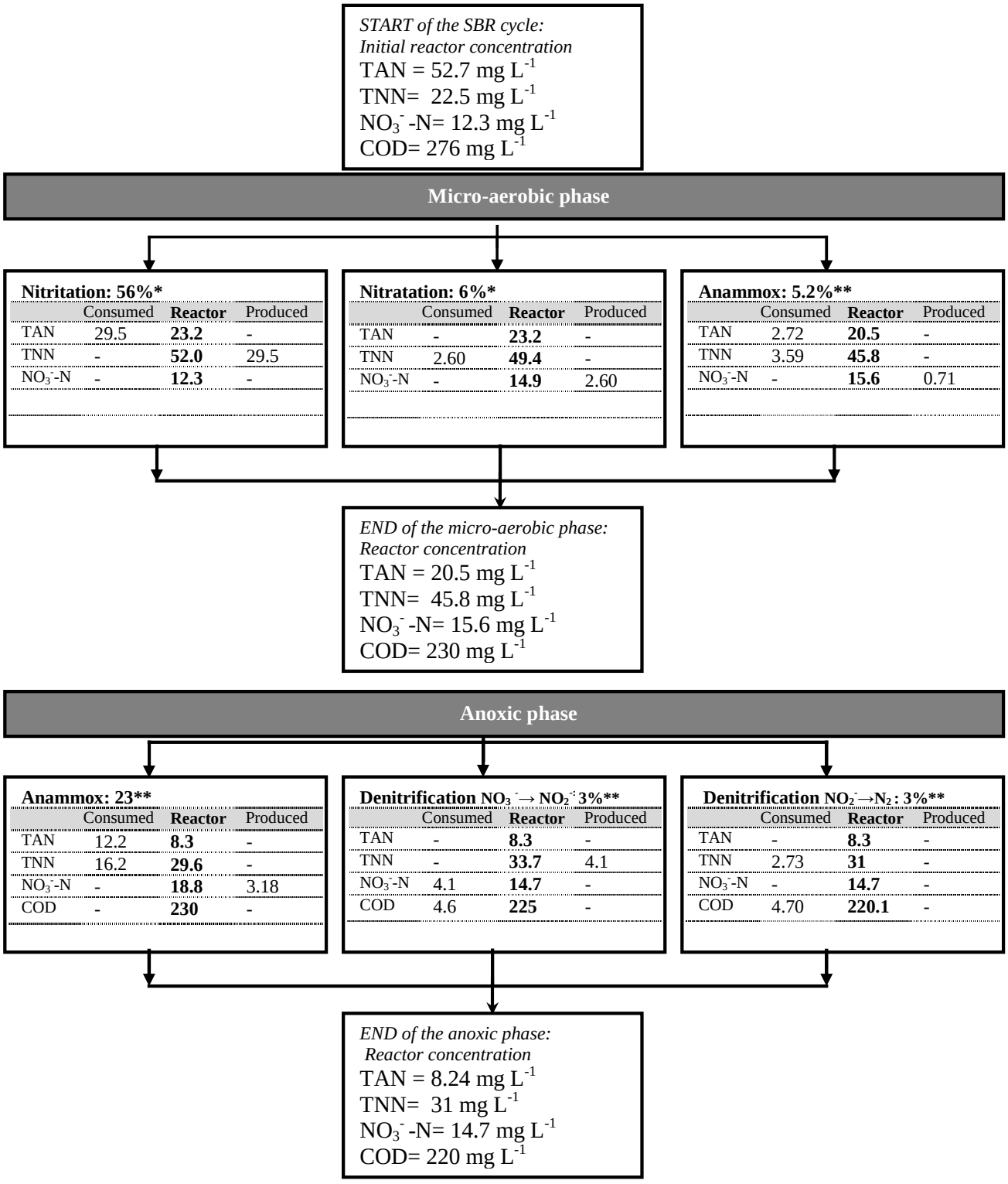


Fig. V.12 Stoichiometry model based evaluation of the SNAD system over the 8h cycle SBR in Period I, step 1).
* attempted values
** calculated values

3.9 Microbial analysis

3.8.1. Specificity of PCR primer

To evaluate the specificity of the designed primer set, targeting the *hao/hzo* gene of anammox bacteria, the extracted DNA from the SNAD biomass, previously analyzed with the conventional PCR method (Langone et al. 2013b), was examined as a template. Negative controls were also used. The SNAD biomass template was positive in the real time PCR assay and produced a mean C_T value of 27.3. Specific PCR products were identified by melting curve analysis and a reproducible distinct melting point (T_M) of 81.6°C was observed for the SNAD biomass amplicon, containing anammox bacteria. The amplification of the DNA from the negative control did not show peaks in T_M that corresponded to 81.6 °C. The results of the real-time PCR analysis are shown in the Supplementary Fig. V-2.

3.8.2. Anammox bacteria detection and distribution in the SNAD biomass

The whole SNAD biomass (0.2 mL) used as inoculums was analyzed using real-time PCR, confirming the presence of anammox bacteria in the whole biomass, containing both red and brown granules and single cell in a net of filamentous bacteria. In our study, separated analysis conducted on the granular and suspended fraction of the SNAD biomass, showed that anammox bacteria were detected in the both fractions. The presence of anammox bacteria in the granules is well reported in literature (Nielsen et al. 2005; Chen et al. 2009; Li et al. 2011b). The presence of anammox bacteria in the suspended flocs is probably due to the abrasion of the granules, caused by the aeration and mixing system, as also previously demonstrated by De Clippeleir et al. (2009) in their 2.5 L SBR performing the OLAND process. Ni et al. (2010) reported that even the brownish sludge, which coexisted with the red granules in a granular anammox reactor, contained 31% anammox bacteria.

In the SNAD biomass two kinds of granules (red and brownish) could be distinguished (Fig. V.13). The two kind of granules have been demonstrated different physical and microbial properties. In our study we showed that the anammox bacteria were present in the granular biomass. Both the total-granule DNA samples and the brownish-granule DNA samples were positive in the real-time PCR assays and a melting point of 81.6°C was observed. However the total-granule DNA samples (extracted from 0.02 g of total granular biomass) produced a mean C_T value of 26, while the brown-granule DNA samples (extracted from 0.02 g of brownish granular biomass) produced a mean C_T value of 36. As the C_T value is inversed related to the DNA number copy, in the SNAD biomass samples analyzed, the anammox bacteria in the brownish granules were significantly lower in respect to the total granules analyzed (Fig. V.14), confirming that anammox bacteria are more abundant in the red granules. De Clippeleir et al. (2009) showed that while in the red granules an equilibrated AOB and anammox activity can be detected, in the brownish granules a lower anammox activity can be detected.

Moreover, in this study after ~3 months of operation, the whole SNAD biomass (0.2 mL) was taken from the reactor and further analyzed using the real-time PCR. The samples analyzed were positive. The produced mean C_T was comparable to the value measured at the beginning of the SBR operation, confirming the low growth rate of anammox bacteria.

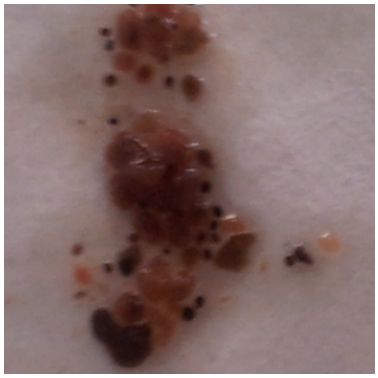


Fig. V.13 Red and brownish SNAD granules.

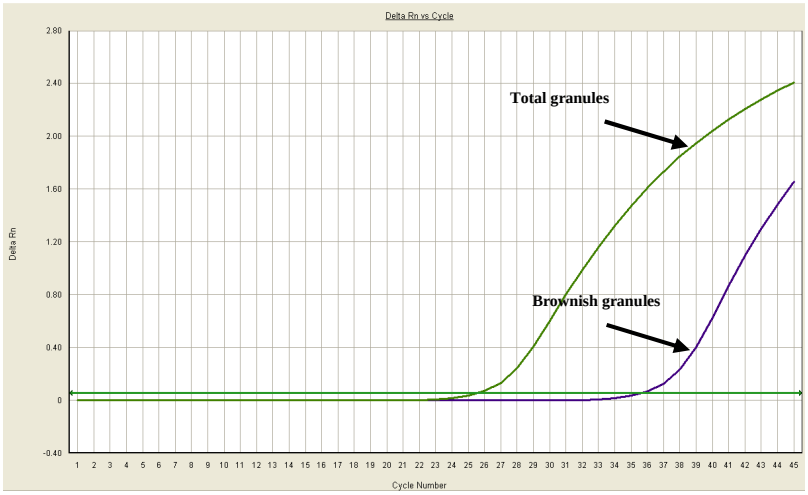


Fig. V.14 Real–time PCR amplification of *hzo/hao* gene of total granules and brownish granules samples

3.10 Influence of FA and FNA

The values of free nitrous acid (FNA) and free ammonia (FA) within suitable ranges are of great importance for the successful operation of the SNAD process, especially as FNA and FA could contribute to the inhibition of the nitratation process. Anthonisen et al. (1976) determined the levels of FNA and FA that are inhibitory to AOB and NOB. FNA became inhibiting to NOB between 0.22 and 2.8 mg HNO_2-N L^{-1}. Further, the author showed that AOB became inhibited at FA levels of between 10 and 150 mg NH_3-N L^{-1}, while NOB became inhibited at FA concentrations between 0.1 and 1.0 NH_3-N L^{-1}.

In this study, according to Eqs. Eq. V-1 and Eq. V-2, the FNA and FA concentrations in the effluent of the SBR were calculated over the whole course of the experiment and presented in Fig. V.15 and Fig. V.16, respectively. The concentrations of FNA in the effluent of the SBR were always lower than 0.006 mg HNO_2-N L^{-1}, not creating either AOB or NOB inhibition. Instead, the concentrations of FA in the effluent were in the range of 0.003 – 1.3 mg NH_3-N L^{-1}, with few peaks. The AOB were not inhibited by such low FA concentrations. On the contrary, FA higher than 0.1 mg NH_3-N L^{-1} may be considered to be inhibitory or partially inhibitory to NOB activity (Anthonisen et al. 1976).

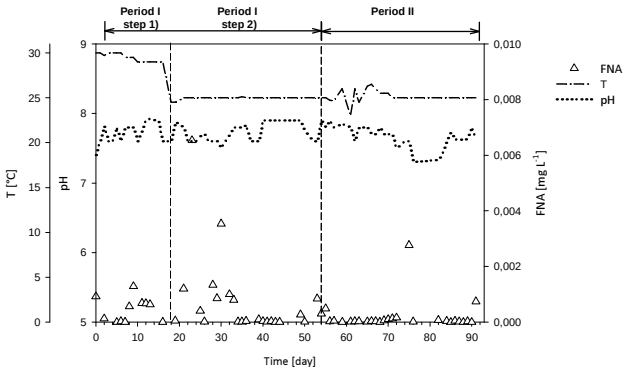


Fig. V.15 FNA concentration in the effluent during period I and period II.

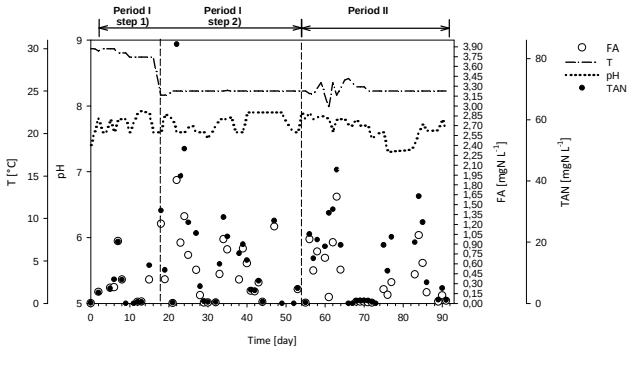


Fig. V.16 FA concentration in the effluent during period I and period II

However, the behavior of the FA in the effluent did not justify the NOB activity increase during the Period II at 25°C, treating anaerobic digester effluent. To better study the evolution of FNA and FA concentration in the system, the single 8 h SBR cycle was analyzed in each period investigated. The pH was continuously monitored

and FA and FNA were calculated over the SBR cycle. Fig. V.17 shows the evolution of FNA and FA concentrations as well as the pH in the mixed liquor over the 8 h SBR cycles in Period I and Period II.

The pH during the SBR cycle followed the biological process development. Generally, the highest pH values (around 8) were reached after the filling phase due to the high alkalinity of the influent (4500 mg $CaCO_3$ L^{-1}). Over the reaction phases in the SBR cycle, the pH first decreased during micro-aerobic phase achieving a minimum value between 7.4 – 7.7, due to the nitritation process which consumed alkalinity and then the pH increased due to the contribution of denitrification process, which produced alkalinity and of anammox process, which consumed hydrogen ions.

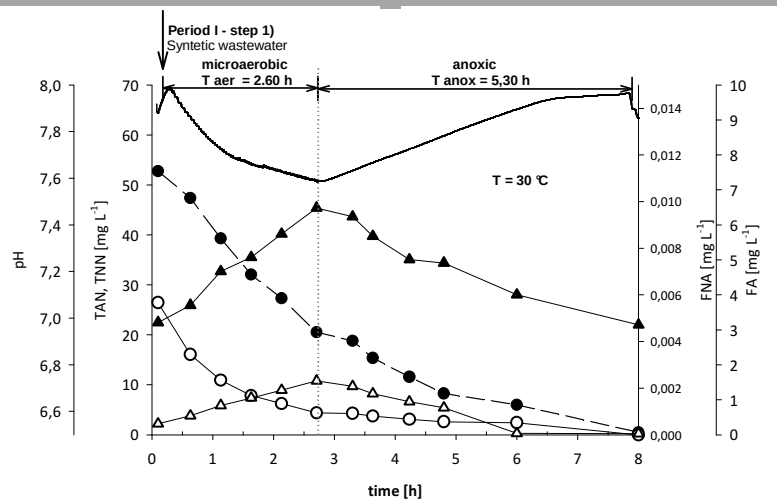
Anthonisen et al. (1976) suggested that FA was the main inhibitor of NOB under high pH conditions (pH ~8.0), which were measured at the beginning and at the end of the SBR cycle. In the SBR cycle, at the beginning of each cycle, when both the TAN concentration (50 – 70 mg L^{-1}) and the pH value reached their maximum value, the FA concentration also reached its maximum value. Further, Anthonisen et al. (1976) suggested that FNA was the main inhibitor of NOB under low pH conditions (pH~7.5), which were reached at the end of the micro-aerobic phase.

Over the Period I the concentration of FA was higher than 3.5 mg NH_3-N L^{-1} both at 30 and 25°C, inhibiting the NOB activity. In the Period II, even having the same amount of TAN, a lower level of FA was found at the beginning of the SBR cycle (<2.0 mg NH_3-N L^{-1}) due to the lower pH in the reactor. The lower pH in the reactor could be caused by the change of the influent, from the sinthetic wastewater to the anaerobic digester effluent. The lower FA levels at the beginning of the SBR cycles in Period II could favour the reactivation of the NOB, explaining the higher nitrate accumulation detected in the SBR treating anaerobic digester effluent at 25°C.

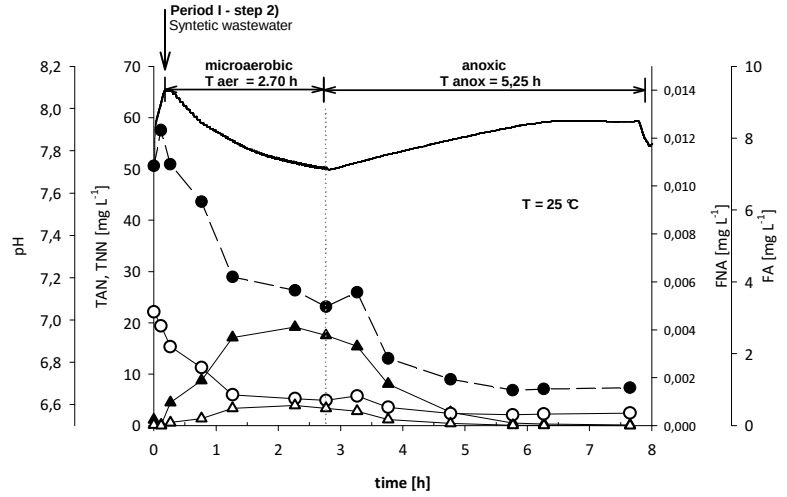
However, literature on FA inhibition on NOB shows disagreement about the threshold FA concentration and its inhibition degree. Vadivelu et al., (2007) stated that the respiration (catabolism) of *Nitrobacter* spp. was inhibited by FA concentrations in the range of 1 – 9 mg NH_3-N L^{-1} at pH of 7.3 and 22°C (100 - 1000 mg TAN/L). However, this inhibitory effect was limited (only inhibited by 12 - 25%). Further, the authors found the biosynthesis (anabolism) of NOBs ceased completely at a FA concentration of 7.5 mg NH_3-N L^{-1}. Kim et al. (2008a) reported different thresholds for *Nitrobacter* spp. and *Nitrospira* spp.. In particular, *Nitrobacter* spp. were inhibited at 30 ~ 50 mg NH_3-N L^{-1} while *Nitrospira* spp. were inhibited at 0.04 ~ 0.08 mg NH_3-N L^{-1}. The authors also showed that FA inhibition of nitrite oxidizing bacteria was reversible. Turk and Mavinic (1989) showed an adaptation of the NOB to FA, confirmed also by other studies (Villaverde 2000).

On the contrary, FNA did seem not influence the SNAD process. At the end of the micro-aerobic phase, when the TNN concentration was maximum and the pH was the lowest, the FNA concentration achieved its maximum value. Over the all experiment, FNA reached values lower than 0.003 mg HNO_2-N L^{-1}. The concentration of FNA never reached the FNA inhibition thresholds reported for NOB. It was indeed reported that FNA starts to affect NOBs activity in the range of 0.011 - 0.07 mg HNO_2-N/L, while the complete inhibition was observed at 0.023 - 0.22 mg HNO_2-N/L (Anthonisen et al. 1976; Vadivelu et al. 2006; Zhang et al. 2010a).

a) Period I – step 1)



b) Period I – step 2)



c) Period II

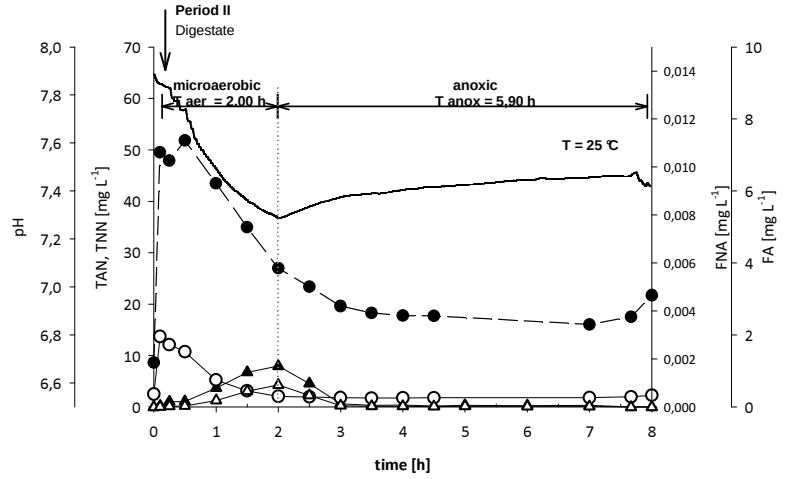


Fig. V.17 Evolution of FNA and FA concentrations coupled with the pH in the mixed liquor over a 8 h SBR cycle: (a) Period I- step 1); (b) Period I- step 2), c) Period II

FNA pH FA TNN TAN

4. Wastewater implications

The SNAD process will offer a great potential for the savings of energy and resource for an efficient nitrogen and carbon removal from wastewater characterized by low C/N ratios at moderate temperature (T=25°C). Treating ammonium rich-streams, the SNAD-SBR couples together the advantages of the autotrophic nitrogen-removal over nitrite with the SBR system, achieving low capital and operative costs.

One of the keys of a properly working SNAD process is the development of suitable environmental conditions which allow the co-existence of aerobic ammonia oxidizing, anammox and denitrifying bacteria. In order to obtain such a suitable co-existence, in this study, experiments were carried out in a granular SNAD-SBR, which represents one of the most appropriate reactor configurations, ensuing different micro- and macro-environmental conditions, in both the bulk and the granules. The SBR cycle was conducted with two reaction phases: a micro-aerobic and an anoxic one. In particular, in the micro-aerobic phase, performed under oxygen limitation in order to inhibit the NOB, ammonium would be converted partly to nitrite by AOB (partial nitritation). Then in the anoxic phase, anammox bacteria would convert ammonia with nitrite to nitrogen gas. Heterotrophic bacteria in aerobic conditions would oxidize the biodegradable organic matter while in anoxic conditions would reduce both nitrate and nitrite to dinitrogen gas using the remain biodegradable organic matter as carbon source.

Nevertheless, we showed that to maintain a long term stable SNAD process a high-rate and stable running partial nitritation process is the critical point. Partial nitritation depends on both the SBR cycle configuration and the SBR operative conditions. In particular, at moderate temperatures treating a real wastewater, besides the effect of the the oxygen limitation on the nitritation reaction, a proper control of the FA is necessary to inhibit NOB. Within the SBR cycle should be ensuing a tolerable FA range of 4-8 mg NH_3-N L^{-1} (lower than the minimum inhibition threshold reported for AOB and in the range of the inhibitory values reported for the NOB). In this study, in particular it was found that, a regulation of the FA concentration at the beginning of the SBR cycles may be a vital factor in inhibiting NOB and assuring a stable SNAD process. Thus, the pH value could be adjusted at the beginning of each cycle to ensure a suitable FA level.

Further, during the anoxic phase denitrification occurred using the organic matter present in the influent, removing either the residual nitrite and nitrate produced in the nitrification process or the produced nitrate of the anammox process. Nevertheless, in order to avoid the anammox and denitrifying bacteria competition in the anoxic phase, a suitable low C/N ratio should be guaranteed. To this end, wastewater with low C/N ratio (< 1) are suitable for the SNAD process. The micro-aerobic phase in the SBR configuration is also useful to partially oxidize the organic compounds and thus reduce the carbon content in the following anoxic phase.

According to the operation experience on the SNAD-SBR process, it is suggested that a suitable start-up strategy to achieve the SNAD process at moderate temperatures could have two steps. The first one would be the stable SNAD process at high temperature (T=30°C), in order to select the AOB over NOB by high temperature and other optimized conditions, such as oxygen limitation and FA inhibition. Then, the second step would be the adaptation of the SNAD biomass to the lower temperatures. Further, a gradual acclimatation of the SNAD biomass to the treatment of real wastewater is proposed, by filling a mixtute (%v/v) of synthetic and real wastewater, controlling the pH of the system in order to inhibit nitratation process.

5. Conclusions

In this study, the SNAD process performed in an SBR was successful applied to treat anaerobic digestion effluent characterized by low C/N ratio with high ammonium concentration at moderate temperature (T=25°C). After a necessary acclimatization period to the moderate temperature and to the digester effluent, the reactor was operated in a stable manner. The SNAD system shows high TAN (96%) and TN (88%) removal efficiency at NLR of 0.15 kg m^{-3} d^{-1}. The system was able to remove ~ 60% of COD at final stage. A nitrate accumulation was observed in the system as low biodegradable COD was present in the digester effluent, minimizing the denitrification effect on the overall SNAD efficiency. Further, at moderate temperature, besides the effect of DO concentration on the NOB inhibition, a proper control of the FA within a favorable range is necessary, especially at the beginning of the SBR cycles, when the TAN and the pH assume the highest value, in order to further inhibit the NOB activity.

This study showed that, under steady-state condition, nitritation, anammox, denitrification and nitratation co-existed in the SBR, synchronizing each other and establishing a relation, which depended on the operative reactor conditions. The cooperation among these bacteria was considered to be responsible for simultaneous nitrogen and COD removal. The shock in the operating temperature, DO, C/N, nitrite concentrations, nitrogen loading rate of the SNAD system greatly affected the relation of these processes. This can be evidence from the poor TAN and TN removal efficiency of the system when a shock occurred. However, the system was always able to recover its efficiency after 3- 4 time the HRT. Finally, a new primer set targeting the *hao/hzo* gene of anammox bacteria was successful designed and tested. The PCR results confirmed the presence of anammox bacteria in the SBR, both in granules and in flocs.

Acknowledgements

The authors gratefully acknowledge Adriano Joss from EAWAG (SWZ) and Mrs Sabine Burger from ERZ Entsorgung + Recycling Zürich, (SWZ), for providing the SNAD sludge and for their helpful information concerning the SBR. We wish to thank Mr. Giuliano and Mr. Saverio from the WWTP of Trento, for providing the anaerobic digester effluent. We thank V. Miotto and E. Sbrissa, master students of the University of Trento for their precious contribution during experimental phases. The “Servizio Elettronico e Progettazione” and the “Sevizio Meccanico” of the Department of Physic of the University of Trento are gratefully acknowledged for their technical support for the reactor assembly and the acquisition and control software development.

Author Contributions

All authors contributed extensively to the work presented in this paper. The acquisition and control software was developed by M.L with the support of L. Penasa from the University of Trento. Experiments for the reactor and the microbial activity were designed and conducted by M.L. G.A. and M.L. conceived the research. M.C. performed the microbial analysis. M.L. wrote the paper with input from all other authors, discussing the results, implications and commenting on the manuscript at all stages.

Supplementary Material

Further details concerning the characteristic of the synthetic wastewater and the anaerobic digested effluent, the reactor equipment, the acquisition and control hardware and software as well as the primer set specificity.

Supplementary Table

Supplementary Table V-1 Characteristics of the synthetic wastewater	
Parameter	**This study [g L^{-1}]**
NH_4 Cl	1.52
K**HCO**$_3$	3.78
CH_3COO Na	0.56
Medium Composition*	
CaCl_2 $2H_2O$	0.30
MgSO_4 $7H_2O$	0.20
FeSO_4	0.00625
EDTA	0.00625
K**H_2PO_4**	0.0250
Trace elements solution*	1.25 mL L^{-1}
Trace element composition (1L)*	
Zn$SO_4 \cdot 7H_2O$	0.43
Mn$Cl_2 \cdot 4H_2O$	0.99
Cu$SO_4 \cdot 5H_2O$	0.25
Na**Mo**$O_4 \cdot 2H_2O$	0.22
Ni$Cl_2 \cdot 6H_2O$	0.19
H_3**B**O_4	0.014

* adapted by * Thirds et al. 2001

Supplementary Table V-2 Characteristics of the anaerobic digestion supernatant from a municipal WWTP, Trento (Italy). Mean values during the whole study.	
Parameter	**This study [mg L^{-1}]**
pH	7.8±0.1
TSS [mg L^{-1}]	260 ± 200
COD_{total} [mg L^{-1}]	338± 70
$COD_{filt\ 0.45\ \mu m}$ [mg L^{-1}]	193 ±84 *
$COD_{soluble}$ [mg L^{-1}]	194 ± 27 $^{+}$
COD_{bio} [mg L^{-1}]	158± 19 **
RBCOB [mg L^{-1}]	48± 57 **
TOTAL AMMONIUM NITROGEN (TAN_{filt}) [mg L^{-1}]	433±65 $^{+}$
TOTAL NITRITE NITROGEN (TNN) [mg L^{-1}]	<0.08 *
NITRATE (NO_3-N) [mg L^{-1}]	0.4±0.6 *
TOTAL KJELDAHL NITROGEN (TKN) [mg L^{-1}]	470±59
ORGANIC NITROGEN (Norg) [mg L^{-1}]	49±23
TOTAL PHOSPHORUS (P) [mg L^{-1}]	10±0.7
ORTHOPHOSPHATE (PO_4) [mg L^{-1}]	4±1.5 *
ALKALINITY ($CaCO_3$) [mg L^{-1}]	5.360 ±460 *

* values after filtration 0.45 µm

** values determined by respirometric analysis

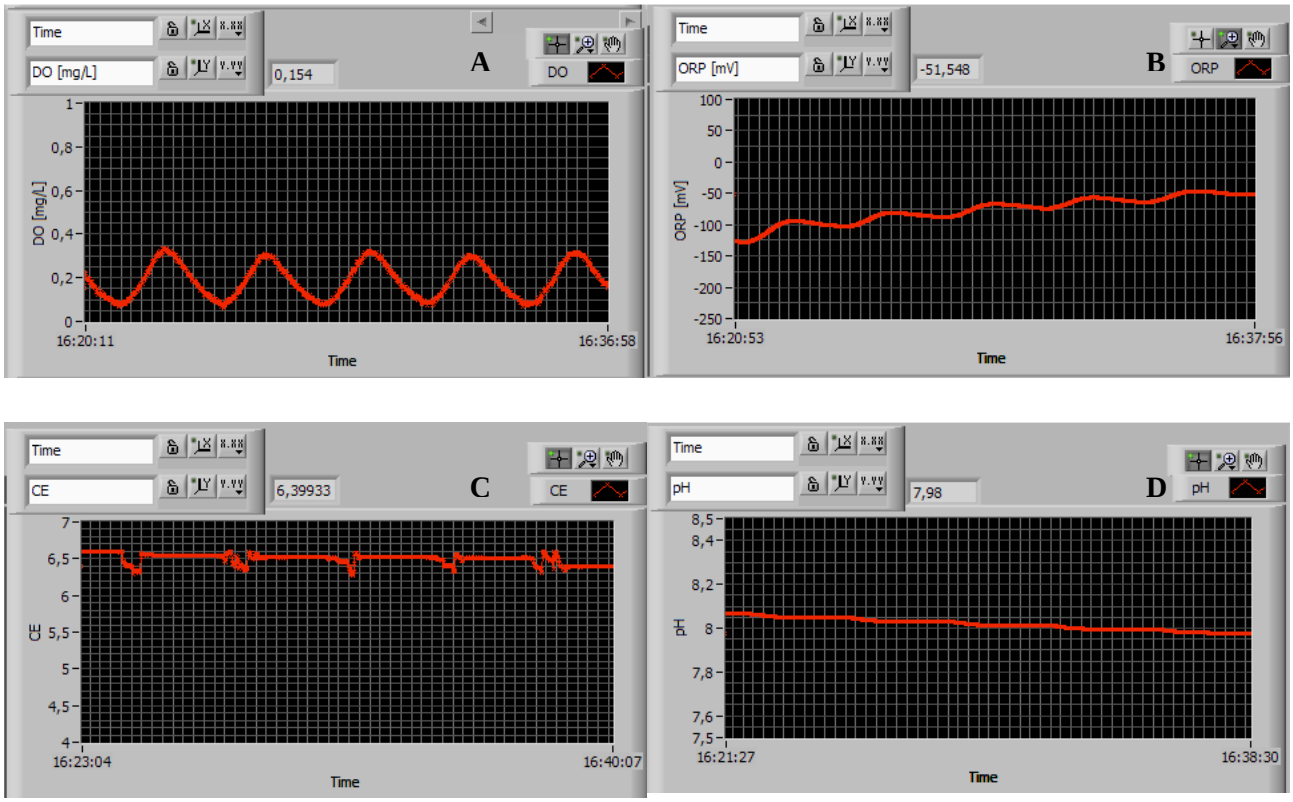
$^{+}$ values determined after ultra filtration 0.45 µm and 0.2 µm

Supplementary Table V-3 cDAQ channel configuration cDAQ – 9174 chassis (4 slot)

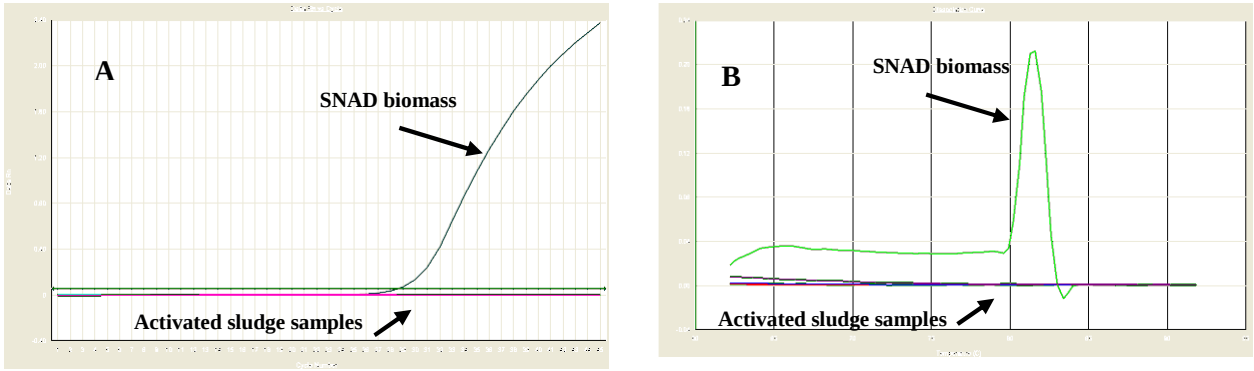
Device	Description	Control	Physical channel	Channel Name	output range	Work range	Work measure
NI 9208 16-Channel Current Input Module							
pH sensor	on-line reactor pH	-	AI0	Numeric 1 pHr	±21.5 mA	4 – 20 mA	4 -10
ORP sensor	on-line reactor ORP	-	AI1	Numeric 2 ORPr	±21.5 mA	4 – 20 mA	-500 + 500 mV
Temperature sensor	on-line reactor Temperature combined with DO sensor	-	AI2	Numeric 3 TDOr	±21.5 mA	4 – 20 mA	5 – 40 °C
DO sensor	on-line reactor DO	-	AI3	Numeric 4 DOr	±21.5 mA	4 – 20 mA	0 – 10 mg L^{-1}
pH sensor	on-line influent tank pH	-	AI4	Numeric 5 pHi	±21.5 mA	4 – 20 mA	4 -10
CE sensor	on-line reactor Conductivity	-	AI5	Numeric 6 CEr	±21.5 mA	4 – 20 mA	0-100 mS
CE sensor	on-line influent tank Conductivity	-	AI6	Numeric 7 CEi	±21.5 mA	4 – 20 mA	0-100 mS
free		-	AI7		±21.5 mA		
Temperature sensor	on-line reactor Temperature combined with CE sensor	-	AI8	Numeric 9 TCEr	±21.5 mA	4 – 20 mA	0 – 40 °C
Temperature sensor	on-line influent tank Temperature combined with CE sensor	-	AI9	Numeric 10 TCEi	±21.5 mA	4 – 20 mA	0 – 40 °C
Level sensor	on-line reactor level	-	AI10	Numeric 11 Levelr	±21.5 mA	0 – 20 mA	5 - 32 cm
free	-	-	AI11		±21.5 mA		
free	-	-	AI12		±21.5 mA		
free	-	-	AI13		±21.5 mA		
free	-	-	AI14		±21.5 mA		
free	-	-	AI15		±21.5 mA		
NI 9485 – 8 Channel C Series Relay, 60 VDC/30 Vrms, 750 mA							
Influent Pump	influent feeding	Time and level On-Off control	AO0	P01	relay for switching voltages up to 60 VDC		
Effluent Pump	effluent discharge	Time and level On-Off control	AO1	P02	relay for switching voltages up to 60 VDC		
Sludge Pump	sludge discharge	Operator, Time and level On-Off control	AO2	P03	relay for switching voltages up to 60 VDC		
Acid pump	alkalinity and basic condition	pH On-Off control	AO3	Pacid	relay for switching voltages up to 60 VDC		
Basic pump	alkalinity and basic condition	pH On-Off control	AO4	Pbase	relay for switching voltages up to 60 VDC		
Aerator	aeration conditions	Time and DO On-Off control	AO5	BL01	relay for switching voltages up to 60 VDC		
Mixer	mixing conditions	Time On-Off control	AO6	MX01	relay for switching voltages up to 60 VDC		
free			AO7	-	relay for switching voltages up to 60 VDC		
NI 9472 - 8 Channel , 24 V Logic, 100 µs Sourcing C Series Digital Output Module							
Over flow alarm In	Detect overflow from bioreactor, safety tank		DIO	Level	6 – 30 V		

AI= analog input; AO= analog output; DO=digital input/output

Supplementary Figures



Supplementary Fig. V-1 Graphical interface of the acquisition and control software developed using LabVIEW 2010 software.
(A) DO signal [mg L-1]; (B) ORP signal [mV] ; (C) Conductivity signal [mS] ; (D) . pH signal.



Supplementary Fig. V-2 *hzo/hao* primer set specificity.
(A) Real –time PCR amplification of *hzo/hao* gene. (B) Melting curve analysis of SYBR Green real-time PCR product of *hzo/hao* gene after 45 cycles.

VI. Chapter

Real time control strategy for Simultaneous partial Nitritation, anammox and Denitrification (SNAD) process in SBR

This chapter was based on:

Langone M., Andreottola G. (2013). *Correlating on-line monitoring parameters, conductivity, ORP pH, and DO with the Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process in* SBRs. Submitted

Real time control strategy for Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process in SBR

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ABSTRACT

A simultaneous partial nitritation anammox (SNAD) process was successful performed in a sequencing batch reactor (SBR) treating a synthetic wastewater and an anaerobic digester effluent from an urban wastewater. The oxidation-reduction potential (ORP), pH, conductivity (EC) and dissolved oxygen (DO) signals were continuously monitored and recorded during the whole experiment. The on-line parameters profiles were well related to the ongoing biological processes and breaking and blending points were also detected, useful to monitor and control the SNAD process. With the beginning of the anoxic–mixed filling phase, EC and pH increased while ORP decreased. Then, with the beginning of the micro-aerobic phase, pH and EC exhibited a decreasing trend while the ORP increased gradually. When the aeration was switched off the ORP showed a sharp decrease whereas the pH increased steadily due to biological conversion processes. The EC continued to decrease slowly. A blending point in the ORP profile in the anoxic phase and the amplitude of the ORP after the blending point were correlated with the complete depletion of nitrite mainly by anammox process and with the denitrification process via nitrate, respectively.

The findings demonstrated that the status of the SNAD process in an SBR, and the ongoing nitritation, anammox and denitrification process, can be successfully monitored and controlled through pH, ORP and conductivity profiles. Further, a successful real-time control strategy has been applied to optimize the SBR cycle, treating anaerobic digester effluents and applying a nitrogen loading rate of 0.15 kg N m^{-3} d^{-1}. Results showed that the average removal efficiencies of ammonium and nitrogen were more than 95% and 88%, respectively, preventing nitrite accumulation and substrates depletion.

Keywords: Anammox, conductivity, denitrification, dissolved ossigen, nitritation, on-line control, oxidation-reduction potential (ORP), pH, SBR cycle, SNAD.

Abbreviations

Parameters		Reactor Configurations and process		Operative	
COD	Chemical Oxygen Demand	CAS	Conventional Activated Sludge	DO	Dissolved Oxygen
CODtot	Total COD	DEMON	Deammonification process	HRT	hydraulic retention times
CODfiltr	Filtrate COD	EBPR	Enhanced Biological Phosphorus Removal	KLa	mass transfer coefficient for oxygen
FA	Free Ammonia (NH_3- N)	MBBR	Moving Bed Biofilm Reactors	NLR	Nitrogen Loading Rate
N	Nitrogen	SNAD	Simultaneous partial Nitritation, Anammox, Denitrification	NRR	Nitrogen Removal Rate
NO_3^--N	Total Nitrate Nitrogen	SBR	Sequencing Batch Reactors	OLR	Organic Loading Rate
TAN	Total Ammonia Nitrogen	WWTP	Wastewater Treatment Plant	VER	Volume Exchange Ratio
TNN	Total Nitrite Nitrogen			ORP	Oxidation-Reduction Potential
TKN	Total Kjeldahl Nitrogen	AOB	Ammonia-Oxidizing Bacteria	EC	Conductivity
TSS	Total Suspend Solid	NOB	Nitrite-Oxidizing Bacteria		
VSS	Volatile Suspend Solid	Anammox	ANaerobic AMMonia -OXidizing Bacteria		

1. Introduction

The simultaneous partial nitritation, anammox and denitrification (SNAD) process has been studied as a suitable and sustainable alternative nitrogen removal pathway for the treatment of high ammonium streams, characterized by low biodegradable organic matter content, such as anaerobic digestion effluents (Joss et al. 2009), old landfill leachates (Wang et al. 2010) and industrial wastewaters (Daverey et al. 2012). In the SNAD process, ammonium would be converted partly to nitrite by oxygen-limited aerobic ammonia oxidizers (AOB), subsequently, the residual ammonium would be directly oxidized to di-nitrogen gas with nitrite as electron acceptor by anammox bacteria, while the excess of nitrate and nitrite would be reduced to di-nitrogen gas by denitrifiers bacteria, which can utilized the low content of organic biodegradable present in the influent.

The SNAD process has specific peculiarities which concern (i) the coexistence in the same reactor of bacteria with opposite environmental conditions (aerobic/anoxic) for which specific chemical compounds act both as substrates and inhibitors and (ii) the presence of autotrophic bacteria, the main actors involved in the process, which are characterized by low growth rates. Hence, reactor configurations based on the occurrence of opposite environmental conditions and a high efficient retention of biomass have been mostly used, such as sequencing batch reactors (SBRs) with granular and suspended biomass (Joss et al. 2009; Xu et al. 2010; Lan et al. 2011; Daverey et al. 2012), sequencing batch biofilm reactors (SBBR) (Zhang et al. 2012), non-woven rotating biological contactors (NRBC) (Chen et al. 2009), anoxic/aerobic granular SBR (Winkler et al. 2012a), moving bed biofilm reactors (MBBR) (Rosenwinkel and Cornelius 2005), granular sludge processes (Abma et al. 2010), granular continuous full-scale aeration tanks (Wang et al. 2010).

Biofilm and granular systems have been adopted, both to enhance the biomass retention in the systems and to create opposite micro-environmental conditions over the granule/biofilm thickness. Among the configurations reported above, the SBR with granular biomass has demonstrated a good potential in performing the SNAD process, as it offer various advantages including: development of sequential and opposite macro-environments where different bacteria can coexist; simultaneous development of micro-environmental conditions thanks to the granular structure, minimal space requirements, low capital and operational costs, management ease and flexibility, implementation of on-line control strategy.

The SNAD process is a very sensitive process, and the equilibrium of the biological processes involved in it plays an important role. Thus, an on-line control of each biological process is crucial for a successful operation strategy implementation, able to optimize the SNAD process in SBRs. Similarly to the conventional nitrification - denitrification processes, the on-line monitoring of the SNAD process through direct real-time measurements of process constituents (NH_4^+, NO_2^-, NO_3^- and N_2) is very difficult and costly to achieve with current sensors. Usually, the lab-scale reactors performing the SNAD process are controlled by timers, while the adjustments of the cycle duration are made after batch measurements of ammonium and nitrite in the effluent. Some installations also include on-line measurement of ammonium and nitrite in granular nitritation/anammox reactor (Abma et al. 2010).

Nevertheless, the biological processes occurrence and dynamic can easily and cheaply be monitored by indirect real-time parameters such as DO (dissolved oxygen), ORP (Oxidation Reduction Potential), pH and conductivity (EC) measurements. Several research groups have studied different SBR operation strategies to carry on the SNAD process. Among them, Wett (2007) proposed an SBR configuration (8 h per cycle), characterized by a continuous feeding over the entire reaction time and a DO set point of 0.3 mg L^{-1} in order to prevent rapid nitrite accumulations and to maintain a continuous aerobic nitrite oxidizing bacteria (NOB) inhibition, thanks to the low DO concentrations and the high temperature. The duration of aeration intervals were ruled by very tight pH-bandwidth (0.01). Until now, several large scale implementation have been developed applying the DEMON process patent filed by Wett (2009) (e.g. Strass in Austria; Heidelberg, Plettenberg in Germany; Thun, Glarnerland in Switzerland, Trento in Italy).

Szatkowska et al. (2007a), conducting an one-stage system with partial nitritation and anammox processes in the moving-bed biofilm reactor (MBBR), showed a strong correlation between removed nitrogen, removed alkalinity and pH value in the effluent. Also conductivity turned out to be a suitable tool for the process monitoring.

Degremont et al. (2009) filed a patent (2/23-FAMPAT-©Questel) on the simultaneous nitritation-anammox process in an SBR (6-8 h per cycle). Each cycle was constituted by a fraction filling phase and two reaction stages: a first aerated stage followed by a non ventilated second phase. In the aerated phase a part (40-60%) of the ammonium (TAN), flow supplied by a volume of influent, was transformed into nitrites (TNN), and the remainder part of the ammonium flow was sufficient to ensure that the produced nitrites and non oxidized ammonium during the first step were converted into gaseous nitrogen. The supply time of influent to be treated was 7-10% of the total duration of the treatment cycle. The inventors introduced a conductivity concentration strategy in order to control the first aerated step (DO= 0.1-0.6 mgL^{-1}). The air supply was stopped if a threshold value in conductivity was reached before one fixed duration, and further an additional filling phase (between 50% and 110% of the volume of initial influent) was introduced at the beginning of the non-ventilated phase in order to ensure a TNN:TAN ratio between 0.9 and 1.5. The inventors measured the concentration of nitrogen ammonium in the system both indirectly by a conductivity (EC) sensor in the tank and directly by a specific ammoniacal sensor. Further, they calculated the nitrogen load to be treated by measuring conductivity (EC) and the flow (Q) in the influent. The on-line measures of the ammonia concentration and conductivity in the tank were taken into account like indicators of conversion of ammonium during the phases of ventilation and not-ventilation in order to control the course of the biological process during each phase and determine their duration. The data were also analyzed in order to calculate the volume of the influent to add at the beginning of the non-ventilated phase.

Joss et al. (2009) successful developed a partial nitritation, anaerobic ammonium oxidation process and denitrification control strategies in an SBR (6-8h per cycle), using on-line measurements of oxygen, ammonium (ion-selective ammonium electrode) or alternatively conductivity. The nitrate concentration was also monitored. The authors combined a discontinuous and short feed at the beginning of each cycle, regulated by a maximum ammonium/conductivity threshold, and an intermittent or continuous aeration controlled by the ammonium or alternatively by the conductivity signal. The aeration phases were under oxygen limitation ($DO < 0.5 mgO_2 \cdot L^{-1}$), allowing the occurrence of the anammox process also in aerobic conditions. The aeration was terminated either after a fixed time interval, chosen to avoid high nitrite concentration, or after a minimum ammonium/conductivity threshold. Finally an anaerobic stirring period was performed in order to remove both the remaining nitrite by anammox process and the N_2 bubbles formed.

Only recently, the oxidation–reduction potential (ORP) profiles have been investigated as process monitoring parameter of the nitritation-anammox process performance in an SBR (Lackner and Horn 2012) and as indicator parameter for a control strategy of SBR, through a combination of interval feeding with interval aeration (Lackner et al. 2012). According to Lackner and Horn (2012), the ORP signal was the most suitable parameter for monitoring a single stage nitritation–anammox process, where denitrification only occur at the beginning of the SBR cycle, during the anoxic feed. The ORP signal was able to detect easily the substrates depletion (ammonium and oxygen). The authors suggested that a control strategy for a full scale plants nitritation/anammox should also include other parameters beside the ORP to monitor the process performance, support failure diagnostic.

However, when denitrification occurs over the process, other on-line parameters should further used to interpret the denitrification contribution. Therefore, the major objective of this research was to correlate the variation in ORP, EC, pH and DO profiles with the biological nitrogen and carbon removal in a SNAD process performed in an SBR system. The influence of different type of wastewaters on the indirect on-line parameters

was also investigated. The experiment was performed using a "fixed time control" strategy to control the SBR and all the on-line parameters were registered. Further, analysing the results of the "fixed time control" strategy it was possible to implement a new "real-time control" strategy, mainly based on the EC and ORP signal in order to achieve a stable SNAD-SBR process. In this contribution the "real time control" strategy is presented, which special focus on the ORP and EC signal as indicator parameters, in order to optimize the SBR cycle pattern for process stability and removal efficiency.

1.1 Meaning of indirect parameters

Dissolved Oxygen (DO) level clearly indicates how well the water is aerated and it is a commonly measured parameter because it is an immediate indicator of the biological processes that could occur, distinguishing between aerobic biological processes and anoxic/anaerobic biological processes.

ORP is related to the concentration of oxidizers or reducers present in the water system. It can successfully indicate the oxidative state of the wastewater, providing information on the variations in DO concentrations, oxidized compounds (such as nitrites, nitrates or sulphates). ORP behaviors in the time could provide useful information both on microorganism activity and bioprocess inhibition, which can be caused by an insufficient chemical oxygen demand (COD) to total Kjeldhal nitrogen (TKN) ratio (COD/TKN) or by the presence of some toxic compound in the influent (De la Vega et al. 2012). Further, ORP may provide information about some operational conditions such as over-loading, under-loading, over- and under-aeration (Tanwar et al. 2008). ORP was used as a monitoring and controlling parameter to manage the conventional biological nitrogen removal pocess in SBR systems (Andreottola et al. 2001; Obaja et al. 2003).

The pH describes the degree of acidity or alkalinity of a water system and it is directly related to the hydrogen ion (H^+) and the hydroxyl ion (OH^-) consumption or production. It is a good indicator of ongoing biological reactions, increasing for ammonification, denitrification and anammox processes and decreasing for nitrification (Chang and Hao 1996; Gut et al. 2007).

Conductivity (EC) variations in wastewater have been found difficult to understand. EC is a general indicator related to the amount of dissolved salts, to cations and anions (mostly inorganic) present in the wastewater. In the case of release of high concentrations of ions (bases, acids, salts), the conductivity variations could be used to monitor processes that causes changes in total ion content, i.e H^+, OH^- and many nutrients, such as NH_4^+, NO_3^-, NO_2^-, $H_2PO_4^-$ and HPO_4^{2-} (Levlin 2009). It has been observed that the conductivity correlates very well with and P-release and P-uptake in the enhanced biological phosphorus removal processes (Maurer and Gujer 1995). Further, has been recently demonstrated that conductivity measurements also correlate well with the ammonium concentration (Levlin 2009) and could be used to monitor the partial nitritation/anammox system for nitrogen removal (Gut et al. 2007).

1.2 pH, DO,ORP and EC variations in a conventional nitrification- denitrification SBR

In general, sequencing batch reactor (SBR) systems have been widely applied to the conventional nitrification and denitrification process and many researches had applied a control method based on indirect parameters to optimize the nitrogen, phosphorous and carbon removal efficiencies (Andreottola et al. 2001; Cui et al. 2009). Usually the SBR configuration used in the conventional nitrogen removal is characterized by step-feed and either by the alternation of aerobic and anoxic stage in order to obtain nitrification and denitrification, or by the alternation of short anoxic and aerobic stages, in order to obtain pre-denitrification and nitrification in sequence.

The behavior of pH, DO and ORP can be used to identify specific control points of the biological processes (Andreottola et al. 2001). The indirect parameters have been studied also for monitoring combined nitrogen and phosphorus removal processes (Chang and Hao 1996). Conductivity has been used to monitor an SBR operated for enhanced biological phosphorus removal (EBPR) (Aguado et al. 2006) and further for controlling nutrient removal processes (Levlin 2009). Thus, typical pH, DO, ORP and EC profiles, characterized by several well defined control points, have been identified carrying on conventional nitrification- denitrification in an SBR (Fig. VI.1).

In a conventional activated sludge (CAS), AI-Ghusain and Hao (1995) showed that, according to the nitrification reaction, the pH value decreases converting ammonia to nitrate, as hydrogen ions are produced. The first step of nitrification, the oxidation of ammonia to nitrite, actually produces hydrogen ions. However in a CAS system, NOBs are not inhibited and the growth rate of NOBs is always higher than AOBs, always achiving a complete nitrification process (nitritation + nitratation) (Wiesmann 1994). Therefore in a CAS system the pH decrease is usually related to the complete nitrification process. When ammonia depletion is achieved, a minimum appears in the pH profile and then the pH value starts to increase due to the CO_2 stripping, also depending on the aeration system energy. The end of ammonia oxidation (nitrification) is known as "*ammonia valley*". By detecting the breaking point in the pH profile, the duration of aerobic phase could be controlled and optimized, avoiding excess of oxygen furniture. After the ammonia valley, in the anoxic phase, the pH increases due to the additional alkalinity produced through ammonification, denitrification or phosphate uptake, and reaches a plateau value. Usually, in denitrification process the complete depletion of NOx corresponds to an apex in the pH profile, called the "*nitrate apex*" and can be used to signal the end of the anoxic cycle.

When aeration is run continuously, a DO blending point can be observed in the DO curve, related to the depletion of ammonia as well as nitrite in the nitrification process (*DO Elbow*). The DO blending point is a flex with a positive slope in the DO curve and corresponds to a decrease in bacterial respiratory activity due to the depletion of substrates (Pavgelj et al. 2001). Thus, in continuous aeration systems where a complete nitrification is required, DO concentration may also be used for the detection of the aerobic phase termination. Nevertheless, not always the DO blending point can be detected at the end of ammonia depletion (Akın and Ugurlu 2005), probably due to a residual nitrite consumption.

Concerning the ORP, according to Peddie et al. (1990) a characteristic and reproducible ORP profile can be associated with the cycled operation of a conventional nitrification – denitrification in an SBR. A first bending point corresponds to the beginning of the aerobic phase when the aeration is switched on. During the aerobic phase, ORP increase as NOx are produced. When the aeration is run continuously, an ORP blending point (*nitrogen break point*) can be observed, coinciding with the DO blending point (*DO Elbow*), due to the dissolved oxygen increase caused by ammonia depletion. When the aeration is switched off, the ORP will decrease quickly, reaching negative values. In anoxic conditions, the ORP is dominated by NOx concentration. Due to nitrate and nitrite consumption, ORP starts to decrease at a slow rate initially, but it falls steeply when the process reaches a blending point, known as *''nitrate knee''* of the ORP profile. The "*nitrate knee*" coincides with the "*nitrate apex*" in the pH profile and indicates the end of anoxic phase (denitrification) and the beginning of the anaerobic phase in the enhanced biological phosphorus removal process. Recognizing this trend, it is possible to reduce the anoxic period, resulting in time saving with respect to the fixed-timing switching scheme.

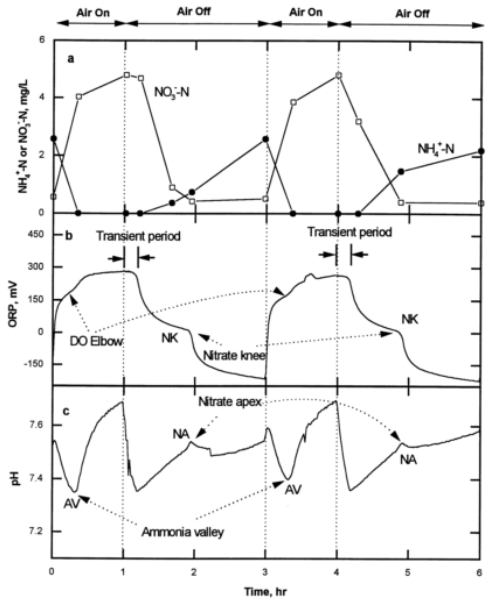


Fig. VI.1 pH and Oxidation–Reduction Potential in an Alternating Aerobic–Anoxic System (Kim and Hao 2001)

Conductivity shows a correlation with P-release and P-uptake in a sequencing batch reactor (SBR) operated for enhanced biological phosphorus removal (EBPR), in both anaerobic and aerobic stages, increasing or decreasing when phosphorus is released or taken up, respectively (Aguado et al. 2006). Spagni et al. (2001), showed that it was difficult, in their experiment, to correlate conductivity decrease and P-uptake in aerobic conditions, due to the stripping of carbon dioxide. On the contrary, over the denitrification process, the conductivity was stable as a result of two opposite and simultaneous phenomena: nitrate removal and P-release. As soon as the denitrification was over, conductivity increased and a plateau was reached when P-release ended. Levlin (2009) showed different changes in conductivity in biological nitrogen removal processes, depending on the alkalinity content of the system. Ammonium, nitrite and nitrate have almost the same molar conductivity. Thus, in nitrification process, if the amount of the available alkalinity is high enough (one mole alkalinity per mole ammonia), biological N-removal through nitrification will give a decrease of conductivity, mainly due to the alkalinity consumption and to the transfer of hydrogen carbonate to carbon dioxide. On the contrary, even if in denitrification process alkalinity is produced, the conductivity decreases due to nitrate and nitrite consumption.

However, the trends of the aforementioned indirect parameters (ORP, pH, DO and EC), the blending and breaking points in ORP, pH and DO curves and their relations with the biological processes depend on the nitritation, nitratation and denitrification rate and can vary from one biological system to another and from one wastewater to another.

As recently pointed out by Guo et al. (2009) in the nitrification process, the pH decrease in the aerobic phase is only related to the ammonia conversion into nitrite (nitritation), while over the nitrite oxidation to nitrate (nitratation) there is no hydrogen ion production and, thus, any further pH decrease. Moreover, the presence of organic matter and other chemical compounds, such as sulphates or nitrates, in raw wastewaters, could determine different behaviors in DO and ORP profiles (Ra et al. 1996). Usually, for low COD/N ratios in the influent, in a conventional nitrification/denitrification process the ‘‘nitrate knee’’ or the ‘‘nitrate apex’’ are unlikely to appear, due to the carbon limitation (De la Vega et al. 2012). Furthermore, the effects of the residual oxygen and the over-aeration have been proved to cause problems when using the ORP to provide an on-line control of the biological system (Han et al. 2008).

2. Materials and Methods

2.1 Reactor set up

SBR reactor. A lab scale Sequencing Batch Reactor (SBR) with a maximum working volume of 8 L was operated with the SNAD sludge from the full-scale SBR in Zürich, Switzerland (Joss et al. 2009). The seed sludge contained granules surrounded by a matrix of brownish flocs. Two kinds of granules (red and brown) could be distinguished. The initial total suspended solids (TSS) concentration of the whole sludge was 3 - 4 g $TSSL^{-1}$. The volatile suspended solids (VSS) were about 70% of the total suspended solids (TSS).

The SBR was first fed with a synthetic wastewater, prepared as reported in the Chapter V (Langone et al. 2013a). Then, the feed was switched to the anaerobic digester effluent from the wastewater treatment plant (WWTP) of Trento, Italy. Fig. VI.2 shows the lab-scale reactor setup. The anaerobic digester effluent was store in a 20 L storage tank and pumped into the SBR automatically at the start of each interval. Aeration was provided by a air pump (Schego M2K3 350) with an air flow rate of 50 nL h^{-1}, through n. 6 air diffusers placed at the bottom part of the reactor. The air flow rate was manually fixed and adjusted in order to achieve a mass transfer coefficient for oxygen (KLa) of 0.15 min^{-1} at 20°C. The DO set-point was fixed at a value of 0.2 ±0.1 mgO_2 L^{-1}. The reactor was equipped with a mechanical stirrer to ensure a complete but mild mixing, at low rpm (150) to avoid size granule reduction. Further, the reactor was also equipped with acid/base pumps and a set of two peristaltic pumps to introduce the feeding solution and to discharge the effluent. The reactor was water-jacketed and the operative temperature was maintained at 30 and 25°C. The pH and ORP were measured using Crison Instruments electrodes, (mod. 53.35 and 53.55, respectively, Crison Instruments Italy). The dissolved oxygen (DO) was measured using a polarographic cell (mod. 60.50 Crison Instruments, Italy). The conductivity (EC) was monitored by using a conductivity meters (mod. 53.88 Crison Instruments, Italy). Finally, the temperature was measured both by the PT 100 included in the DO sensors $T_{(DO)}$, and by the PT 1000 included in the conductivity meters $T_{(CE)}$.

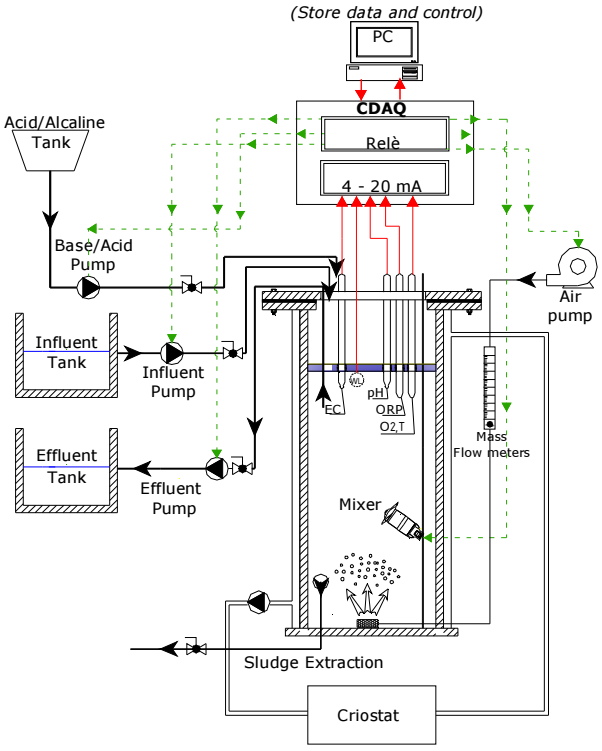


Fig. VI.2 Schematic representation of the 8L lab-scale SBR.

2.2 SBR operation

The SBR technology operates from a sequence of fill, reactions, settle and draw phases which define a cycle to be repeated over the time. A typical cycle of these characteristics was defined by a previous lab-scale study (Langone et al. 2013a) in order to achieve a SNAD process (Fig. VI.3).

Filling phase. A short one time feed strategy was adopted. Thus, the filling phase was carried out under mixing and anoxic conditions in order to create a suitable environment under which the growth rate was higher for floc-formers than for filaments (Artan and Orhon 2005), in order to improve the granulation process. The short filling phase ensured that the biomass was exposed initially to a high nitrogen load, and consequently to a high concentration of Free Ammonia (FA), inhibiting the nitrite oxidizing bacteria (NOB) (Anthonisen et al. 1976). The filling phase was mixed and non aerated and inserted into a short idle period where no oxygen was added, in order to allow the consumption of (i) residual oxygen or alternative electron acceptors, such as nitrite, nitrate and sulphate thanks to heterotrophic bacteria which may use the organics present in the influent (ii) residual nitrite thanks to autotrophic anammox bacteria which may use the ammonium present in the influent. The filling was considered to occur in an instant.

Reaction phase. The reaction phase consists of either one aeration/mixing phase per cycle or multiple aeration/mixing phases per cycle in order to permit a complete nitrogen and carbon removal. The micro-aerobic phase, under oxygen limitation, allowed the developed of nitritation process, inhibiting the activity of the nitrite oxidizing bacteria (NOB) over the ammonia oxidizing bacteria (AOB), due to the lower oxygen affinity of NOB (Wiesmann 1994). The length of the micro-aerobic phase should be chosen in order to oxidize only the 50% of the ammonium present at the beginning of each cycle to nitrite, achieving a suitable TNN/TAN ratio for the following anammox process. The length of the mixing phase should be enough long to deplete the residual ammonium and nitrire by anammox process and any residual nitrate and nitrite by denitrification.

Settling phase. After the completion of the reaction phase, the biomass was allowed to settle. In order to obtain a high biomass retention, a low minimum biomass settling velocity of 0.7 m h^{-1} was fixed, which is the ratio between the vertical distance from the water surface to the effluent discharge point and the settling time (Vlaeminck et al. 2009a). Depending on the pilot plant configuration, a short settling phase (7 min) was fixed, also to improve the granulation phenomena (Liu et al. 2005).

Draw phase. The cycle ended with a window for the effluent discharge and eventually for the sludge wastage. The sludge wastage phase could be set by choosing how often, the volume and the mode of the sludge discharge: either at the end of the settling phase, as a concentrated sludge or at the end of the last mixing phase as a mixed sludge. **Idle**. A final idle interval was optional.

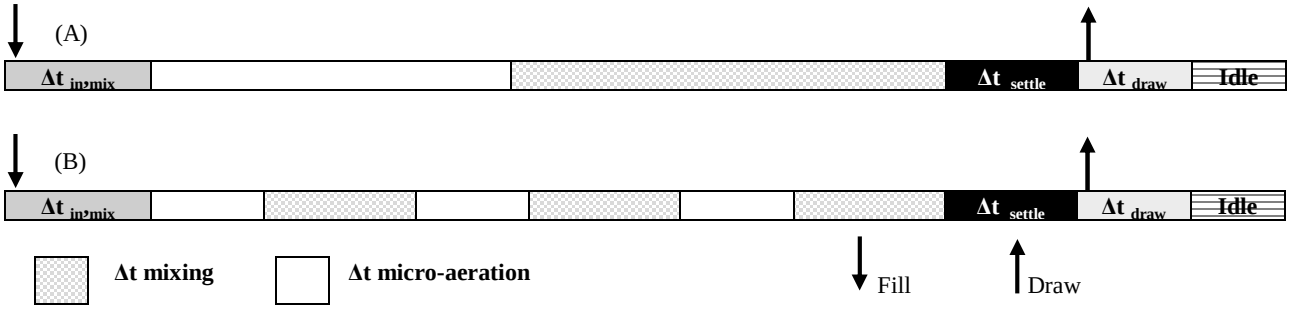
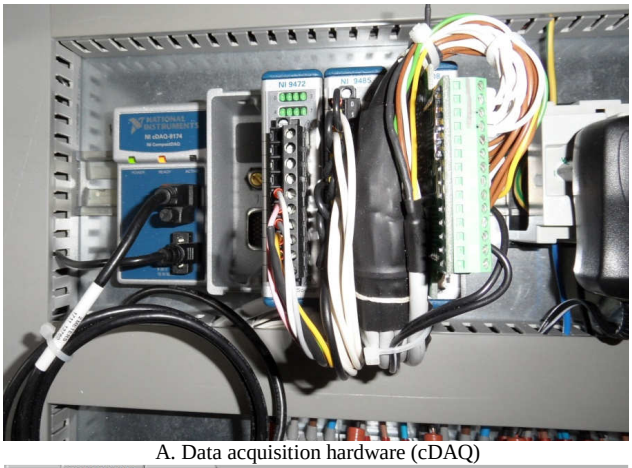


Fig. VI.3 Schematic representation of the possible SBR cycle configurations.
(A) one aeration/mixing phase (B) multiple aeration/mixing phases

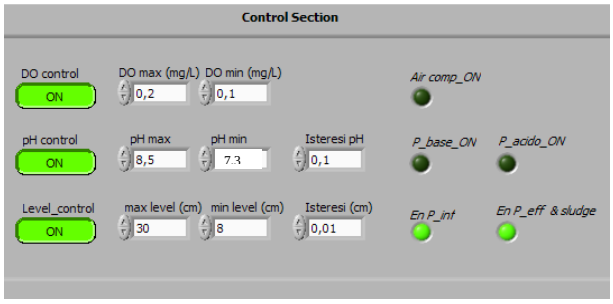
2.3 Monitoring and control system.

The monitoring and control systems of the SBR consisted in a data acquisition system (cDAQ-9174, National Instruments, Austin, TX, USA) and our own software developed using LabVIEW 2010 software (National Instruments, NI, Austin, TX, USA) (Fig. VI.4). The cDAQ-9174 (four slots) USB chassis was used to measure analog I/O signals over a Hi-Speed USB 2.0 interface, through two NI C Series modules: a 16 analog voltage or current input channels, 16-bit resolution (NI9208) and a 8 Channel C Series Relay, 60 VDC/30 Vrms, 750 mA (NI 9485). The cDAQ channel configuration is summarized in the Supplementary Table VI-1.

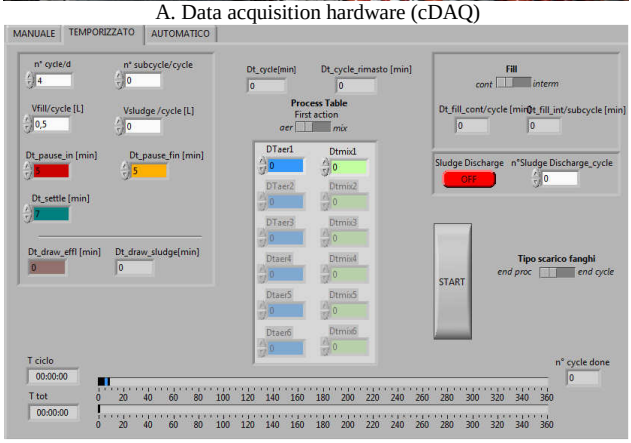
Ten analog input (AI) channels were used to obtain the analog input signals, i.e., level, pH, DO, ORP, CE and $T_{(DO)}$, $T_{(CE)}$ in the reactor and pH, CE ,$T_{(CE)}$ in the influent tank. The acquisition and control software developed was able to acquire the on-line parameters, presenting them in a graphical interface. Signals were recorded and stored in a simple *.xls* file for further processing. Control actions were transmitted by the cDAQ connected to the relay output board (AO), which controlled the on/off switch of the electrical devices.



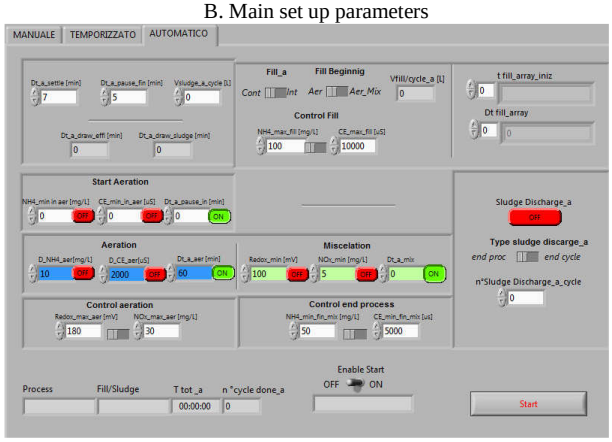
A. Data acquisition hardware (cDAQ)



B. Main set up parameters



C. Fixed time control



D. Real time control

Fig. VI.4 SBR monitoring and control system.

(A) Data acquisition and control hardware (cDAQ). From (B) to (D) Screenshots of the data acquisition and control software developed using LabVIEW 2010 software: (B) main control section: pH, DO and Level; (C) “fixed time control” strategy; (D) “real time control” strategy.

2.4 Control strategies

Two different control strategies were applied in this experiment to control the duration time of each phase of the SBR cycle: a “fixed time control” and a “real-time control” strategy. The “fixed time control” strategy served to evaluate the suitability of the SBR cycle pattern and the performance of the SNAD process. When the fixed time control was adopted, the correlations of nitrogen and carbon removal with pH, ORP, EC and DO profiles were investigated. During the fixed time control both the synthetic wastewater and the anaerobic digester effluent were treated in order to see the influence of different wastewaters on the indirect parameters. Then, the implementation of a “real-time control” strategy based on indirect parameters for a continuous SNAD-SBR operation was examinated. The acquisition and control software developed was able to repeat over the time a previously defined cycle operation by controlling the switching on/off devices.

In both strategies, the same control approach on the pH, DO and level was adopted. The pH was controlled, allowing to vary in the wide pH range of 7.3 - 8.5 by the addition of acid (1 N HCl) and alkaline (1 N NaOH) solutions through the on/off control of the acid/base pumps. The CO_2 stripping phenomena and the inorganic carbon limitation in micro-aerobic phase were reduced both by working at pH higher than 7.3 (Wett and Rauch 2003) and by using a low aeration energy system. During the micro-aerobic phase, the DO was controlled (DO = 0.2 ±0.1 $mgO_2\ L^{-1}$), ensuing a low DO concentration by the on/off control of the aerator. The SBR had a level control, which stopped either the influent pump, when a maximum water level was reached (8.0 L maximal reactor volume) before the stop criteria of the influent pump had not yet been occurred, or the effluent and sludge pumps, when a minimum water level was reached (7.0 L minimal reactor volume) before the stop criteria of the effluent pumps had not yet been occurred.

2.4.1. Fixed time control.

Initially, a control strategy of the SBR operation based on the timers and on a simple on-line measurements of DO, pH and liquid level of the bulk liquid was implemented (Fig. VI.4 -C). The lenght of the whole cycle and the duration of each single phase within the time SBR cycle were fixed and adjusted manually in order to achieve a stable SNAD process and high nitrogen removal efficiencies.

The SBR was operated with three 8 h operational cycles per day, regulated by a timer. The reactor was operated with a fixed residual volume of ~7.0 L, which resulted in a volumetric exchange ratio (VER) of ~12% per cycle and a hydraulic residence time (HRT) of 2.7 - 3 d. Each cycle consisted of 480 min and of six different phases: fill, micro-aerobic react, anoxic react, settle, draw and idle. The cycle started with a short feeding (6.6 min) during a mixing period for 10-15 min followed by a 120 - 210 min of micro-aeration and mixing phase and a 234 - 325 min of anoxic and mixing phase. After the completion of these 3 intervals the biomass was allowed to settle for seven minutes. The cycle ended with a 12 min window for the effluent discharge. A final idle interval was optional. During the whole cycle the on-line parameters were monitored, stored and further analyzed. The pH was controlled in a wide range (7.3 – 8.5); the DO was fixed during the micro-aerobic phase (0.2 ±0.1 $mgO_2\ L^{-1}$) while ORP and EC were completely left free to vary.

2.4.2. Real-time control.

In a second step of the experiment, thanks to the results of the first step, it was possible to implemented a "real-time control" strategy, mainly based on the on-line measurements of CE and ORP (Fig. VI.4 -D). The real-time strategy was able to automatically adjust the lenght of the whole cycle and the duration of each single phase within the time cycle. Further, the control on DO during the microareobic phase, on pH and level was always present. The logic of the "real-time control" strategy is following described.

Feeding. The feeding started at the beginning of the cycle and ended either i) when a maximal conductivity value has been reached, CE_{max}, corresponding to the maximum ammonium threshold: (i.e. 80 $mgTAN \cdot L^{-1}$) or ii) when the maximal fill level of the reactor has been reached (8 L). The stirring unit was switched on during the feeding.

Initial pause. An initial non aerated idle phase, prior to the aeration stage, had been designed both to avoid aerating without the presence of sufficient ammonium substrate in the reactor according to Joss et al. (2009) and to eventually remove the excess of nitrate and nitrite of the previous cycle, through anammox and denitrification processes, being both NH_4^+ and COD present in the influent.

Aeration. The start of the first aeration phase occured either when a set conductivity value ($CE_1 < CE_{max}$) has been reached or after a fixed delay has occurred. During the micro-aerobic phase, the DO was controlled, ensuing a low DO concentration by the on/off aerator controling. The DO set-point was fixed at a value of 0.2 ±0.1 $mgO_2\ L^{-1}$. The aeration phase stopped either when the conductivity value decreased of a fixed ΔCE with respect to its value at the beginning of the aeration phase or alternatively when the ORP control limit (ORP_{max}) has been reached. The ΔCE was chosen in order to have only a part of ammonium oxidation (i.e. 50%, partial nitritation) while the maximum ORP value (ORP_{max}) was set in order to avoid high nitrite concentrations in the reactor (i.e. maximum 20 – 30 mg $TNNL^{-1}$), preventing the short and long effect of nitrite inhibition on anammox bacteria (Bettazzi et al. 2010). A suitable TNN/TAN ratio for the sequent anammox process should be achieved. An additional timer control of the aeration phase (Δt_{aer}) has been defined to ensure a continuous operational SBR cycle, avoiding problems due to the malfunction of the conductivity or ORP sensor. Thus after a fixed maximum duration (Δt_{aer}), the aeration terminated even if the other stop criteria had not yet been occurred. Due to the low airflow rate, the stirring unit was switched on during the aeration in order to ensure a suitable mixing inside the SBR.

Mixing. A mixing phase followed the aeration phase, where both anammox and denitrification processes occured. The mixing phase stopped when a minimum ORP ($ORP_{min} < ORP_{max}$) control limits has been reached. Also in this case, an additional timer control of the mixing phase has been defined (Δt_{mix}) to ensure a continuous operational SBR cycle.

Number of repetitions. The software had been developed taking into account that SBR configuration could be characterized either by one aeration/mixing phase per cycle or by multiple aeration/mixing phases per cycle. The number of repetitions depended on the conductivity value measured at the end of the mixing phase. The cycle terminated when the set minimal conductivity value has been reached ($CE_{min} < CE_1 < CE_{max}$). The number of repetition implicitly depended on the ΔCE or ORP_{max} chosen in the aeration setting. Small ΔCE or low ORP_{max} easily implied multiple aeration/mixing phases per cycle. For an increased operating reliability, a timer control of the total reaction phase (aeration + mixing) has been defined, after which the cycle proceeded to settling phase even if the other stop criteria had not yet been reached.

Settle. The settling phase was time-controlled and its duration depends on the reactor and sludge characteristics, having fixed a low minimum biomass settling velocity of 0.7 m h^{-1}.

Draw. The effluent discharge phase was also time-controlled and has been setted to times that allow discharging the volume filled during the feeding, calculated from cycle to cycle. An additional sludge wastage phase, based on time, could be set.

Final pause. The final idle between cycles was optional.

2.5 Analytical methods

Total suspended solid (TSS), volatile suspended solid (VSS), total ammonium as nitrogen (TAN), total nitrite as nitrogen (TNN), nitrate as nitrogen (NO_3-N), total Kjeldahl Nitrogen (TKN) and chemical oxygen demand (COD) were determined according to the Standard Methods (APHA 2005). The COD was analyzed taking into account the chloride interference and further recalculated considering the interference due to the presence of nitrite (nitrite exerts a COD of 1.142 mgO_2/mg TNN). Alkalinity determinations were conducted using the kit USEPA Buret Titration Method 8221 (Hach Company). The KLa was estimated following the standard method of measurement of the transfer of oxygen in clean water published by the American Society of Civil Engineering (ASCE 1992)..

3. Results and discussion

3.1 Reactor performance (fixed time control)

The concentration of nitrogen species and carbon in the influent and in the effluent of the SBR, over the entire experimental period using the fixed time control, as described in the section 2.4.1, are shown in Fig. VI.5 and Fig. VI.6 respectively. The SBR cycle pattern defined in the section 2.2 was able to achieve high nitrogen and carbon removal performances treating synthetic wastewater at 30°C. High nitrogen removal efficiencies were also achieved at moderate temperature (T=25°C). Finally, after a gradual acclimatation to the anaerobic digester effluent, good results were achieved treating anaerobic digester effluent at 25°C. Our results showed that the SBR cycle pattern defined was suitable for a SNAD process. However, the control strategy based on the "fixed time control" required a constant supervision of the process and manual adjustments of the SBR cycle confifuration. Further, often indesiderable situation occurred, such as the accumulation of nitrite and nitrate in the system as well as the complete depletion of ammonium.

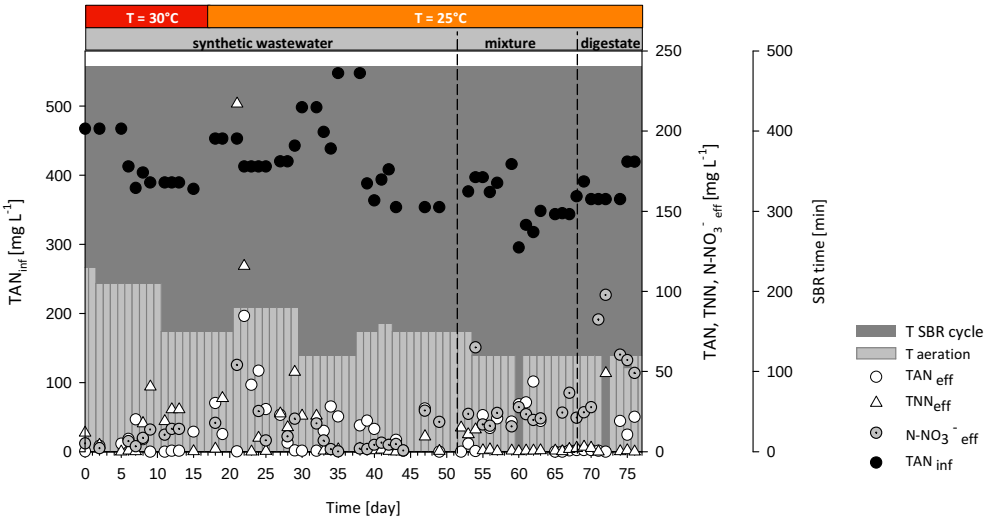


Fig. VI.5 Concentration of the nitrogen species in the influent and effluent of the SBR during the fixed time control strategy.

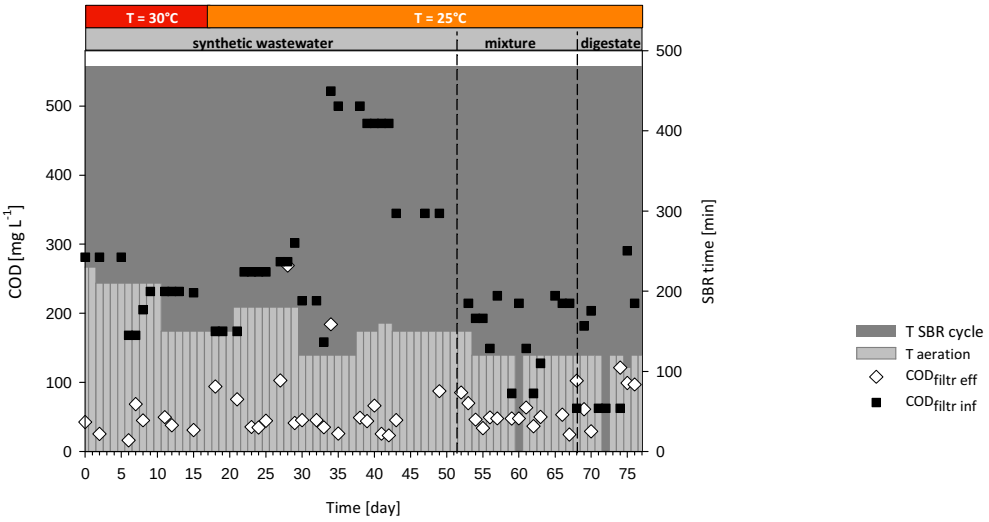


Fig. VI.6 Concentration of the COD in the influent and effluent of the SBR during the fixed time control strategy.

Influent and effluent characteristics are presented in Table VI-1. With an average hydraulic retention time (HRT) of 3 d and a reactor TSS concentration of 3-4 g L^{-1}, the achieved nitrogen removal rate (NRR) was 0.125

kg-N m^{-3} d^{-1} at maximum. The total ammonia and the total nitrogen removal efficiencies were comparable to the efficiencies of other SBR systems performing the SNAD process at 30°C, achieving values of 93% and 81% respectively. The nitrogen removal efficiencies at 25°C achieved in this study were significantly higher than other studies at moderate temperatures. The C/N ratio in the influent was lower than 1, between 0.4 - 0.9. The COD removal was in the range of 70 – 80%, suggesting that nitrogen removal via denitrification occurred. A summary of several experimental studies described in literature is given in Table VI-2.

Table VI-1 Characteristic of the nitrogen removal over the fixed time control experiment
Average influent and effluent specifications of the SBR

Parameter	**Period I**		**Period II**		**Period III**	
	Influent	**Effluent**	**Influent**	**Effluent**	**Influent**	**Effluent**
Wastewater	Synthetic wastewater		Synthetic wastewater		Digester effluent	
Period [d]	1-17		18-53		68-77	
Temperature reactor [°C]	30		25		25	
NLR [kgN m^{-3} d^{-1}]	0.134±0.026		0.136±0.036		0.131±0.011	
OLR [kgCOD m^{-3} d^{-1}]	0.077±0.019		0.11±0.05		0.06±0.03	
TAN removal rate [%]	98.5±1.7		96.1±3		98.4±2	
TN removal rate [%]	93.2±4.2		91.8±5		88.6±4	
COD removal rate [%]	81.7±11.9		77.0±2.1		70.1±12	
NRR[kgN m^{-3} d^{-1}]	0.125		0.125		0.116	
TAN [mgN L^{-1}]	415±36	5±6	428±55	15±14	374±27	6±8
TNN [mgN L^{-1}]	0	13±14	0	9±12	0	6±15
NO_3-N [mgN L^{-1}]	0	9±5	0	10±9	0	37±21
COD tot[mgCOD L^{-1}]	236±44	44±23	319±100	93±82	323±73	93±73
CODfiltr [mgCOD L^{-1}]]	231±40	40±18	310±90	68±57	156±85	73±36
TSS [g L^{-1}]	-	0.05	-	0.09	-	0.08

During the “fixed time control” experiment, the length of each phase of the SBR cycle was manually adapted in order to achieve high nitrogen and carbon removal efficiencies in the SNAD process. In particular, the duration of the micro-aeration phase, key factor to achieve a partial nitritation over the whole cycle, was regulated cycle by cycle after batch analysis on the SBR effluent, requiring time and chemical costs.

Table VI-2 Overview of typical innovative nitrogen removal processes

Wastewater	CODbio/N	Reactor type	Volume	SBR configuration	Fill	Aeration mode	DO [mg L^{-1}]	Inoculum	HRT [d]	pH	T [°C]	VSS [gL^{-1}]	OLR [gCOD $L^{-1}d^{-1}$]	NLR [gTN $L^{-1}d^{-1}$]	Removal rate [g TN $L^{-1}d^{-1}$]	%TAN	% TN	%COD	Reference
Synthetic wastewater	0	SBR	2.5 L	1h cycle	instantaneous **beginning cycle**	continuous	0.3-0.7	OLAND biomass	0.2	>7.4	33±1	2.3	0	1.5*	1.1	73%*	-	0	De Clippeleir et al. 2009
Synthetic wastewater	1:2	GSBR	18 L	24h cycle	continuous	continuous	0.5 – 1.0	SNAD sludge	3	7-8	35	-	0.033	0.066	0.061*	92,6% (85% PN+ Amx; 7,3% Den)	93%	72%	Lan et al. 2011
Synthetic wastewater	0	SBR	3 L	12h cycle	intermittent	intermittent	1.0 – 1.5	active sludge+ anammox biomass	1.5	7.2-8	30	1.2	0	0.187	0.140*	65%	70%	0	Xu et al. 2010
Synthetic wastewater	**1:1.8**	**SBR**	**8 L**	**8 h cycle**	**instantaneous beginning cycle**	**intermittent**	**0.2-0.3**	**SNAD sludge**	**3**	**7.5-8.3**	**25**	**1.9**	**0.11**	**0.136**	**0.125**	**96%**	**91.8%**	**77%**	**This study**
Synthetic wastewater	1:2	NRBC	small	nd	nd	continuous	0.4 – 0.6	nitrifying biomass+ anammox biomass	0.25	nd	35	nd	0.34	0.69		70%	79%	94%	Chen et al. 2009
Digester effluent	1:1.2	SBR	140L	8 h cycle	interval	continuous	0.3-0.5	SNAD sludge	2.5*	-	30 -26	3.3*	0.31	0.4	0.36*	95%	90%	71-74%	Lackner, et al 2012
Digester effluent	1:3	SBR	500 m3	8h cycle	continuous	intermittent	0.2-0.3	activated sludge	-	7 -7.1	28 ±1	3	0.14*	0.43*	0.37*	85.8%	90.3%	56%*	Wett 2007
Digester effluent	1:2	SBR	1400 m3	6-8h cycle	instantaneous **beginning cycle**	continuous	<0.8	activated sludge + anammox biomass	1.45*	-	30±3	3.6	0.2*	0.45*	0.42*	94.6*	95%*	37%*	Joss et al 2009
Digester effluent	1:1.2	SBR	1.5L	5.75h cycle	continuous	Air pulsing	0.1 -2	granular nitrifying + anammox biomass	0.5	7.5-7.9	20-24	1.5-4.5	-	0.7	0.25-0.45	35 - 64%	nd	nd	Vázquez-Padín et al 2009b
Digester effluent	nd	GSBR	1.5L	3 h cycle	continuous	Air pulsing	2.7	granular heterotrophic	0.25	-	20	5	-	1.6*	1.0	60%	nd	nd	Vázquez-Padín et al 2009a
Digester effluent	**1:2.5**	**SBR**	**8 L**	**8 h cycle**	**instantaneous beginning cycle**	**intermittent**	**0.2-0.3**	**SNAD sludge**	**3**	**7.3-8.3**	**25**	**1.9**	**0.08**	**0.15**	**0.14**	**96%**	**88%**	**58%**	**This study**
Landfill Leachate	1:2.6	SBR	3 L	12h cycle	intermittent **beginning cycle**	intermittent	1.0 – 1.5	active sludge+ anammox biomass	1.5	7.2-8	30	1.5	0.36*	0.961	0.896*	92%	92%	95% BOD (6,7% COD)	Xu et al. 2010
Landfill Leachate	nd	SBBR	3 L	24h cycle	intermittent **beginning cycle**	intermittent	0.6	active sludge	0.16	7.8-8.54	25-30	nd	-	-	-	97%	86%	-	Xiao et al 2009
Landfill Leachate	1:10	Aeration tank	384 m3	nd	continuous	continuous	0.3	active sludge	1.26	7.4	30	1.5	0.44	0.5	0.38*	75% (%68 PN+ Amx; 8% Den)	80%	28% (Den)	Wang et al. 2010
Landfill Leachate	**1:1.15**	**SBR**	**4 L**	**6 h cycle**	**instantaneous beginning cycle**	**intermittent**	**0.2-0.3**	**SNAD sludge**	**3.8**	**7.3-8.3**	**30**	**1.9-2.3**	**0.33**	**0.34**	**0.24**	**87%**	**74%**	**34%**	**This Study**
Opto-electric	1:5	SBR	2.5 L	24h cycle	continuous 12h	continuous	0.1	SNAD sludge	2.5	7.8-8.0	25	2.3	0.04	0.23	0.197	85%	90%	70%	Daverey et al. 2012

PN=Partial Nitritation, Amx= Anammox, Den = Denitrification

* value calculated

3.2 Variation of CE, pH, DO and ORP over a SNAD-SBR cycle (fixed time control)

During the "fixed time control" strategy, the variation of nitrogen species for the typical cycle pattern of the SBR, performing the SNAD process, have been studied and compared to the profile of pH, ORP, DO, EC in order to define a correlation with the ongoing biological processes.

In an SBR cycle, it was observed that TAN concentration gradually decreased with elapsed time under micro-aerobic conditions, while a corresponding increase of nitrite was detected. During the micro-aerobic phase, an increase of nitrate concentration can be observed both due to the not complete washout/inhibition of nitrite oxidized and to the low contribution of the anammox process under oxygen limited conditions (Yan et al. 2012). At the end of the micro-aerobic phase a consistent nitrite concentration and a residual ammonia content was measured. Then, in the mixed-anoxic phase a decrease of both ammonia and nitrite was observed, which was referred to the anammox activity. Further, analyzing the COD profiles over the SBR cycle (data not shown), it can be deduced that the aerobic biodegradable organic compounds oxidation was combined with the nitritation process during the micro-aerobic phase while the denitrification was coupled with the anaerobic ammonium oxidation during the anoxic phase. Denitrification could both reduce nitrate to nitrite and nitrite to di-nitrogen gas, if biodegradable carbon source is available. The decreasing COD profiles in the anoxic phase showed that heterotrophic denitrifiers are active during the anoxic phase, especially using synthetic wastewater where more biodegradable organic matter was present.

CE, pH, DO and ORP over typical cycles were on-line monitored in a time-course experiment and a complete set of the on-line data is provided in the following sections. Each signal was analyzed, in order to monitor the SNAD process in an SBR and further to establish a "real-time control" strategy. An in-depth understanding of the relation between degradation and conversion of $NH_4^+/NO_2^-/NO_3^-$ with variations of the indirect on-line measurements was detected. A good relation between the on-line parameters and the development of the SNAD process has been observed in the fixed time control strategy, treating both the synthetic wastewater and the anaerobic digester effluent,

3.2.1 pH correlation

As the pH was left free to vary in a wide pH range (7.3 - 8.5), it follows the processes behavior over the SBR cycle (Fig. VI.7). During the whole experiment, the pH value remained stable in the range of 7.3 – 8.3 with no external addition of base or acid. The pH value in the reactor was mainly determined by the balance of the added alkaline influent, the hydrogen ions production during nitritation, the hydrogen ions consumption during anammox processes and OH^- production during denitrification process. In the normal operative conditions, the pH increased towards the upper pH set-point during the feeding event, due to the high alkalinity content in the influent and a possible denitrification in the short anoxic fill period, reaching a maximum value (α_{pH}) at the end of the filling phase. According to the nitritation process, during the micro-aerobic phase, the pH decreased linearly in the course of converting ammonia to nitrite as hydrogen ions are produced until the aerator was switch off, and a minimum pH value (β_{pH}) was measured at the end of the micro-aeration phase. On the contrary of the nitritation process (aerobic oxidation of ammonia to nitrite), the nitratation process (aerobic oxidation of nitrite to nitrate) does not produce hydrogen ions. Thus, any pH variation in micro-aerobic phase can be associated with the nitratation process. When a maximum concentration of nitrite of 20-40 mg L^{-1} was achieved in the reactor, ensuing a proper TNN/TAN ratio for anammox process, the aeration was turn off manually. As consequence of the switch off of the aerator, an increase in pH was observed in the next anoxic phase. The pH

increase depends (i) on the OH^- production by the denitrification process, which depends on the availability of biodegradable organic matter; and (ii) on the H^+ consumption by anammox process during cell synthesis.

In this experiment performing the SNAD process, a minimum of the pH profile (β_{pH}) appeared but it did not occurred spontaneously. The minimum β_{pH} occurred when the aeration was turn off, as programmed in the "fixed time control" strategy. At the end of the micro-aeration phase in the SNAD-SBR, only a part of ammonia was removed, while a residual part was available for the anammox process in the following mixing phase. As consequence, the breaking point in the pH profile cannot be interpreted as the breaking point known as "*ammonia valley*" in the convetional nitrification/denitrification SBR, which is related to the complete depletion of ammonia. The minimum in the pH profile may be used to monitor the development of the whole SNAD process rather than to control and adjust the duration of the micro-aerobic phase.

At the end of the anoxic phase, another breaking point in the pH profile can be observed (γ_{pH}), which was related to the complete depletion of NO_2^-, while nitrates were still present in the system. This breaking point recalls the apex in the pH profile in conventional nitrification/denitrification SBR, even if "*nitrate apex*" was related to the nitrate depletion. However, γ_{pH} similarly to the "*nitrate apex*" could be used to signal the end of the anoxic phase. In the SNAD system, γ_{pH} can be associated with the end of anammox reaction due to nitrite depletion. After the occurrence of the breaking point (γ_{pH}), pH continued to increase with a lower slope, which depends on the ongoing denitrification process.

Hence, the pH behaviors could be used mainly to monitoring the end of the micro-aerobic and anoxic phases. In addition, the pH profile after the breaking point (γ_{pH}) could be also used to regulate the length of anoxic phase in order to deplete nitrite through anammox process and further nitrate through denitrification process, if COD_{bio} is available.

3.2.2 ORP correlation

In the normal operative conditions, after the filling period, the ORP decreased reaching a minimum (α_{ORP}), as a result of sludge mixing with the influent to treat, and, probably, also due to the consumption by anammox or denitrification process of residual nitrite and nitrate in the systems, accumulated from the previous cycle. When the aeration was switched on, the ORP value sharply increased and reached a maximum value (β_{ORP}), due both to the presence of the dissolved oxygen in the systems and to the progressively oxidation of ammonium to nitrite, and eventually to nitrate, as nitrates were also produced during the aeration phase as reported above. During the SBR micro-aeration phase, the ORP value increased reaching maximum values between + 50 - +100 mV, depending on the nitrite and nitrate concentration achieved in the bulk liquid. In Fig. VI.8, a maximum ORP (β_{ORP}) of + 50 mV was achieved correspondent to about 20 mg TNN L^{-1} and 8 mg NO_3^--N L^{-1}. When anoxic period started, the ORP value quickly decreased due to the aeration switch-off and to the complete oxygen depletion. Then, the ORP showed a sharp decrease reaching negative values whereas nitrite were consumed mainly by anammox process and nitrate by denitrification process.

In the anoxic phase, when nitrite were depleted, a blending point could be detected (γ_{ORP}), corresponding to the breaking point in the pH profile (γ_{pH}). The blending point (γ_{ORP}) could be related to the *''nitrate knee''* of the ORP profile of the conventional SBR. However, unlike in the conventional SBR, here, the γ_{ORP} indicates the depletion of nitrite, rather than nitrate, and, thus, the end of anammox process due to nitrite limitation.

As nitrate were still present in the system (5 mg NO_3^--N L^{-1}), after this blending point a further decrease of ORP can be detected. The amplituted of the further ORP decrease (ΔORP_{den}) depended on the availability of biodegradable COD, necessary to carry on the reduction of nitrate by denitrification process. The ORP reached a

minimum value at the end of the anoxic phase (-100 mV). A complete depletion of nitrate and nitrite was observed at around – 300 to -400 mV (Ra et al., 1998).

Recognizing this trend, it is possible to reduce the micro-aerobic and the anoxic period, resulting in time saving with respect to the fixed-timing switching scheme. Under stable SNAD conditions, the maximum ORP value could be used as indicator of high nitrite concentrations. Further, the minimum value of the ORP signal, could be used to signal the end of the anoxic cycle, due to the nitrite and nitrate depletion, either prior to another aerobic phase or prior to the end of the cycle, depending on the residual ammonia concentration.

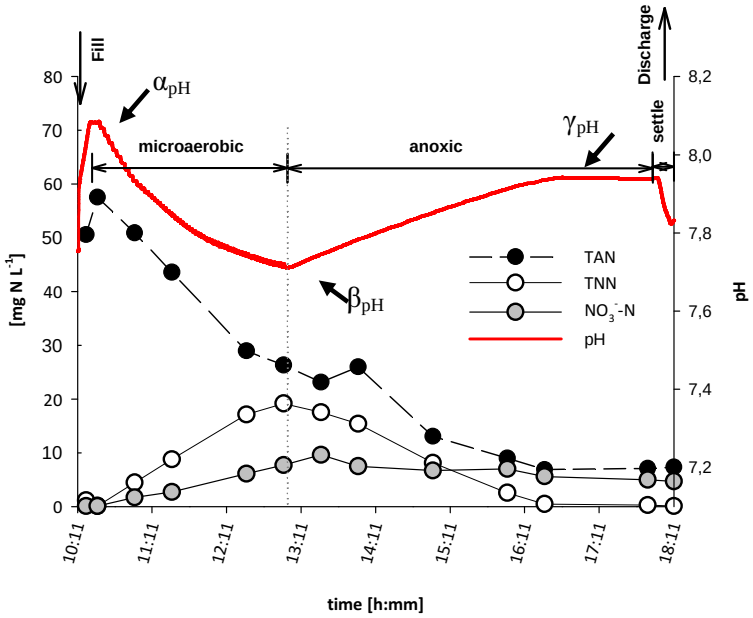


Fig. VI.7 On line data of the pH and nitrogen species over an SBR cycle

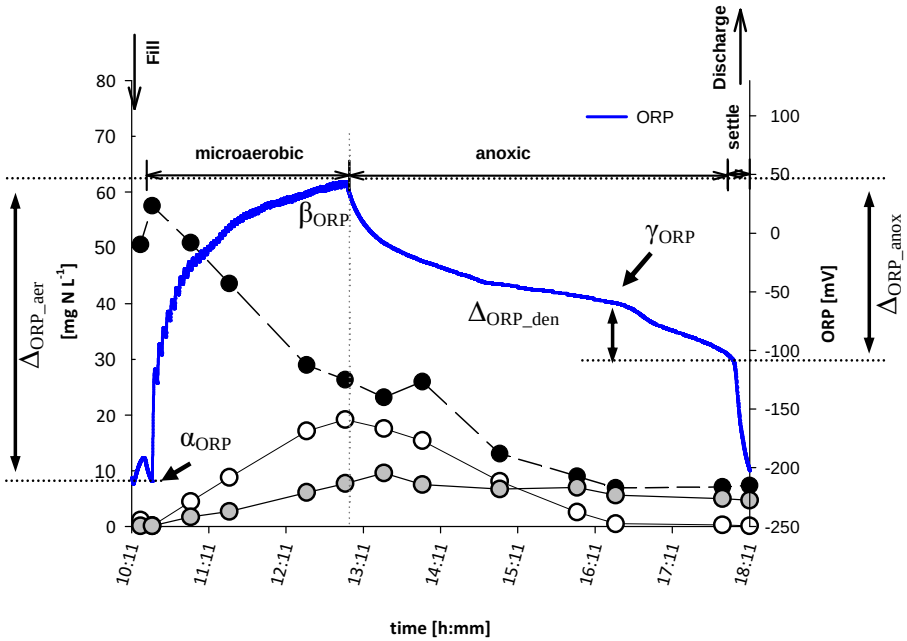


Fig. VI.8 On line data of the ORP and nitrogen species over an SBR cycle

3.2.3 CE correlation

At the beginning of each cycle, influent was fed and a sharp increase of conductivity was observed (α_{EC}), as a result of sludge mixing with a high salinity influent. Then, the aeration was switched on and the EC decreased due to the biological conversion. The EC value takes into account both the decrease of salinity due to the ammonia consumption and increase of the salinity due to the nitrite and nitrate production. Ammonium, nitrite and nitrate have almost the same molar conductivity. However, if in a system there is enough alkalinity, the biological N-removal through nitrification will give a decrease of conductivity, mainly due to the alkalinity consumption, according to Levlin (2009). When the aeration was switched off, the EC again decrease reaching a minimum (γ_{EC}) at the end of the anoxic phase due to the anammox and denitrification processes. Even if denitrification process produces alkalinity, conductivity profile decreased due to ammonium and nitrite consumption by anammox process and nitrate and nitrite consumption by denitrification. The amount of nitrate produced in the anammox process is only a little part of the ammonium anaerobically oxidized, thus the overall effect was an EC decrease in the anoxic phase. At the end of the anoxic phase, in the time fixed control strategy, the EC value was constant, as no anammox and denitrification processes occured. Over the whole SBR cycle a decrease in EC can be detected from a maximum value (α_{EC}) at the end of the filling phase to a minimum value (γ_{EC}) at the end of the anoxic phase. The EC value, at the beginning and at the end of the SBR cycle, indirectly follows the ammonium profile. EC could be used as an useful signal to detect both the end of the filling phase and the end of the anoxic phase.

3.2.4 DO and OUR correlation

As a DO set point was fixed during the micro-aerobic phase, using the on/off control on the aerator, the classical DO profile is reported in Fig. VI.10. The DO signal was only detectable during the micro-aerobic phase and concentration were an overage around 0.2 – 0.4 mg L^{-1}. During the anoxic phase no oxygen was detected. A DO breaking point (β_{DO}) occurred as consequence of the aerator switch off, corresponding to the minimum of the pH value (β_{pH}) and to the maximum of the ORP value (β_{ORP}). Also in this case like for the pH, the breaking point in the DO profile cannot be effectively used to adjust the duration of the micro-aerobic phase, but only as a monitoring parameter, indicating the turn off of the aeration and the passage from the micro-aerobic to the anoxic phase.

Further, in this experiment the slopes of the decrease in DO concentration during the micro-aerobic period of the SBR (during the aeration off period of the DO control) were measured by determining the oxygen uptake rate (OUR, mg O_2 $L^{-1}h^{-1}$) (Fig. VI.11). According to Andreottola et al., (2002), the OURs were calculated from the on-line DO drop measurements when no air flow was supplied. The OUR values indicated the biological activity in the reactor during micro-aerobic step. In the micro-aerobic phase, the DO concentration available in the system is utilized to oxidize both COD and ammonia, by aerobic heterotrophic and autotrophic bacteria, respectively. This is confirmed by the COD and TAN decrease in micro-aerobic phase. The OUR assumed a lower value at the beginning of the micro-aerobic phase, then it increased and it was approximately constant (15 mgO_2 L^{-1} h^{-1}) until the end of the micro-aerobic phase. Nevertheless, as also previously reported in other studies (Blackburne et al. 2008c), it was difficult to find an easily implementable correlation between the OUR value and the length of the micro-aerobic phase of a SNAD-SBR system, as partial nitritation and carbon oxidation occurred simultaneously.

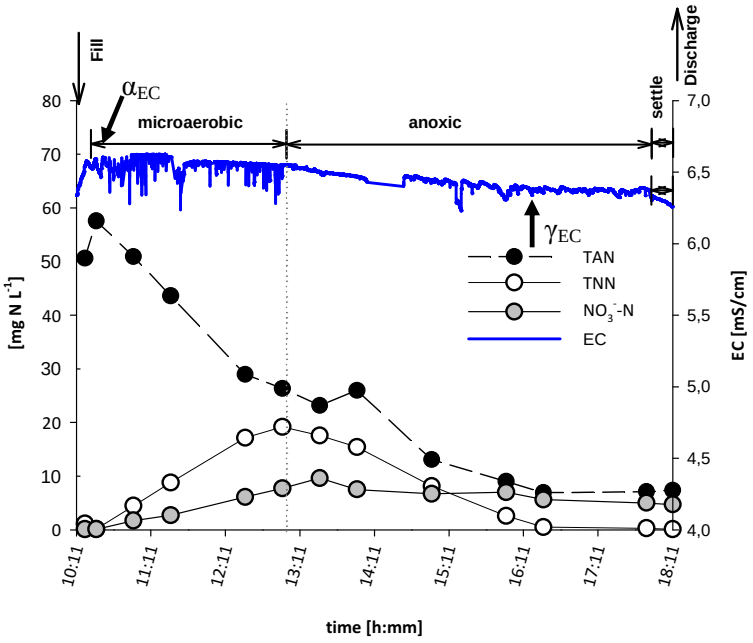


Fig. VI.9 On line data of the EC and nitrogen species over an SBR cycle

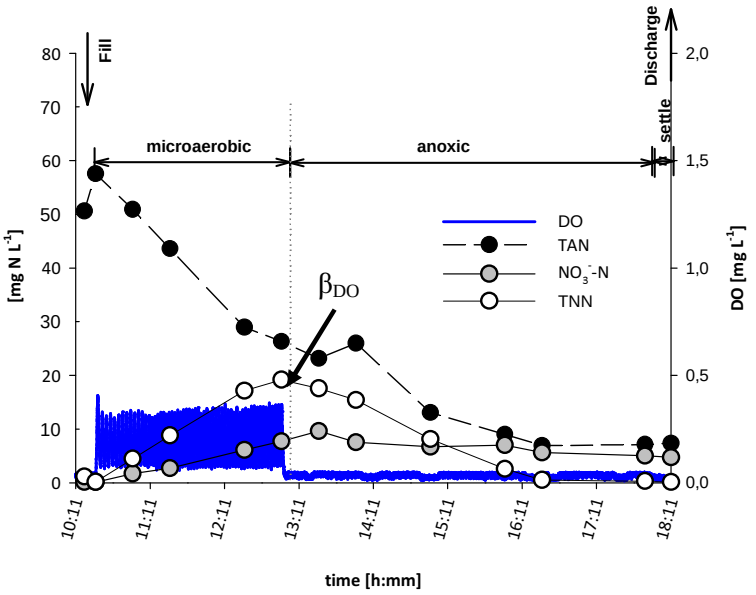


Fig. VI.10 On line data of the DO and nitrogen species over an SBR cycle

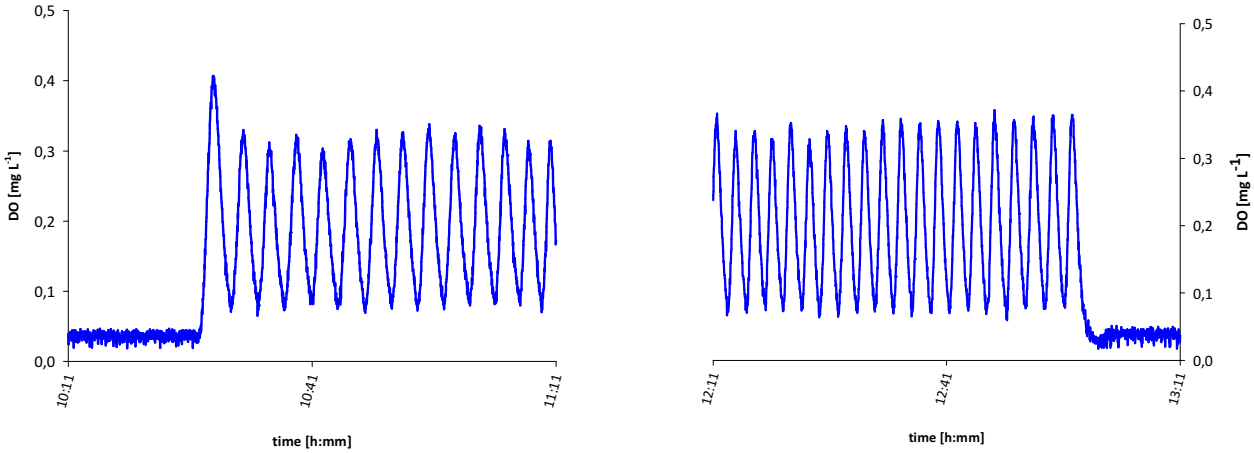


Fig. VI.11 Slope of the DO during the aeration off period of the DO control

3.3 Reactor operation during the fixed time control strategy

During normal conditions of the "time-fixed control" strategy, the reactor was operated with the cycle strategy described in Section 2.4.1. The characteristic profiles during the three periods are reported in the following figure: (I) SNAD-SBR treating the synthetic wastewater at 30°C, (II) SNAD-SBR treating the synthetic wastewater at 25°C and (III) SNAD-SBR treating the anaerobic digester effluent at 25°C. EC, ORP, pH and DO profile patterns are provided in Fig. VI.12, Fig. VI.13 and Fig. VI.14 for period I, II and III., respectively.

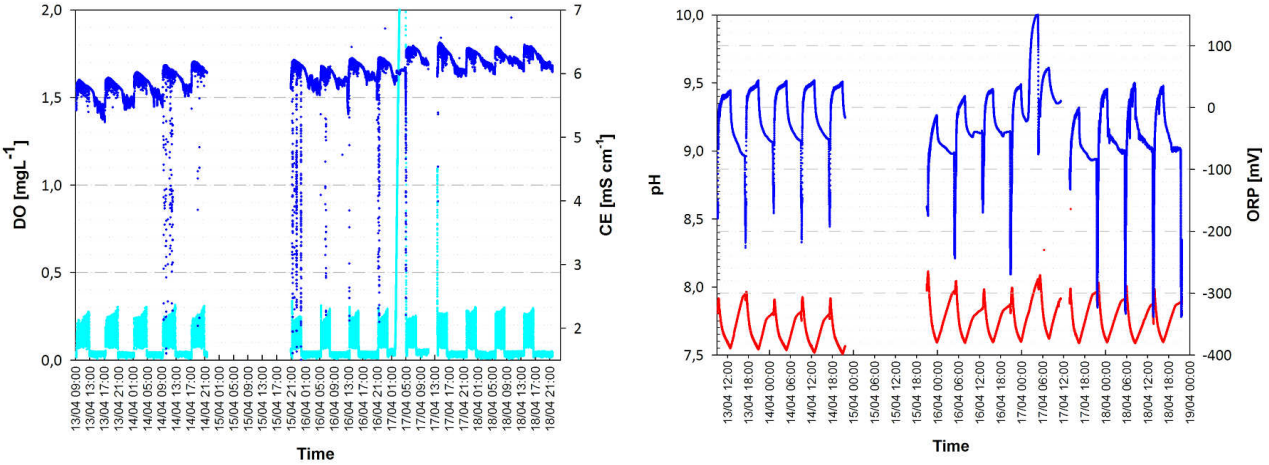


Fig. VI.12 Period I: SBR cycles from the day 8 to 13, treating synthetic wastewater at 30°C

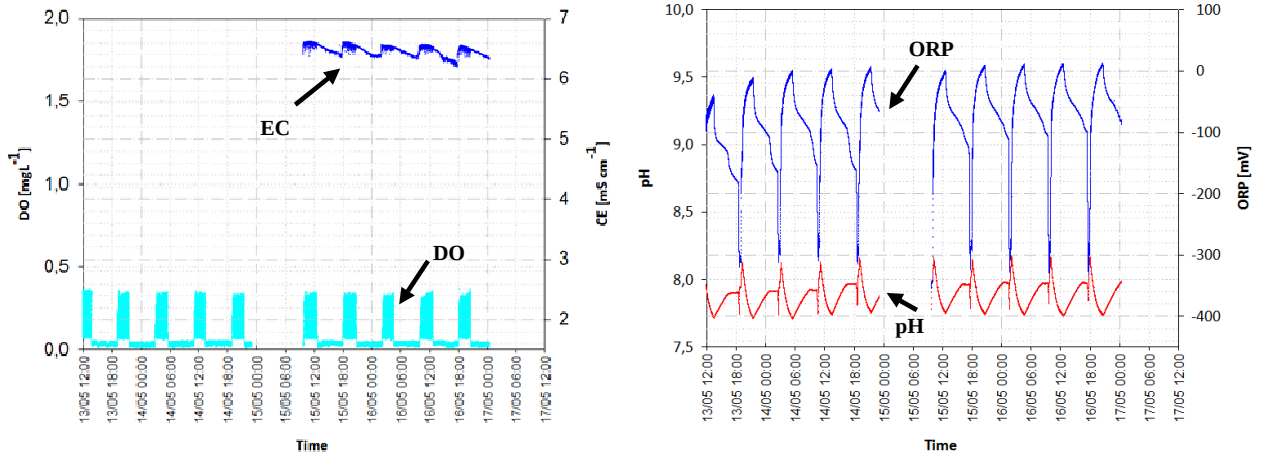


Fig. VI.13 Period II: SBR cycles from the day 38 to 42, treating synthetic wastewater at 25°C

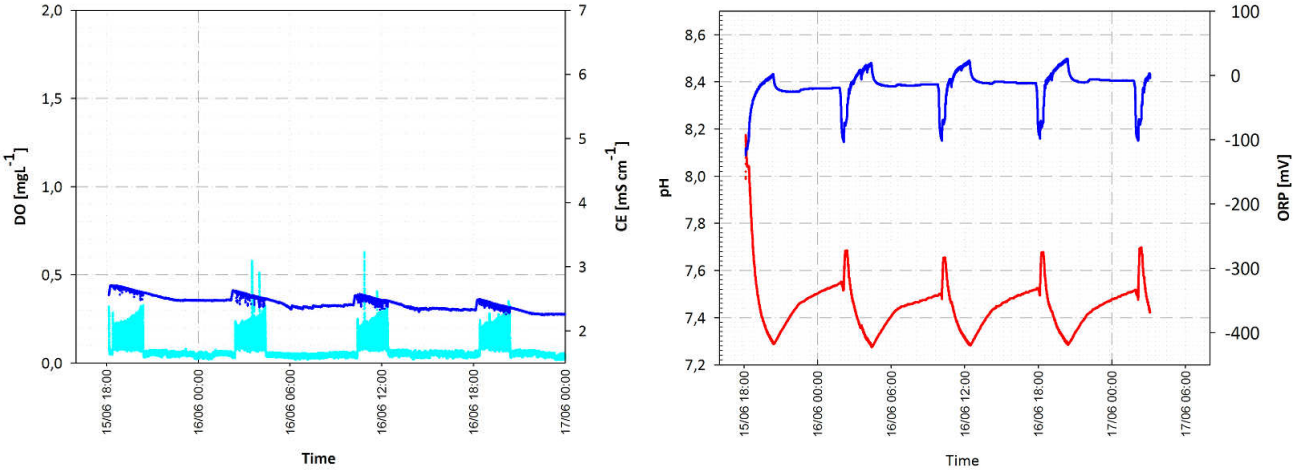


Fig. VI.14 Period III: SBR cycles from the day 71 to 73, treating anaerobic digester effluent at 25°C

In the whole experiment the on-line parameters patterns were reproducible. The anoxic filling phase followed by a short mixed-anoxic idle, determined an increase of EC and pH and a decrease of ORP. When the aeration was switched on, the ORP showed a sharp increase whereas the pH and EC decreased steadily due to biological conversion processes. During the anoxic phase, EC continued to decrease slowly, and whereas the pH increased the ORP decreased due to denitrification and anammox processes.

Fig. VI.12 and Fig. VI.13 shows examples of the characteristic profiles patterns of the indirect parameters from the day 8 to 13 and from the day 38 to 42, treating the synthetic wastewater at 30°C and 25°C, with 3 cycle per day (Period I and Period II). Treating the synthetic wastewater, conductivity values were in the range of 6.5 – 5.5 mS cm^{-1}. Each cycle starts with the addition of synthetic wastewater to the reactor under anoxic condition. The conductivity reaches its maximum value (α_{CE}) due to the addition of ammonium. Then, the conductivity decreased both in micro-aerobic phase and anoxic phase. At the end of the cycle the conductivity values were constant, mainly indicating the end of anammox process and a minimum EC value was reached (γ_{CE}). However, the values of conductivity in the system are wastewater specific. Treating the anaerobic digester effluent, the conductivity gradually decreased. When stable conditions were achieved (day 71-73) the conductivity values varied between 3 – 2 mS cm^{-1} (Fig. VI.14). However, appliying similar nitrogen loading rates (NLR), Fig. VI.15 shows that, even if the value of conductivity depends on the kind of wastewater, the delta values of conductivity obtained during the micro-aerobic and anoxic phase are constant over the whole experiment. A constant difference (ΔEC) of 0.3 mS cm^{-1} was measured over a complete SBR cycle, whereas a ΔEC_{aer} of 0.1 mS cm^{-1} was detected during the microaerobic phase and a ΔEC_{mix} of 0.2 mS cm^{-1} during the anoxic phase.

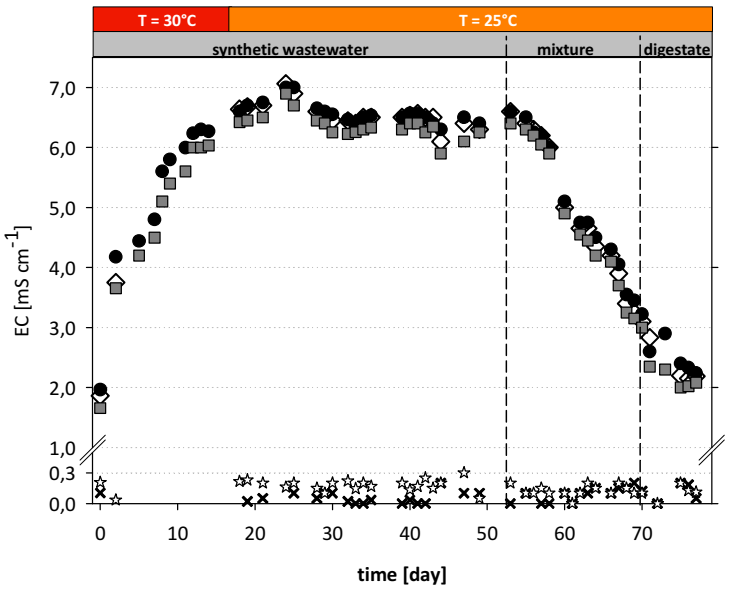


Fig. VI.15 Breaking and Blending points in EC profile during the whole experiment
α point(black circle); β point (white rumble); γ point (gray square); ΔEC in micro-aeration phase (cross); ΔEC in anoxic phase (star)

The pH in period I and II varied between 7.5 and 8 (Fig. VI.16). The maximum value was reached at the beginning of the micro-aerobic phase, when the ammonium concentration was the highest. As consequence, the nitrite oxidizing bacteria (NOB) were partially inhibited by the Free Ammonia (Anthonisen et al. 1976). In period III, treating the digester effluent, when a stable nitrogen removal was achieved, the pH naturally decreased and varied between 7.3 and 7.6. This implied that the inhibition of NOB was lower compared to the period I and II, due to the lower pH at the beginning of SBR cycles, and thus to the lower Free Ammonia

concentrations. A nitrate accumulation in the system in period III confirmed the higher activity of nitrite oxidizing bacteria.

Also in this case a constant difference (ΔpH) in micro-aerobic and anoxic phases can be detected, independent of the initial and final pH values. An average ΔpH_{aer} of 0.3 was detected during the microaerobic phases whereas a ΔpH_{mix} of |-0.1| during the anoxic phases.

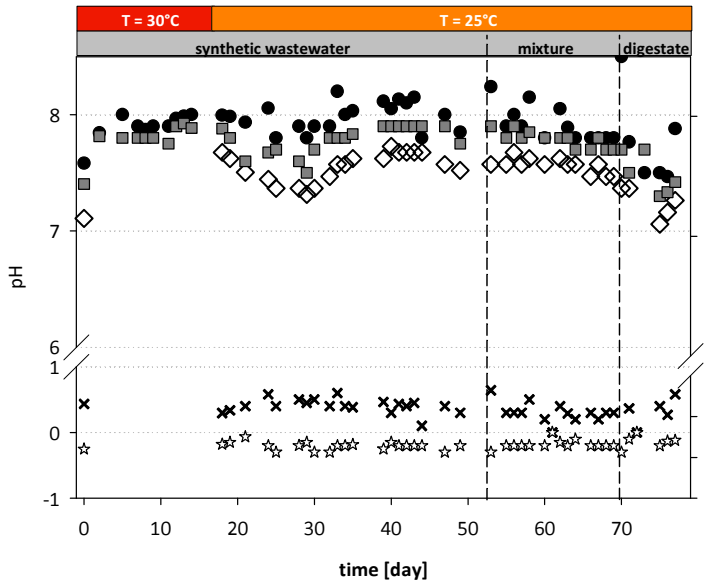


Fig. VI.16 Breaking and Blending points in pH profile during the whole experiment
α point (black circle); β point (white rumble); γ point (gray square); ΔpH in micro-aeration phase (cross); ΔpH in anoxic phase (star)

The ORP signal followed each phase of the SBR cycle. The ORP always increased during micro-aerobic phase and a maximum ORP value could be imposed in the micro-aerobic phase to avoid high nitrite concentrations in the system, which could inhibit anammox bacteria. Further, during the anoxic phase, the ORP decreased and the deplete of nitrite (thus the end of anammox process) can be detected observing the ORP profile and the occurrence of a blending point (γ_{ORP}). After γ_{ORP} has been achieved, the ORP could further decrease. Fig. VI.17 shows the breaking and blending points in the ORP profiles during the whole experiment and the amplitude of the ORP during both the microaerobic (Δ_{ORP_AER}) and anoxic phases (Δ_{ORP_ANOX}). In the aerobic phase the amplitude of the ORP depended on the occurrence of nitritation and nitratation process and the nitrite and nitrate accumulation in the system. On the contrary the amplitude of ORP during the anoxic period depended both on anammox and denitrification processes. In particular, the amplitude of the ORP in the anoxic phase after the blending point γ_{ORP} ($\Delta_{ORP\_denitrification}$) depended on the denitrification process. In period III, treating the anaerobic digester effluent, the denitrification contribution was lower than that measured treating the synthetic wastewater, due to the lower COD content and the amplitude of the ORP in the anoxic phase after the blending point (γ_{ORP}) was very small (Fig. VI.14).

In Fig. VI.17 can be seen that, treating the sysntetic wastewater, the lower nitrite and nitrate accumulation in the effluent, were achieved when the ORP value at the end of the microaerobic phase (β_{ORP}) was about + 30 mV and at the end of anoxic phase reached a minimum of -80 ÷ -100 mV. Treating the anaerobic digester effluent, the ORP at the end of the anoxic phase was higher (- 30 ÷ -10 mV), as nitrate accumulation occurred, due to the lower biodegradable organic content in the digestate effluent and the reactivation of NOB bacteria.

According to Lackner and Horn (2012), the ORP is one of the best parameters to control the development of biological process, especially in pH controlled systems, as both pH and EC are strongly influenced by the pH

adjustment, resulting in a constant values. The authors showed that in the pH controlled systems, the characteristic ORP pattern still remained and the relative breaking and blending point stills are detectable and visible for the process control.

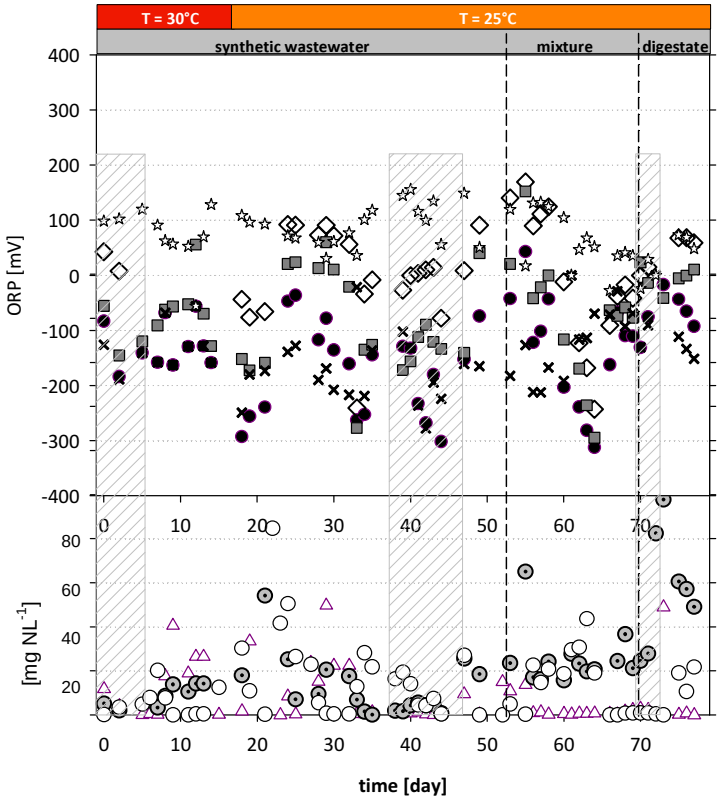


Fig. VI.17 Breaking and Blending points in ORP profile during the whole experiment
α point (black circle); β point (white rumble); minimum OPR value (gray square);
ΔORP_{AER} in micro-aeration phase (cross); ΔORP_{ANOX} in anoxic phase (star)
ammonium effluent (white triangle), nitrite effluent (while circle), nitrate effluent (gray circle)

3.4 Reactor operation and cycle optimization (real-time control)

After the study of the relevant points in the ORP, EC, pH and DO profiles, the overall treatment was optimized implementing a "real-time control strategy", as described in the section 2.4.2. The "real-time control" strategy was validated for 10 days, after more than two months of a stable SNAD process.

During the real time control strategy, the total ammonia and nitrogen removal efficiencies stayed high, 96,5% and 88.6% respectively (Fig. VI.18). Inhibition due to high nitrite accumulation in the system was prevented. The COD efficiency was comparable with the efficiency obtained in the "time fixed control" strategy.

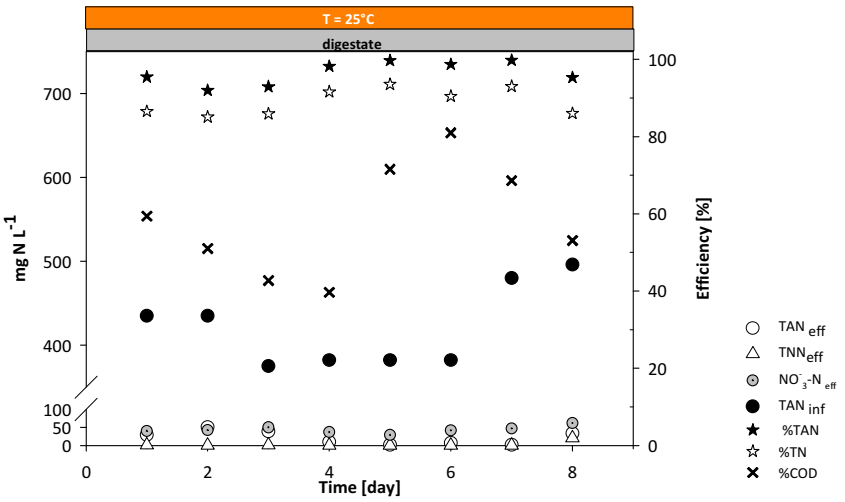


Fig. VI.18 Concentration of the nitrogen species in the influent and effluent of the SBR during the real time control strategy

The optimization allowed to achieve the high results measured in the "fixed time control" strategy, preventing any critical situations in the reactor: (i) the risk of high Free Ammonia inhibition for AOB at the end of the filling phase were minimized by fixing a maximal EC value (α_{EC}) during the feed; (ii) the anammox inhibition by high nitrite concentrations in the system was minimized by fixing a maximal ORP value (β_{ORP}) at the end of the micro-aerobic phase (iii) a high degree of ammonium removal was ensured by fixing a proper minimum EC (γ_{EC}) and ORP (ORP_{min}) thresholds at the end of the process.

For a specific wastewater, the maximum EC value (α_{EC}) was correlated with the ammonium concentration at the beginning of the SBR cycle whereas the minimum EC value (γ_{EC}) was correlated with the ammonium concentration at the end of the anoxic phase. Thus, a maximum and minimum values of conductivity (α_{EC} and γ_{EC}) were fixed treating anaerobic digester effluent: 2.2 mS cm^{-1} and 2.0 mS cm^{-1}, respectively. The maximum value was used to control the end of the filling phase while the minimum value was used to control the length of the whole reaction phase (aerobic+ anoxic) of the SBR.

The maximum ORP value in the SBR cycle was correlated at the nitrite concentration in the reactor at the end of the micro-aerobic phase. Thus, the aeration length was regulated by a maximum ORP value (β_{ORP}). A value of +30 mV was fixed, avoiding to reach high nitrite concentration at the end of the micro-aerobic phase. Further, at the end of the anoxic phase, an additional minimum ORP threshold was fixed (-40 mV) to ensure the complete depletion of nitrite by anammox process and the depletion of nitrate by denitrification process, if organic carbon is available in the system. The control strategy considered that if the minimum ORP value was achieved in the system, but the EC was still higher than γ_{EC}, a micro-aerobic phase restarted, in order to have a partial nitritation, followed by another anoxic period.

The imposed thresholds in the SBR cycle, automatically optimized the performance of the SBR cycle compared to the "time fixed control" strategy. In particular, the "real-time control" strategy allowed to optimize the lengh of the cycle, avoiding inhibitions and wastes of time. Further, a lower supervision of the systems was required, thus, reducing the operational costs.

The "real-time control" strategy naturally developed a pattern characterized by multiple aeration/mixing phases per cycle. Fig. VI.19 and Fig. VI.20 show the on line data of two SBR cycles. Within a single SBR cycle, from the DO profile can be observed two micro-aerobic phases, as at the end of the first anoxic phase the minimum ORP value was achieved but the conductivity value was still higher than the minimum conductivity threshold. The EC profiles decreased with different slope during the micro-aerobic and the anoxic phases. The pH profile nicely followed the ongoing biological processes.

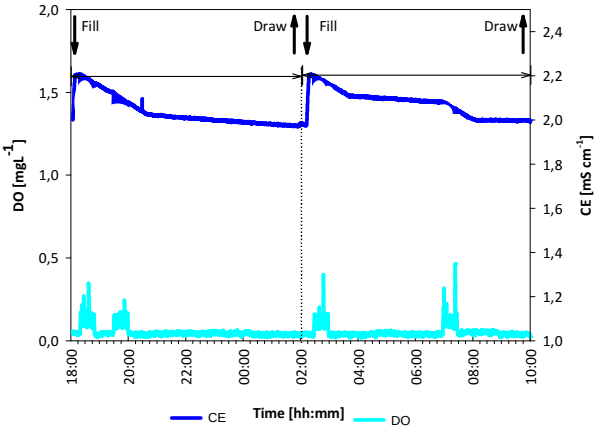


Fig. VI.19 On line data of the EC and DO for the cycles controlled by the real time control strategy

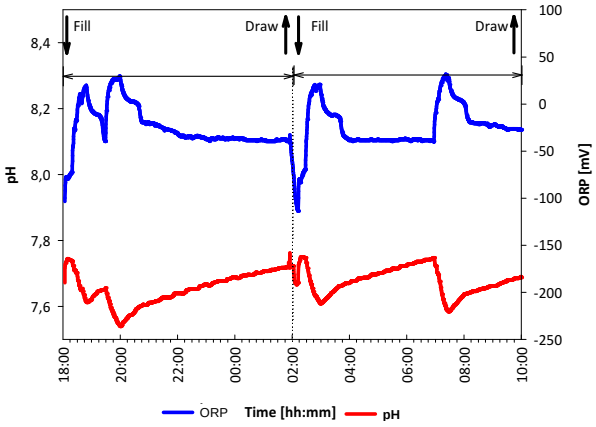


Fig. VI.20 On line data of the ORP and pH for the cycles controlled by the real time control strategy

4. Conclusion

In this work the suitability of a "real-time control" strategy in an SBR system for the simultaneous partial nitritation, anammox and denitrification (SNAD) process has been analyzed for the nitrogen and carbon biological removal from wastewaters with high ammonia concentration and low biodegradable organic content. The continuous monitoring of pH, ORP, DO and EC allowed the analysis of the dynamic of the nitrogen forms. Similarly to the conventional nitrification/denitrification in an SBR, the pH, ORP, DO and EC profiles followed the ongoing biological processes. Results of this study showed that:

- conductivity value was correlated with the maximum ammonium concentration at the beginning of the SBR cycle and with the minimum ammonium concentration at the of the SBR cycle;

- conductivity value depended on the type of wastewaters treated, but as a stable process occurred, the EC values are reproducible over the time;

- ORP value was correlated to nitritation process and to the nitrite accumulation during the micro-aerobic phase;

- a ORP blending point in the anoxic period was detected and it was related to the nitrite depletion mainly by anammox bacteria, while the OPR amplitude after the blending poit was correlated with the contribution of denitrification process via nitrate in the SNAD process.

- pH nicely followed the ongoing processes, however it is preferable to set the pH in the range of 7.5 – 8.5.

Nevertheless, the control strategy based on the “fixed time control” required a constant supervision of the process and manual adjustments of the SBR cycle configuration. The length of each phase of the SBR cycle was manually adapted in order to achieve high nitrogen and carbon removal efficiencies in the SNAD process. In particular, the duration of the micro-aeration phase, key factor to achieve a partial nitritation over the whole cycle, was regulated cycle by cycle after batch analysis on the SBR effluent, requiring time and chemical costs.

We showed that indirect on-line paramenters could be used to implement a “real time control” strategy to automatically determine the length of each phase in a SBR cycle. Adjudgments on the ending of micro-aerobic and anoxic phase can be based on the breaking and blending points on the curve of ORP, EC, pH, throughout each SBR cycle rather than manually regulations. A “real-time control” strategy based mainly on the ORP and EC measurements was designed and applied in this study. High nitrogen and carbon removal efficiencies were obstained, comparable to the efficiency measured during the “fixed time control” strategy. Nevertheless, the “real-time control” strategy has some advantages compared to a “fixed time control” strategy. It allowed the optimization of the lengh of the reaction phases of the SBR cycle, thus, reducing aeration and mixing time for saving energy source. Further, it allowed to avoid inhibition of the process, due to nitrite accumulation, high loads, complete substrate depletion, by saving time and by treating higher nitrogen load. Finilly, the real-time control strategy required a lower supervision of the systems and thus operational costs saving.

This study reveales that the SNAD process in a SBR allowed the implementation of a control strategy, mainly based on the EC and ORP signals to determine the duration of the filling and reaction phases of the SNAD-SBR. The SNAD process performed in an SBR allows energy saving, low cost operation. A easy control strategy makes SNAD- SBR an interesting option for the biological treatment of ammonium rich streams, such as old landfill leachates and anaerobic digester effluents, to name a few. Interesting future wastewaters implication would be the implementation of the SNAD-SBR control strategy besed on the on-line direct parameters, such as ammonium, nitrite and nitrate.

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Author Contributions

All authors contributed extensively to the work presented in this paper. The acquisition and control software was developed by M.L with the support of L. Penasa, from the University of Trento. Control strategies and experiments for reactor were designed and conducted by M.L. G.A. and M.L. conceived the research. M.L. wrote the paper with input of G.A. discussing the results, implications and commenting on the manuscript at all stages.

Supplementary Material

Further details concerning the acquisition and control hardware and software.

Supplementary Table

Supplementary Table VI-1 cDAQ channel configuration cDAQ – 9174 chassis (4 slot)							
Device	**Description**	**Control**	**Physical channel**	**Channel Name**	**output range**	**Work range**	**Work measure**
NI 9208 16-Channel Current Input Module							
pH sensor	on-line reactor pH	-	AI0	Numeric 1 pHr	±21.5 mA	4 – 20 mA	4 -10
ORP sensor	on-line reactor ORP	-	AI1	Numeric 2 ORPr	±21.5 mA	4 – 20 mA	-500 + 500 mV
Temperature sensor	on-line reactor Temperature combined with DO sensor	-	AI2	Numeric 3 TDOr	±21.5 mA	4 – 20 mA	5 – 40 °C
DO sensor	on-line reactor DO	-	AI3	Numeric 4 DOr	±21.5 mA	4 – 20 mA	0 – 10 mg L^{-1}
pH sensor	on-line influent tank pH	-	AI4	Numeric 5 pHi	±21.5 mA	4 – 20 mA	4 -10
CE sensor	on-line reactor Conductivity	-	AI5	Numeric 6 CEr	±21.5 mA	4 – 20 mA	0-100 mS
CE sensor	on-line influent tank Conductivity	-	AI6	Numeric 7 CEi	±21.5 mA	4 – 20 mA	0-100 mS
free		-	AI7		±21.5 mA		
Temperature sensor	on-line reactor Temperature combined with CE sensor	-	AI8	Numeric 9 TCEr	±21.5 mA	4 – 20 mA	0 – 40 °C
Temperature sensor	on-line influent tank Temperature combined with CE sensor	-	AI9	Numeric 10 TCEi	±21.5 mA	4 – 20 mA	0 – 40 °C
Level sensor	on-line reactor level	-	AI10	Numeric 11 Levelr	±21.5 mA	0 – 20 mA	5 - 32 cm
free	-	-	AI11		±21.5 mA		
free	-	-	AI12		±21.5 mA		
free	-	-	AI13		±21.5 mA		
free	-	-	AI14		±21.5 mA		
free	-	-	AI15		±21.5 mA		
NI 9485 – 8 Channel C Series Relay, 60 VDC/30 Vrms, 750 mA							
Influent Pump	influent feeding	Time and level On-Off control	AO0	P01	relay for switching voltages up to 60 VDC		
Effluent Pump	effluent discharge	Time and level On-Off control	AO1	P02	relay for switching voltages up to 60 VDC		
Sludge Pump	sludge discharge	Operator, Time and level On-Off control	AO2	P03	relay for switching voltages up to 60 VDC		
Acid pump	alkalinity and basic condition	pH On-Off control	AO3	Pacid	relay for switching voltages up to 60 VDC		
Basic pump	alkalinity and basic condition	pH On-Off control	AO4	Pbase	relay for switching voltages up to 60 VDC		
Aerator	aeration conditions	Time and DO On-Off control	AO5	BL01	relay for switching voltages up to 60 VDC		
Mixer	mixing conditions	Time On-Off control	AO6	MX01	relay for switching voltages up to 60 VDC		
free			AO7	-	relay for switching voltages up to 60 VDC		
NI 9472 - 8 Channel , 24 V Logic, 100 µs Sourcing C Series Digital Output Module							
Over flow alarm In	Detect overflow from bioreactor, safety tank		DIO	Level	6 – 30 V		

AI= analog input; AO= analog output; DO=digital input/output

Summary and outlook

Summary and outlook

Different features of the Simultaneous partial Nitritation, Anammox and Denitrification (SNAD) process are described in this thesis. Literature review and experimental work carried out in this research confirmed the suitability and sustainability of the SNAD process for the treatment of ammonium-rich wastewaters, characterized by a low biodegradable organic matter content, such as anaerobic digester effluents and old landfill leachate.

The community composition and spatial distribution of the SNAD biomass, used as inoculums of lab-scale reactors, were evaluated. The SNAD seed sludge, investigated in this study, was collected from an SBR for the digester supernatant treatment at the WWTP of Zürich, Switzerland, where a successful nitrogen and carbon removal was achieved. The SNAD sludge contained granules surrounded by a matrix of brownish flocs. Two kinds of granules (red and brown) could be distinguished with diameters between 0.5 and 2.0 mm.

The 16S rRNA gene and functional gene approach suggested this SNAD biomass had a diverse microbial community. The main groups of microorganisms were found to be ammonia-oxidizing bacteria of the N*itrosomonas europaea/eutropha* group, anaerobic ammonium-oxidizing bacteria of the "*Candidatus* Brocadia fulgida" type and denitrifying bacteria related to the betaproteobacteria *Thauera*, *Pseudomonas*, *Dechloromonas aromatic* and *Aromatoleum aromaticum*. Nitrite-oxidizing bacteria from the genus *Nitrobacter* were detected while no nitrite-dependent anaerobic methane oxidizing bacteria were found. The high microbial diversity detected, expecially in the ammonia-oxidizing- and denitrifying bacteria species, supported the hypothesis that in the SNAD process, carried out in an SBR, different macro- and micro-environments strongly affected the population structure, which contribute to the stability of the SNAD process.

FISH analysis of SNAD biomass samples clearly showed that long filamentous microorganisms were dominant in the outer layer of the granules, with single cells as well as aggregates trapped in a net of these filamentous bacteria. Filamentous microorganisms are considered to be responsible for the construction of web-like structures while bacteria such as *Thauera* spp. may contributes to the granular process, thanks to their abundant production of extracellular polymeric substances (EPS). Aerobic nitrifiers were located at the outside of the aggregates as single cells and in clusters, while anammox bacteria were abundant in the inner part of the granules in agreement with the expectation that the interior of the aggregates would be anoxic.

Then, a thorough characterization of the ammonium-rich wastewaters has been performed. The COD fractions were divided based on the solubility and the biodegradability in order to perform a proper COD fractionation, useful for biological process simulation models and evaluate the truly biodegradable COD to Nitrogen (COD_{bio}/N) ratio. The COD_{bio}/N is one of the key parameters to the applicability of the SNAD process, in order to allow an equilibrated co-existence of anammox and denitrifiers bacteria. In particular, due to its complex composition, the characterization of old landfill leachates required the combination of appropriate physical-chemical and respirometric methods. In this study the interference due to chemical compounds, present in the leachate, were taken into account during sampling, experimental analysis and data processing procedures.

The SNAD process was validated to treat both old landfill leachates and anaerobic digester effluents in a Sequencing Batch Reactor (SBR) under a sequence of micro-aerobic (oxygen-limiting) and anoxic conditions.

Referring to the characteristics of the reactor, the SBR configuration was chosen to carry on the SNAD process, as it provides efficient biomass retention (over 90% of biomass retention), ensuring the enrichment of very slow-growing microbial community, such as the aerobic and anaerobic autotrophic nitrifiers (AOB and

anammox bacteria). Further, both the SBR technology and granular biomass allow the co-existence of several bacterial populations. The dynamic operative conditions of SBRs can dictate different metabolic activity by sequencing each phase of the mixing, aeration and non-mixing/non-aeration pattern during each cycle, ensuring different macro-environmental conditions for bacteria (aerobic/anoxic conditions or high/low loading). On the other hand, bio-granulation technology have a compact microbial structure and a strong microbial stratification that develop different micro-environments from the outer to the inner layer of aggregates. Both of these technologies create substrate gradients that improve the system resistance to shock and toxic loadings, preventing inhibition effects.

With regards to operational conditions, special attention must be paid to SBR cycle pattern. In this study, the SBR cycle was conducted with a short filling phase, two reaction phases (a micro-aerobic and an anoxic one) and a short settling phase. From experimental results over the whole research, a stable and high-rate running partial nitritation process has been found to be the critical point of the SNAD process. Several conclusions on SBR cycle pattern have been drawn which lead to practical suggestions for the SNAD process implementation.

Subsequent batch tests at high temperature (T=30°C), simulating one cycle of the operation of the SBR, were conducted on synthetic wastewater and leachate with a nitrogen loading rate of 0.27 kgTAN $m^{-3}d^{-1}$ and organic loading rate of 0.30 kg $COD_{filtrate}$ $m^{-3}d^{-1}$. Treating leachate ($COD_{bio}/N=0.2$), a total ammonium nitrogen and a total nitrogen removal efficiencies respectively of 76-87% and 64-79% were observed. A carbon (COD) removal efficiency of 34% was achieved. The efficiencies were lower than the values obtained using the synthetic wastewater, due to the presence of inhibitors compounds in the leachate for the aerobic ammonia oxidizing bacteria and to the lower biodegradable organic carbon content which reduces the overall total nitrogen and COD efficiencies removal. This study was carried out as an initial step towards development of a continuous SNAD process performed in an SBR under oxygen limited conditions for treating old landfill leachates. Influent characteristics, biomass activities and the operative reactor conditions (i.e. pH, DO, T) influenced the SBR configuration set up. In this study, a good relation between the SBR reaction phases lenght and the nitrogen loading rates was determined, in order to achieve a suitable partial nitrification during the micro-aerobic phase followed by a proper anammox process during the anoxic phase.

Then, the integration of the partial aerobic ammonia oxidation (nitritation), anaerobic ammonium oxidation (anammox) and heterotrophic denitrification via nitrate and nitrite was successfully developed in a lab-scale sequencing batch reactor (SBR) treating a syntetic wastewater with a COD_{bio}/N in the range of 0.4 -0.9.

The effects of high (T=30°C) and moderate (T= 25°C) temperature were evaluated. The Nitrogen Loading Rate (NLR) was set within 0.13-0.15 $kgNm^{-3}d^{-1}$ while the applied Organic Loading Rate (OLR) was between 0.08-0.14 $kgCODm^{-3}d^{-1}$. The HRT was ~3 d. At 30°C, the total ammonia removal and the total nitrogen efficiencies were higher than 98% and 93%, respectively. The COD removal was also high (81%). When the lower temperature was applied, the system was stabilized after ~10 days (3-4 times of the HRT). At 25°C, after a necessary acclimatization period to the lower temperature, the reactor was operated in a stable manner, achieving nitrogen and carbon removal efficiency comparable to the performance of the SNAD process at 30°C.

Then, the feasibitilty to treat anaerobic digester effluents at moderate temperature (T=25°C), was investigated. An ammonium conversion efficiency of 96%, a total nitrogen removal efficiency of 88% and COD removal efficiency of 58% were obtained with the nitrogen and COD loading rate of 0.15 kg Nm^{-3} d^{-1} and 0.08 kg $m^{-3}d^{-1}$, respectively. A mean nitrogen removal rate of 0.13 g $NL^{-1}d^{-1}$ was registered. The SNAD sludge in the

reactor was gradually adapted to the anaerobic digester effluent. Nevertheless, treating the anaerobic digester effluent at moderate temperature, the contribution of the nitrite oxidizing bacteria (NOB) increased. Under those conditions, both the oxygen-limiting conditions and a proper control of the Free Ammonia (FA) levels seemed to be the key factors of the NOB inhibition. Thus, in order to achieve a stable partial nitritation in a SNAD process, besides the effect of DO concentration on the nitritation reaction, a proper control of the FA would be necessary, considering a tolerable FA range of 4-8 mg NH_3-N L^{-1} (lower than the minimum inhibition threshold reported for AOB and in the range of the inhibitory values reported for the NOB). In this study it was found that, a regulation of the FA concentration at the beginning of the SBR cycles within a favorable range may be a vital factor in inhibiting NOB and assuring a stable SNAD process, even at moderate temperature.

A stoichiometric model was developed in order to evaluate the contribution of each biological process involved in the SNAD process during the micro-aerobic and anoxic phases of the SBR. The results of the stoichiometric modeling indicated that, during the micro-aerobic phase, nitritation was the prevalent TAN removal process (40 – 56%). The anammox process was showed to contribute to the TAN removal efficiency in very low percentage (5 – 7%) in micro-aerobic phase. On the contrary, during the anoxic phase, anammox process was the prevalent process, contributing 23 – 35 % to the total TAN removal. The influent carbon-nitrogen ratio governed the contribution of denitrification over the anammox process in the anoxic phase.

During the whole experiment, the oxidation-reduction potential (ORP), pH, conductivity (EC) and dissolved oxygen (DO) signals were continuously monitored and recorded. The on-line parameters profiles were well related to the ongoing biological processes in the SNAD reactor. Breaking point and blending points were also detected in the on-line parameters profiles, useful to monitor and control the SNAD process. With the beginning of the anoxic–mixed filling phase, EC and pH increased while ORP decreased. Then, with the beginning of the micro-aerobic phase, pH and EC exhibited a decreasing trend while the ORP increased gradually. When the aeration was switched off, the ORP showed a sharp decrease whereas the pH increased steadily due to biological conversion processes. The EC continued to decrease slowly. A blending point in the ORP profile in the anoxic phase and the amplitude of the ORP after the blending point were correlated with the complete depletion of nitrite mainly by anammox process and with the denitrification process via nitrate, respectively.

The analysis of the on-line parameters profiles allowed to develop and successfully applied a “real-time control” strategy mainly based on the conductivity and oxidation-reduction potential signal. The “real-time control” strategy can automatically determine the duration of the filling and reaction phases of the SNAD-SBR, optimizing and reducing the aeration and mixing time for saving energy source. The “real-time control” strategy allowed to reach high nitrogen and carbon removal efficiency, comparable to the efficiency measured during the “fixed time control” strategy. Further, it allowed to avoid inhibition of the process, due to nitrite accumulation, high load, complete substrates depletion. A low supervision was required, thus reducing operational costs.

Experimental procedures to investigate the potential activity of each processes involved were defined. Activity assays were conducted in order to determine the potential activities of aerobic ammonia- and nitrite-oxidizing bacteria (AOB and NOB), anaerobic ammonium oxidizing bacteria (anammox) and denitrifying bacteria, considering both denitrification via nitrate and via nitrite. The procedures were employed to evaluate the activity of the single biological processes involved in the SNAD process, minimizing the interference of other biological processes that could simoultaneously occurr. The results of these assays can be useful to monitor the SNAD biomass activity during a running experiments. Further, a new primer set targeting the hao/hzo gene was designed and successful applied for the identification of the anammox bacteria.

The findings of this research showed that, under steady-state condition, nitritation, anammox, denitrification and nitratation co-existed in the SBR, synchronizing each other and establishing a relation, which depended on the operative reactor conditions. The cooperation among differen bacteria, with opposite environmental conditions, was considered to be responsible for simultaneous nitrogen and COD removal. The shock in the operating temperature, DO, C/N, nitrogen loading rate of the SNAD system greatly affected the relation of these processes. However, the system is characterized by a high resistance and after short-term shock events always restored its performance after 3-4 times of the HRT.

According to the operation experience on the SNAD-SBR process, it is suggested that a suitable start-up strategy to achieve the SNAD process at moderate temperatures could have two steps. The first one would be the stable SNAD process at high temperature (T=30°C), in order to select the AOB by high temperature or other optimized conditions, such as oxygen limitation and other NOB inhibitors (FA). Then, the second step would be the adaptation of the SNAD biomass to the lower temperatures. Further, a gradual acclimatation of the SNAD process to the treatment of the real wastewater is proposed, by filling a mixtute (%v/v) of a synthetic and a real wastewater.

The SNAD process is a sustainable process for the treatment of ammonium-rich wastewater, characterized by a low biodegradable organic matter content and represents an interesting solution to enhance the treatment of the urban wastewater streams (Verstraete and Vlaeminck 2011; Winkler et al. 2012a) as well as side-streams (Jetten et al. 1997; Joss et al. 2009; Wang et al. 2010; Daverey et al. 2012).

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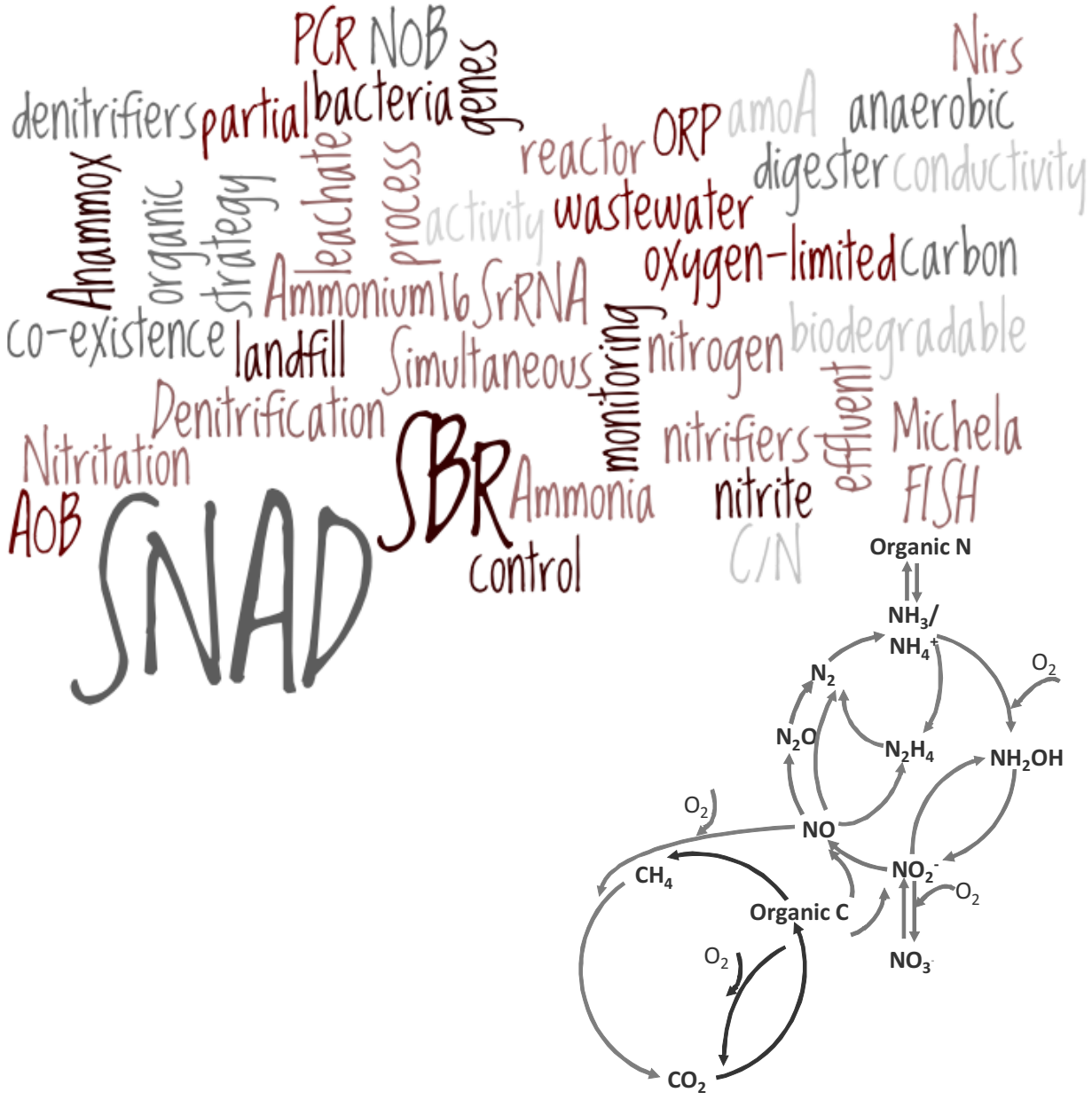
Curriculum vitae



Michela Langone was born on the 5th of April 1982 in Cava dei Tirreni, Italy. In 2006, she received the master's degree in Environmental Engineering from the University of Naples, Italy. During her master, she worked at Italian National Agency for New Technologies, Energy and Sustainable Economic Development (ENEA). Here, she studied the Microbial Fuel Cell, a new approach of wastewater treatment with electric power generation, under the supervision of dr. eng. Andrea Giordano and dr. Roberto Farina (ENEA) and Prf. Francesco Pirozzi (University of Naples). She spent the following years in an environmental engineering company, Envis Srl, where she worked as a consultant engineer. In 2009, Michela Langone started her PhD research at the University of Trento under the supervision of Prof. dr. eng. Gianni Andreottola, at the department of Environmental Engineering. Her research focuses mainly on the interactions between nitrifying, denitrifying and anammox communities and the application of those processes in the nutrient and carbon removal from wastewater streams. She has presented and discussed the results obtained during her research at various national and international scientific meetings.

Publications & relevant work

Journal Articles

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Invited speaker

Langone M., Andreottola G. May 2011. "*Trattamenti innovativi per la rimozione dell'azoto"*. Conference "Soluzioni e tecnologie Ladurner per il territorio", organized by Durner. 19 May 2011, Torino (IT).

Andreottola G., **Langone M.**, 2011. "*Processi Biologici Innovativi per la rimozione dell'azoto dai surnatanti della digestione anaerobica"*. Rapporti G.S.I.S.R. N. 251 – 02/11. Conference "Settimana Ambiente 2011 - Depurazione delle Acque e Trattamento dei Fanghi Aspetti gestionali e nuove tecnologie". Milano (IT), 25 February 2011.

Master Thesis Co-Supervisor

Completed

Burli, R. 2013. (Environmental Engineering). *Trattamento del percolato di discariche mediante il processo di Simultanea parziale Nitritazione, Anammox e Denitrificazione*. Supervisor: Andreottola G.

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