Università degli Studi di Cagliari

PHD DEGREE

Earth and Environmental Sciences and Technologies

Cycle XXXI

AUTOTROPHIC NITROGEN REMOVAL FROM HIGH AMMONIUM CONTAINING WASTEWATER

Scientific Disciplinary Sector:
ICAR/03 – Sanitary and environmental engineering

PhD Student	Giaime Tocco
Coordinator of the PhD Programme	Prof. Aldo Muntoni
Supervisors	Prof. Alessandra Carucci Dr. Stefano Milia (IGAG-CNR)

Final exam. Academic Year 2017 – 2018
Thesis defence: January – February 2019 Session

Abstract

The present thesis is framed within a wider project, promoted by the University of Cagliari and the Italian National Research Council, which pursued as its general objective the development of a novel integrated system for the biological combined anaerobic production of H_2 and CH_4 from the organic fraction of municipal solid waste (OFMSW), the valorization of solid residues by aerobic bio-oxidation (composting) and the biological treatment of ammonium-rich liquid by products by two-stage partial nitritation (PN)/anaerobic ammonium oxidation (anammox) process.

According to the project plan, a biological combined anaerobic production of H_2 and CH_4 from the OFMSW took place in two different bioreactors, in a double-step configuration. The effluent from the second reactor underwent a liquid/solid separation. The assessment of feasibility of the treatment of the liquid fraction by means of two-stage autotrophic nitrogen removal was the research question that tracked the main investigation line of the work described in the present thesis. The application of the coupled PN/anammox process, also referred to as fully autotrophic nitrogen removal, would allow to increase the sustainability of the entire OFMSW treatment chain, since the need for carbon addition (and concomitant increased sludge production) is omitted, oxygen consumption (i.e., energy requirement) is reduced, and the emission of nitrous oxide (a significant factor in the greenhouse gas footprint of the total water chain) can be cut significantly.

To date, few researches focused on the application of partial nitritation/anammox process for the treatment of the liquid fraction originated by the anaerobic digestion of organic substrates such as OFMSW. Moreover, the two-stage anaerobic digestion of such substrates is a relatively novel technology which has not deeply investigated yet in terms of its potential environmental impact, and few studies focused on characterization and management of the liquid residues.

To the best of our knowledge, no previous study was performed concerning the application of the two-stage PN/anammox process for the treatment of the anaerobic supernatant of a two-stage anaerobic digestion of organic fraction of municipal solid waste and food waste.

Table of Contents

Objectives and summary

The present thesis is framed within the field of wastewater treatment, and specifically in the field of nitrogen removal from high ammonium-containing wastewater originating from the anaerobic digestion of solid waste.

The discovery of anaerobic ammonia oxidation (anammox) organisms, more than 20 years ago, represented a revolution in biological nutrient removal (BNR) processes. Anammox metabolism allows the oxidation of ammonium in the absence of oxygen, using nitrite as final electron acceptor. Thus, a previous step to anammox is required in order to provide a partial aerobic oxidation of ammonium to nitrite, i.e. partial nitritation (PN).

The development of the coupled PN/anammox process, also referred to as fully autotrophic nitrogen removal, allowed to increase the sustainability of wastewater treatment as the need for carbon addition (and concomitant increased sludge production) is omitted and oxygen consumption is reduced, as well as the emission of nitrous oxide, which has become a significant factor in the greenhouse gas footprint of the total water chain. Such coupled process was first operated in two distinct reactors (two-stage PN/anammox); subsequently, simultaneous PN and anammox process was achieved in a single reactor.

Several different anammox-based technologies have been rapidly and widely investigated and developed, and almost 100 full-scale plants were established by the end of 2014. Also, many reactor configurations have been proposed, such as continuous stirred tank reactor, sequencing batch reactor, membrane bioreactor, etc.

Anammox-based treatments were applied to different influent streams, usually characterized by high ammonium content and low biodegradable organic matter concentration such as landfill leachate and supernatant from anaerobic digestion of waste sludge; more recently, the range of treated wastewater widened, including industrial wastewater from petrochemical, pharmaceutical, fish canning industry, as well as supernatant from anaerobic digestion and co-digestion of different organic substrates, such as organic waste originated from urban environment.

In Europe and Italy, several problems concerning the correct management of the organic fraction of municipal solid waste (OFMSW) are still far from being solved. The most applied treatment technologies, i.e. composting and anaerobic digestion (AD), are characterized by important weaknesses which make them not attractive as stand-alone processes. In particular, operative costs related to composting are not counter-balanced by incomes deriving from

entering the final product into the market; on the other hand, conventional anaerobic digestion (AD) of the OFMSW lacks in terms of process stability due to the intrinsic heterogeneity of organic residues, the difficulty of hydrolysis of solid and complex substrates in the early stage of the process, the need for water recirculation in wet systems and the high ammonium concentration (>500 mgNH_4-N/L) in the final digestate, which is unsuitable to be treated in conventional wastewater treatment plants. Therefore, it becomes essential to look at the management of the OFMSW from a wider perspective.

Within such framework, the HyMeCA (Hydrogen Methane Compost Ammonia) project was promoted by the Department of Civil-Environmental Engineering of the University of Cagliari (DICAAR) and by the Institute of Environmental Geology and Geoengineering of the Italian National Research Council (IGAG-CNR), and funded by Autonomous Region of Sardinia (Law 7/2007) in order to pursue, as its General Objective, the development of a novel integrated system for the biological combined anaerobic production of H_2 and CH_4 from the OFMSW (and particularly from food waste), the valorization of solid residues by aerobic bio-oxidation (composting) and the biological treatment of liquid byproducts by an advanced process based on double stage partial nitritation/anaerobic ammonium oxidation.

The biological combined anaerobic production of H_2 and CH_4 from the OFMSW took place in two different bioreactors, in a double-step configuration, under mesophilic and wet conditions; in particular, the effluent from the hydrogenogenic reactor (first step) was fed to the methanogenic reactor (second step). The effluent from the methanogenic reactor underwent a liquid/solid separation; the solid fraction was addressed to a different research group, which investigated the valorization of such residue through aerobic stabilization (composting), while the assessment of feasibility of the treatment of the liquid fraction by means of two-stage autotrophic nitrogen removal was the research question that tracked the main investigation line of the work described in the present thesis.

To date, few researches focused on the application of partial nitritation/anammox process to the treatment of the liquid fraction originated by the anaerobic digestion of organic substrates such as OFMSW, food waste (FW) or kitchen waste (KW). Moreover, the two-stage anaerobic digestion of such substrates is a relatively novel technology which has not been deeply investigated yet, and few studies focused on characterization and management of the liquid residues.

To the best of our knowledge, no previous study was performed concerning the application of the two-stage PN/anammox process for the treatment of the anaerobic supernatant of a two-stage anaerobic digestion of organic fraction of municipal solid waste and food waste.

On the basis of all the aforementioned, the feasibility of the proposed treatment was first preliminarily evaluated by starting and operating a partial nitritation continuous flow stirred tank reactor (CSTR) and a granular anammox sequencing batch reactor (SBR) using a synthetic influent, in order to determine the best operating conditions. In a second phase, the target wastewater, i.e. the supernatant originating from the two-stage AD of FW, was fed into the PN/anammox process; the experiment aimed at the identification and adjustment of the key operational parameters, and at a general assessment of the feasibility of the process.

The main content of each chapter of the present thesis will be detailed in the following sections.

In Chapter 1, a literature survey concerning the management of OFMSW and FW in Italy and the management of liquid residues is reported, together with an insight on the two-stage AD aimed at hydrogen and methane production, and the difference in supernatant characteristics.

In Chapter 2, the partial nitritation process and the anammox process are described, with particular concern to inhibiting factors that might have been present in the target wastewater. A survey on different applications of anammox-based technologies is also provided.

In Chapter 3, the experimental activity aimed at the preliminary evaluation of the feasibility of the proposed treatment is described and the results reported. A presentation synthesizing the work, accompanied by a poster, was presented at the Frontiers International Conference on Wastewater Treatment and Modelling (FICWTM2017) which took place in Palermo (Italy) on May, 2017. A full paper was then published on the special issue of Lecture Notes in Civil Engineering book series, (Springer).

Part of the experimental activity carried out using the synthetic influent was aimed at the assessment of nitrous oxide (N_2O) emissions from the partial nitritation reactor. Results from this experiment were presented at the 15th International Conference on Environmental Science and Technology (CEST2017) which took place in Rhodes (Greece) on September, 2017. In Chapter 4, aim, methodology and results of the experiment are described. Starting from the data collected in the experiment, a full paper was also written and submitted to Desalination and Water Treatment journal for peer review, and recently published online.

In Chapter 5, the experimental activity carried out using the real target wastewater is described and results reported. Main issue faced during the experiment concerned the adjustment of the

operational parameters in order to produce a suitable effluent, especially in terms of nitrite to ammonium molar ratio. Different solutions were proposed and tested. Such issue directly affected the subsequent anammox treatment: two solutions were proposed, one of which was, to the best of our knowledge, experimentally tested only once. Moreover, a novel biomass characterization based on the digital measurement of biomass color was tested as potential quick, simple and cost-effective indirect measurement of process performance, metabolic activity and biomass enrichment. Such technology was recently proposed and tested on biomass fed with synthetic influent only; no previous application of this approach was reported on biomass operating with real wastewater. This resulted in a specific biomass preparation and measurement protocol that was elaborated and tested in order to evaluate the potential influence of the presence of dyed real wastewater on biomass color characterization.

A research stay abroad was conducted during the PhD course, primarily aimed at achieving direct experience in operating a single-stage PN/anammox system. Thus, a four-month stay, from January to May, 2018, was carried out at the University of Santiago de Compostela (Spain), under the supervision of Prof. Anuska Mosquera Corral. During such productive period, an experiment was planned and performed in the framework of a research project co-funded by European Commission and Government of Galicia (Spain). In the experiment, two lab-scale single-stage PN/anammox SBRs were operated at room temperature, and the effects of repeated prolonged starvation-reactivation alternating periods on process performance and biomass characteristics were assessed. Background, methodology and results of the experiment are reported in Chapter 6.

Chapter 1

Nitrogen emissions related to anaerobic digestion of food waste

1.1 Organic fraction of municipal solid waste (OFMSW) and food waste (FW) in Italy: production and management

Production of municipal solid waste in Italy accounted for 30.1 million tons (Mt) in 2016, corresponding to a +2% increase with respect to 2015. Sorted waste accounted for 52.5% of the total collected, and organic fraction (i.e., food waste, FW, and "green" waste, GW - garden cuttings, hedge trimmings, fallen leaves, dead plant matter, etc.) represented 41% of them, i.e. ~6.5 Mt [1].

Composting, integrated anaerobic/aerobic treatment and anaerobic digestion are the most common biological treatment applied to both municipal and industrial organic waste in Italy. Almost 5.7 Mt of OFMSW were sent to biological treatments in 2016 (+10% with respect to 2015). Composting represented the most used technology for the treatment of OFMSW (3.4 Mt: FW, 1.95 Mt; GW, 1.44 Mt), which provided 82.3% of total composted waste [1]. Composting is an energy-consuming technology aimed at the recycling of organic matter by producing valuable products such as fertilizers and soil improvers; its economic and environmental sustainability as the main process for organic waste recovery and valorization has been recently revised. In a recent study [2] technical, economic, and environmental aspects of composting and anaerobic digestion (AD), and their potential to improve the sustainability of waste management, were examined: authors concluded that AD is environmentally favorable in terms of lower greenhouse gas (GHG) emissions due to production of biogas as a renewable energy source, while in composting, no single aeration scheme or additive has been found to be effective in reducing odor and GHG emissions simultaneously; moreover, AD was considered economically more advantageous than composting, depending on plant scale and valorization of end products, while composting appeared more profitable at smaller scales (e.g. <20,000 t). Therefore, AD may be favored for centralized treatment. Such results confirmed those reported in another 2006 study [3].

The number of AD plants in Italy constantly increased in the last decade. By 2015, 46 AD plants were realized with a total authorized capacity of 2 Mt; most plants (26) integrate AD with (post)

composting of digestate (i.e., the mixed liquid/solid residual fraction of the anaerobic digestion treatment) [4]. Such integrated aerobic/anaerobic waste treatment plants increased in number up to 31 in 2016, with a total authorized capacity of 2.5 Mt: major contribution came from FW, with ~1.9 Mt treated (81% of the total) [1]. Anaerobic digestion as solely waste treatment technology was lesser used in Italy, treating 0.89 Mt in 2016; OFMSW accounted for 0.25 Mt, 36.3% of the total, while the rest of the inflow was mostly constituted of waste sludge (WAS) from municipal wastewater treatment plants [1].

Waste treated through anaerobic digestion altogether, both as stand-alone treatment and coupled in an anaerobic/aerobic integrated process, amounted to 2.97 Mt in 2016: as depicted in Figure 1.1, food waste largely represented the major contribution, and OFMSW overall accounted for 81% of total [1].

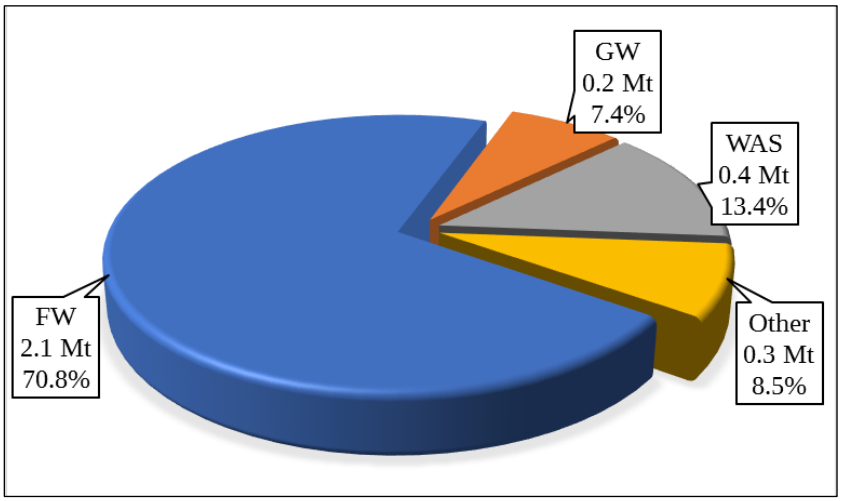

Figure 1.1: Variety of waste treated by AD, both as only treatment and coupled with aerobic post treatment, in Italy, year 2016. Elaboration from 2017 ISPRA official data [1].

In conclusion, to date organic matter from MSW is still mainly (~59%) recovered and valorized through composting, in particular regarding its lignocellulosic fraction (green waste); on the other hand, anaerobic digestion utilization for biogas and biomethane production from organic waste increased during last years (+33% from 2015 to 2016). This is reflected in the recent efforts made to improve AD efficiency, optimize process economics, and mitigate adverse environmental impacts: the number of publications regarding AD has increased dramatically in the past decade, while publications on composting have remained at a similar level [2].

1.2 One-stage and two-stage anaerobic digestion of organic waste

In current applications of anaerobic digestion (AD) systems, organic matter is converted into a mixture of gaseous compounds, mainly methane (CH_4) and carbon dioxide (CO_2), via acid fermentation and volatile fatty acids (VFAs) degradation, and through the activity of two groups of microorganisms: acid-forming and methane-forming bacterial biomass, respectively [5].

In a single-reactor system, namely one-stage anaerobic digestion, those microorganisms are kept together in a balance, which is delicate, because both groups differ widely in terms of physiology, nutritional needs, growth kinetics, and sensitivity towards environmental conditions [5]. Considering these aspects, the two-stage AD system, where the sub-processes organic matter hydrolysis and its fermentation to organic acids are physically separated from the methane production process, was proposed since many years [5].

In two-stage AD systems, the physical separation of the reactors responsible for the two independent processes enables optimal conditions for the acidogenic and the methanogenic bacterial biomass to be established, thus optimizing specific metabolic activities and ultimately maximizing methane generation [6]. Moreover, the issue of operating AD in the two-stage configuration has become topical in recent years as a result of the interest aroused by the possibility of producing bio-hydrogen from organic substrates [7].

As alternative energy carriers to fossil fuels, hydrogen represents one of the most attractive solution because of its high energy content, environmental friendliness (as the only final by-product of its combustion is water), and also because it can give substantial social, economic and environmental credentials [8].

The major challenge in the use of this promising energy carrier lies in its sustainable production and storage. In commercial applications, hydrogen has been produced from natural gas, oil, and by other industrial methods which are highly energy intensive and use non-renewable sources of energy which makes them less attractive from an environmental point of view [8]. Alternatively, hydrogen can be produced from biological processes which are less energy intensive and more environment-friendly, i.e. in terms of global reduction of CO_2 [8].

Biohydrogen can be produced by both autotrophic and heterotrophic microorganisms: in autotrophic conversions (also known as direct or indirect biophotolysis), solar energy is directly converted to hydrogen via photosynthetic reactions mediated by photosynthetic microorganisms, i.e. microalgae, protists and photosynthetic bacteria, while in heterotrophic conditions the organic substrates are transformed into simpler organic compounds with

simultaneous production of molecular hydrogen [8]. There are two types of heterotrophic conversions: photo-fermentation carried out by photosynthetic bacteria and dark fermentation (DF) carried out by anaerobic bacteria that convert carbohydrates into biohydrogen [8].

In dark fermentation, the produced H_2 is recoverable, provided that a harsh environment for hydrogenophylic methanogens is guaranteed [5]; in addition, a mix of volatile fatty acids (VFAs) and reduced end products including alcohols is generated as well. Thus, the second anaerobic digestion stage for CH_4 production, although mostly applied, is not the only viable option as the subsequent treatment phase downstream of DF. Alternative paths may possibly include: a photo-fermentation stage aimed at H_2 production; a microbial electrolysis cell devoted to H_2 production; a microbial fuel cell for direct electricity generation; a biochemical stage for biopolymer production [5].

Two-stage AD aimed at the sequential H_2 and CH_4 production is, from a theoretical point of view, energetically more favourable than single-stage AD [5]. Second generation biomass sources, such as waste biomass, are abundant and can thus support the supply of renewable substrates for DF [8]; in particular, organic fraction of municipal waste (OFMSW), generally constituted of food waste (FW), containing a high biodegradable carbohydrates fraction, represents a good substrate for DF [8]. Thus, FW has been used extensively in DF experiments [7].

DF followed by AD has shown technical and economic feasibility of the integrated process up to pilot scale [8]. According to Schievano et al. [13], several studies eventually confirmed the supremacy of two-stage DF+AD process on single-stage AD in terms of energy recover at both thermophilic and mesophilic conditions; however, overall advantages of DF+AD in comparison with single-stage AD, concerning effluent stream and its subsequent treatment, are still investigated.

In a recent study, De Gioannis et al. [5] performed several one- and two-stage anaerobic digestion batch tests of a standardized food waste, aimed at recovering methane and hydrogen plus methane, respectively, in order to achieve a direct comparison between the two configurations and assess the benefits associated with the two-stage approach in terms of overall energy recovery. The results obtained suggest that a two-stage process where the first reactor is properly operated in order to achieve a significant net hydrogen production, may display a 20% comparatively higher energy recovery yield as a result, mainly, of enhanced methane generation, as well as of the associated hydrogen production [5]. With regard to the digestate characteristics, the 25% increase in volatile solids removal achieved in the two-stage anaerobic

digestion system (66.7% VS removal vs. 53.3%) implied a higher degree of digestate stabilization [5].

Different results were reported in a 2012 study by Schievano et al. [9]. The authors compared a two-stage (DF+AD) and one-stage AD lab-scale CSTR systems, fed with identical organic substrates (mixture of swine manure and market biowaste) and loading rates. Despite no significant differences in overall energy recovery were found for the two-stage and one-stage, the chemical characterizations suggested remarkable dissimilarities between the two AD systems: in particular, the two-stage process seemed to be slightly less efficient in degrading organic matter and this was linked to a partial inefficiency of the methanogenic reactor of the two-stage process. Digestate from single-stage AD system showed total VFA concentrations approximately 10 times lower than those in DF+AD; moreover, while no significant difference was measured for TN and NH_4 content in liquid fraction, slightly lower alkalinity/N ratio was observed in two-stage effluent stream.

Cavinato et al. [10] studied the optimization of a two-phase DF+AD thermophilic process treating biowaste, carried out at pilot scale using two stirred reactors (CSTRs). Fed biowaste consisted of ground OFMSW diluted with water. Effluent stream was characterized by a relatively high content in VFA (90-650 mg/L) and a total alkalinity/TKN molar ratio around 1.3.

In conclusion, two-stage AD aimed at separated biohydrogen and biomethane production was proved to represent a valid solution for the valorization of second generation biomass source, namely organic waste such as food waste, agricultural waste, etc. During dark fermentation, enhanced hydrolysis and fermentation of organic matter produce a high-concentrated stream of mixed volatile fatty acids (VFAs) and reduced end products including alcohols; although such compounds may potentially enhance methanogenic activity, environmental conditions in AD reactor should be strictly controlled, in order to avoid inhibition of methanogenic bacteria, due to low pH/acid or to the accumulation of toxic compounds such as alcohols or aromatic compounds (i.e., phenols). An (even partial) inhibition of methanogenic activity may result in a certain amount of relatively rapidly degradable organic matter left undegraded in the effluent stream, and also to lower alkalinity level [9]. Such characteristics are to be taken into account when coupled partial nitritation (PN)/anammox treatment is proposed for nitrogen removal from supernatant originating from two-stage AD of OFMSW.

1.3 Nitrogen content and management of the liquid fraction from AD of OFMSW

The main advantages of AD include energy generation through the use of biogas and the production of an effluent stream that is rich in nutrients and can be suitably exploited, under certain conditions [11]. Use of the digested effluents in agriculture as organic fertilizer according to the current legislation, e.g. the nitrate directive (91/676/EEC, The Council of the European Communities, 1991), and based on crop needs is advised [12], but this management strategy may be limited by factors like transport requirements, water content, or presence of heavy metals and pathogenic microorganisms [13]; moreover, the main criticality in digestate final use is nitrogen release into the environment (up to 30% of ammonia nitrogen can be lost by volatilization, due to an enhancement in soil pH) [14]. Consequently, adequate post-treatment is required in order to remove nutrients from the anaerobic effluent [11]. However, the management of nitrogen dosage is sometimes difficult because of the variability of the feedstock, as the composition of whole digestate is mainly influenced by the input materials [14,15].

The anaerobic effluent usually undergoes a solid/liquid separation process, thus creating two different streams: the solid stream and the liquid waste stream that is known as the anaerobic supernatant [11]. As stated in section 1.1, most of the OFMSW treated through AD is food waste (FW), which is characterized by low C/N ratios (~10) and low pH (4−5), and high amounts of soluble organic matter that can be easily converted to intermediates. FW mainly consists of carbohydrates, fat, and protein [2]. The anaerobic digestion of such substrates results in the transfer of nutrients from the solid to the liquid phase. Consequently, the resulting anaerobic supernatant is characterized by very high ammonium nitrogen concentrations [11]. Direct discharge of the anaerobic supernatant to the municipal sewers is not an option as it would unbalance the chemical oxygen demand / total Kjeldahl nitrogen / total phosphorus (COD/TKN/TP) ratio of municipal wastewater [16].

In the literature, there are few studies that examine the characteristics and post-treatment of the anaerobic supernatant produced from the treatment of OFMSW. According to the in-depth study by Malamis et al. [11], which represents the main source regarding this topic, the characteristics of the supernatant produced from the anaerobic treatment of the OFMSW and from the co-digestion of OFMSW with other biodegradable organic waste streams (waste sludge, agri-food industrial waste) depend on the substrates compositions, on the operating conditions of the anaerobic digestion process and on the efficiency of the solid/liquid separation

process. Despite the heterogeneous nature of OFMSW and the fact that the operating conditions of the anaerobic processes employed by researchers are different, common characteristics arise:

- the anaerobic supernatant is characterized by very high NH_4-N content (0.5-7.5 gN/L) and high COD/biochemical oxygen demand (BOD_5) ratio (2-6). The high COD/BOD_5 ratio is characteristic of effluents from fermentation processes showing that effective biodegradation has taken place in the digester;
- in most cases, anaerobic supernatant is also characterized by high ortho-phosphate and total phosphorus concentration. Nutrient levels are high as the feed to the anaerobic digester is usually rich in nutrients and at the same time the anaerobic digestion process cannot effectively remove nutrients;
- the ratio of BOD_5/TKN is low (~1) and thus the biological nitrogen removal through the conventional process of heterotrophic denitrification requires the addition of readily biodegradable organic matter;
- alkalinity in the supernatant from anaerobic digestion of OFMSW can vary significantly depending on the hydraulic retention time (HRT), the temperature and the characteristics of the feed waste. The molar ratio of alkalinity to TKN is usually low (<1.5-2); thus, the effluent alkalinity is not usually sufficient to achieve complete nitrification.

With regard to nitrogen removal/recovery from the supernatant, various physicochemical and biological processes are reported [15]. The usual physicochemical processes include:

- ammonia stripping;
- struvite precipitation;
- membrane filtration processes (ultrafiltration or microfiltration potentially coupled with reverse osmosis);
- evaporation.

In general, biological processes are less expensive than the physicochemical ones and have the advantage that nitrogen is removed, i.e. converted into a gaseous form [11].

1.3.1 Biological treatment of the anaerobic supernatant originating from the treatment of OFMSW

In the past years, both suspended and attached growth systems have been employed at different scales (lab, pilot and full) for the treatment of supernatant produced by AD of OFMSW and FW, including continuous flow stirred tank reactors (CFSTRs), sequencing batch reactors

(SBRs), membrane bioreactors (MBRs), conventional activated sludge (CAS) processes, moving bed biofilm reactors (MBBRs) and their combinations (i.e. MBBR–SBR) [11].

The anaerobic supernatant contains a significant amount of organic compounds that cannot be degraded under anaerobic conditions. The post-treatment of the supernatant through aerobic biodegradation can remove several of these compounds [11], even though some recalcitrant compounds such as humic acids resist to degradation [14].

The biological processes employed for nitrogen removal from the anaerobic supernatant produced by the treatment of OFMSW include: (i) conventional nitrification/denitrification, (ii) short-cut nitrogen removal through partial nitrification (i.e., nitritation) combined with heterotrophic denitritation and (iii) the completely autotrophic nitrogen removal process, i.e. combined (partial) nitritation and anaerobic ammonium oxidation (anammox) [11].

Compared to completely autotrophic nitrogen removal, nitritation/denitritation may be more robust and reliable as it is less sensitive to environmental and operating parameters [17]; in addition, coupled to certain short-chain carbon sources, it may allow the contemporary denitrifying phosphorus removal via-nitrite (DPRN), e.g. via external addition from fermentation liquids [17]. Thus, as documented in literature, nitritation/denitritation is to date the most common biological treatment process for the removal of nitrogen from supernatant produced by AD of OFMSW [11], while the only application of anammox bacteria is reported by Caffaz et al. [16].

1.4 References

[1] ISPRA, Rapporto Rifiuti Urbani - Ed. 2017 - Rapporto 237/2017, Roma, 2017.

[2] L. Lin, F. Xu, X. Ge, Y. Li, Improving the sustainability of organic waste management practices in the food-energy-water nexus: A comparative review of anaerobic digestion and composting, Renew. Sustain. Energy Rev. 89 (2018) 151–167. doi:10.1016/j.rser.2018.03.025.

[3] J.D. Murphy, N.M. Power, A technical, economic and environmental comparison of composting and anaerobic digestion of biodegradable municipal waste, J. Environ. Sci. Heal. - Part A Toxic/Hazardous Subst. Environ. Eng. 41 (2006) 865–879. doi:10.1080/10934520600614488.

[4] M. Centemero, Italian composting and anaerobic digestion report - 2016, Rome, 2016. doi:10.13140/RG.2.1.4578.6485.

[5] G. De Gioannis, A. Muntoni, A. Polettini, R. Pomi, D. Spiga, Energy recovery from one- and two-stage anaerobic digestion of food waste, Waste Manag. 68 (2017) 595–602. doi:10.1016/j.wasman.2017.06.013.

[6] A. Schievano, A. Tenca, S. Lonati, E. Manzini, F. Adani, Can two-stage instead of one-stage anaerobic digestion really increase energy recovery from biomass?, Appl. Energy. 124 (2014) 335–342. doi:10.1016/J.APENERGY.2014.03.024.

[7] G. De Gioannis, A. Muntoni, A. Polettini, R. Pomi, A review of dark fermentative hydrogen production from biodegradable municipal waste fractions, Waste Manag. 33 (2013) 1345–1361. doi:10.1016/j.wasman.2013.02.019.

[8] A. Ghimire, L. Frunzo, F. Pirozzi, E. Trably, R. Escudie, P.N.L. Lens, G. Esposito, A review on dark fermentative biohydrogen production from organic biomass: Process parameters and use of by-products, Appl. Energy. 144 (2015) 73–95. doi:10.1016/j.apenergy.2015.01.045.

[9] A. Schievano, A. Tenca, B. Scaglia, G. Merlino, A. Rizzi, D. Da, R. Oberti, F. Adani, Two-Stage vs Single-Stage Thermophilic Anaerobic Digestion: Comparison of Energy Production and Biodegradation Efficiencies, Environ. Sci. Technol. 46 (2012) 8502–8510. doi:10.1021/es301376n.

[10] C. Cavinato, D. Bolzonella, F. Fatone, F. Cecchi, P. Pavan, Optimization of two-phase thermophilic anaerobic digestion of biowaste for hydrogen and methane production through reject water recirculation, Bioresour. Technol. 102 (2011) 8605–8611. doi:10.1016/j.biortech.2011.03.084.

[11] S. Malamis, E. Katsou, S. Di Fabio, D. Bolzonella, F. Fatone, Biological nutrients removal from the supernatant originating from the anaerobic digestion of the organic fraction of municipal solid waste., Crit. Rev. Biotechnol. 8551 (2013) 1–14. doi:10.3109/07388551.2013.791246.

[12] R. Nkoa, Agricultural benefits and environmental risks of soil fertilization with anaerobic digestates: a review, Agron. Sustain. Dev. 34 (2014) 473–492. doi:10.1007/s13593-013-0196-z.

[13] A. Magrí, F. Giovannini, R. Connan, G. Bridoux, F. Béline, Nutrient management from biogas digester effluents: a bibliometric-based analysis of publications and patents, Int. J. Environ. Sci. Technol. 14 (2017) 1739–1756. doi:10.1007/s13762-017-1293-3.

[14] C. Pieroni, L. Maggioni, M. Gabini, F. Petracchini, F. Liotta, V. Paolini, D. A., S.P.

(CNR), S. Mannelli, M. Monni, B. Croce, K. Eroe, B. Croce, Deliverable D2.1: State of the art and best practices collection, 2016.

[15] W. Fuchs, B. Drosg, Assessment of the state of the art of technologies for the processing of digestate residue from anaerobic digesters, Water Sci. Technol. 67 (2013) 1984–1993. doi:10.2166/wst.2013.075.

[16] S. Caffaz, E. Bettazzi, D. Scaglione, C. Lubello, An integrated approach in a municipal WWTP: Anaerobic codigestion of sludge with organic waste and nutrient removal from supernatant, Water Sci. Technol. 58 (2008) 669–676. doi:10.2166/wst.2008.683.

[17] N. Frison, S. Di Fabio, C. Cavinato, P. Pavan, F. Fatone, Best available carbon sources to enhance the via-nitrite biological nutrients removal from supernatants of anaerobic co-digestion, Chem. Eng. J. 215–216 (2013) 15–22. doi:10.1016/j.cej.2012.10.094.

Chapter 2

Anammox-based technologies for ammonium-rich wastewater treatment

2.1 Introduction

Anaerobic ammonia oxidation (anammox) has been one of the most innovative developments in biological wastewater treatment in recent years [1]. In the anammox reaction, ammonium nitrogen is anaerobically oxidized to molecular nitrogen with nitrite used as the electron acceptor. Its impact on biologic nutrient removal (BNR) systems is still expanding.

Coupling of partial nitritation (PN) and anaerobic ammonia oxidation (anammox) was first tested by Jetten et al. in 1997 [2] using two different reactors, while in 2002 Sliekers et al. described the one-stage completely autotrophic nitrogen removal over nitrite (i.e., CANON) system [3]. Main advantages recognized to the complete autotrophic nitrogen removal (short, PN/anammox) over conventional nitrification/denitrification (N/DN) and short-cut biological nitrogen removal via nitrite (SBNR, also: nitritation/denitritation) are: no need for organic substrates; negligible waste sludge production (0.08 kgVSS/kgN, compared to ~1 kgVSS/kgN of conventional N/DN [4]); lower oxygen demand (1.9 kgO_2/kgN instead of 4.6 kgO_2/kgN of N/D [5]); reduced CO_2 emissions (actually, PN/anammox is a CO_2-consuming process [4]).

Within the last decade several technologies have been developed and successfully implemented in full scale, e.g. sequencing batch reactors, granular sludge reactors, and moving bed biofilm reactors. Early PN/A implementations used two-stage reactor configurations or made use of already existing nitritation systems (e.g. SHARON type reactors). With more full-scale experiences, focus has shifted mainly to single-stage systems. Until 2014, almost 100 full scale PN/anammox plants were established worldwide [1].

2.2 Partial nitritation: process description and influencing factors

2.2.1 Introduction

Nitrification process, i.e. the aerobic ammonia oxidation to nitrate, consists of two subsequent reactions carried on by different bacterial group. The first step is the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-) carried out by the ammonia-oxidizing bacteria (AOB), while the second

step is the oxidation of nitrite (NO_2^-) to nitrate (NO_3^-) carried out by the nitrite-oxidizing bacteria (NOB). Effective nitritation relies on stimulating the first step of nitrification while inhibiting the second step and by consequence accumulating ammonia-oxidizing bacteria (AOB). Successful AOB accumulation depends upon the knowledge of their microbial characteristics and kinetics parameters as well as the main parameters that can selectively inhibit NOB growth or allow AOB to outcompete them [6]. Moreover, anammox process requires an influent a NO_2^-/NH_4^+ molar ratio around 1.0-1.3 [5]: such influent is produced when the oxidation of ammonium to nitrite under aerobic conditions account for roughly 50-55% of initial ammonium, i.e. when partial nitritation process is achieved.

Several bioreactors configurations either in suspended or attached growth have been used towards achieving full and partial nitritation, using different operating conditions [6].

Microbiology and metabolism of ammonia-oxidizing bacteria have been extensively investigated in the past years [6–9]. To date, five AOB genera have been recognized and classified in the Proteobacteria class, four of which lies in the β-Proteobacteria subclass, while one cluster of *Nitrosococcus* belongs to the γ-Proteobacteria subclass [6].

Ammonia oxidation to nitrite (Eq. 2.1) is carried on by AOB within two steps with hydroxylamine (NH_2OH) as an intermediate product [6]. The first step is the oxidation of ammonia to hydroxylamine, catalyzed by the membrane bound ammonia monooxygenase (AMO) enzyme: this step requires a molecular oxygen and a pair of electrons. In the second step, hydroxylamine is further oxidized to nitrite catalyzed by the hydroxylamine oxidoreductase (HAO) enzyme using oxygen from water and an additional molecular oxygen as a terminal electron acceptor. This step generates two pairs of electrons, one pair of which is compensated for the support of the first step of ammonia oxidation, whereas the other pair is passed to the terminal oxidase via an electron transport chain, generating a proton motive force [9]. Such process serves as energy-yielding reaction for AOB which utilize ammonia as their sole source of energy. Besides being AOB energy source, part of the ammonia is used for their cell growth as nitrogen source while carbon dioxide serves as their chief carbon source (Eq. 2.2) [6,9]. The energy released as a result of (Eq. 2.1 is utilized in reaction (Eq. 2.2. The two can be combined to form an overall synthesis-oxidation reaction. Several different stoichiometries were proposed, depending on observed biomass yields [9]; one of the most used is reported in (Eq. 2.3 [10].

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + 2H^+ + H_2O + (58 \div 84)\ \text{kcal} \qquad \text{(Eq. 2.1)}$$

$$13NH_4^+ + 15CO_2 \rightarrow 10NO_2^- + 3C_5H_7O_2N + 23H^+ + 4H_2O \quad \text{(Eq. 2.2)}$$

$$NH_4^+ + 1.985HCO_3^- + 0.074CO_2 + 1.403O_2 \rightarrow 0.985NO_2^- + 1.985CO_2 + 0.015C_5H_7O_2N + 2.941H_2O \quad \text{(Eq. 2.3)}$$

Remarkably, stoichiometric coefficients imply that per mole of ammonium removed, such process requires a significant amount of oxygen, produces a small amount of biomass, and results in substantial destruction of alkalinity through the production of hydrogen ions [9].

The AOB and NOB kinetics have been thoroughly studied: however, an evident variation has been observed in the reported kinetics values, as summarized by Soliman et al. [6]. This wide range in the values of kinetics parameters is due to the different conditions that vary from a study to another such as: wastewater characteristics (low or high strength), operational conditions (temperature, pH, DO), reactor configuration (suspended or attached growth), identification technique (experimental or model based) [6]. Strategies for achieving and maintaining nitritation and partial nitritation are thus based on the control of such different conditions, aimed at achieving AOB accumulation and NOB inhibition or washout [8].

2.2.2 Dissolved oxygen concentration

Ammonia oxidizing bacteria were proved to be less inhibited by low dissolved oxygen (DO) concentration, compared to NOB, and DO concentration below 1.0 ppm is supposed to be sufficient to induce the dominance of the ammonia oxidizers [9]. Such behavior is generally explained by higher energy yield provided by ammonium oxidation than by nitrite oxidation, resulting in a greater oxygen affinity of AOB, compared to NOB, i.e. in a lower half saturation constant (K_O) value [8,11]. However, experimentally determined values of K_O reported in literature widely vary, i.e. 0.2–1.5 mgO_2/L and 1.2–1.5 mgO_2/L for AOB and NOB, respectively [8]. Other authors [12] suggested NOB are inhibited by toxic ammonia oxidation intermediates such as hydroxylamine, which accumulate at low DO, more than by different oxygen affinity. Limiting DO levels (~0.5 ppm) as well as aeration patterns (such as quick alternance between aerated and not aerated conditions) were first suggested and subsequently proved to be a suitable strategy to achieve inhibition of NOB activity [13,14]. Several SBR deammonification full-scale plants have implemented their own aeration strategies; most of them have used intermittent aeration [1].

2.2.3 pH and free ammonia/free nitrous acid concentration

Both AOB and NOB microbial activity is directly influenced by pH of the environment. The pH range for AOB growth is commonly 7.0–8.6, while that for NOB activity was 6.0–7.5 [14]. Such different optimum pH ranges may allow to exert selective pressures against NOB activity, although pH influence on AOB selection over NOB is mainly ascribed to change in free ammonia and free nitrous acid concentration.

Ammonium (NH_4^+) and nitrite (NO_2^-) represent the ionized forms of ammonia (NH_3, also identified as Free Ammonia, FA) and nitrous acid (HNO_2, also identified as Free Nitrous Acid, FNA), respectively. The laws that regulate the equilibrium between dissociated and undissociated forms of each compound were expressed by Anthonisen et al. [15], and depend on pH and temperature. Generally, when pH raises, FA increases and FNA decreases, and vice versa. FA and FNA concentration are of great concern because they are supposed to represent actual substrate for AOB and NOB, respectively, rather than ammonium and nitrite, moreover, they are recognized as inhibiting compounds both to AOB and NOB, at different concentrations [11]. A wide range of FA/FNA inhibiting values of AOB/NOB activity are reported in literature: such variability can be explained by differences in microbial communities; however, NOB always showed either FA or FNA inhibition at much lower concentrations compared to AOB [14].

2.2.4 Alkalinity and inorganic carbon

AOB and NOB are autotrophic organisms that use inorganic carbon (IC) as the essential assimilative carbon source: thus, IC is a key factor to affect the stable and effective removal rate of the nitrification process [14]. According to some studies, AOB bioactivity would be inhibited under IC limited conditions [14,16,17].

Additionally, IC serves as the bicarbonate alkalinity to compensate the acidity produced by nitritation process (almost two moles of H^+ are produced per mole of oxidized ammonium, (Eq. 2.1 and (Eq. 2.2). Thus, if initial alkalinity/ammonium molar ratio (Alk/N ratio) is <2, acidity accumulates and pH decreases, leading to the AOB activity decrease and inhibition (pH<6.5). In this sense, Alk/N ratio was proved to be a key factor to achieve a partial nitritation and regulate the ammonium oxidation rate to nitrite [18,19], which is a crucial aspect when PN is coupled to a subsequent anammox process.

2.2.5 Temperature and sludge retention time

Hellinga et al. [20] concluded that temperatures above 25°C lead to an increase of the specific growth rate of ammonia oxidizing bacteria, which become higher than that of nitrite oxidizing bacteria. The SHARON process (Single reactor High activity Ammonia Removal Over Nitrite) is based on this principle. In this process, nitrification of ammonium to nitrite is established in a chemostat by working at high temperature (above 25°C) and maintaining an appropriate sludge retention time (SRT) of 1–1.5 days, so that ammonium oxidizers are maintained in the reactor, while nitrite oxidizers are washed out and further nitrification of nitrite to nitrate is prevented [11]. However, partial nitritation process was also successfully started up and maintained at lower temperatures (≤25°C) [8]. These results suggested that the application of the partial nitritation process could be not restricted to effluents with temperatures ≥30°C, such as the effluents from methanogenic reactors, but could be applicable to many kinds of industrial wastewater treatments [11]. In recent years, many researchers began to pay more attention to application of nitritation/denitritation and PN/anammox to mainstream municipal wastewater treatment, with lower temperature and lower ammonium concentrations [21].

2.2.6 Organic matter

Presence of biodegradable organic matter may lead to the growth of ordinary heterotrophic organisms (OHO) which could consume oxygen and uptake ammonium at a higher rate than AOB, thus causing a decrease in nitritation effectiveness. Mosquera Corral et al. [22] observed that AOB were outcompeted by OHO in a SHARON reactor for acetate dosage above 0.2 gC/gN, leading to the deterioration of PN process. However, the types of influent carbon sources also affected partial nitrification, since the biochemical reactions involved in nitrification are driven by enzymes, whose activities are largely depending upon carbon sources [8].

2.2.7 Other influencing and inhibiting factors

The presence of phosphates could assume a crucial role in achieving AOB to outcompete NOB, since NOB were proved to be unable to perform nitrite oxidation in absence of phosphates (“phosphate block”) [11].

Many industrial wastewaters are characterized by high salinity, which can potentially represent an inhibitory factor; however, stable partial nitritation was achieved in a SHARON reactor operated under a salt concentration of 400 mM NaCl (>23 g/L) [23].

Light is inhibiting to both AOB and NOB, through the oxidation of cytochrome c caused by light in the presence of oxygen [9].

Many other organic and mineral compounds were tested and reported to exert inhibitory effects on AOB and NOB, often at different levels.

Benzene, toluene and xylene induce a significant decrease in the values for nitrification specific rates affecting mainly the ammonia oxidation pathway [11].

Formic, acetic, propionic and n-butyric acid all inhibited nitrite oxidation, but exhibited no significant effect on ammonium oxidation [24].

Chlorate, cyanide, azide and hydrazine were proved to be more inhibitory to the oxidation of nitrite than to the oxidation of ammonium. Other toxic components that influence nitrite oxidation are the disinfectants bromide and chloride [11]

Heavy metals chromium, nickel, copper, zinc, lead and cadmium might inhibit both steps of nitrification reaction but the inhibition effects are different [25]. Contradictory results were found, e.g. inhibition effects by nickel towards NOB were observed at low concentrations (0.7 mg/L); however, *Nitrosomonas* sp. was found to be equally or even more sensitive than *Nitrobacter* sp. towards nickel and copper, while other studies testing a set of heavy metals reported NOB inhibition by cadmium, but not by copper, or even no inhibitions effect at all [9].

Hydroxylamine has been found to severely inhibit *Nitrobacter*: no nitrite oxidation occurred when 0.42 mg NH_2OH-N/L was present. Addition of 2.5–5 mg NH_2OH-N/L to a submerged filter system significantly enhanced nitrite accumulation during nitrification. Moreover, this inhibitory effect of hydroxylamine on NOB was found to be irreversible [9]. Yang and Alleman [12] noted that the nitrite build-up in activated sludge batch cultures, correlated with the accumulation of free hydroxylamine, and not necessarily with FA nor with low DO concentrations [9].

Adding inhibitors for NOB is another approach for partial nitrification, such as sulfide, hydroxylamine, salt, heavy metals, chlorate, cyanate, halide, azide, hydrazine, and organic chemicals [9].

2.3 Anaerobic ammonia oxidation: process description and influencing factors

2.3.1 Introduction

In 1995, Mulder et al. [26] observed in a denitrifying fluidized bed reactor losses in nitrogen balances which could be explained by the occurrence of anaerobic ammonia oxidation. Van de Graaf [27] demonstrated that the process was biologically mediated by microorganisms, labelled as anammox (anaerobic ammonia oxidation) organisms. Later, nitrite was proved to be the final electron acceptor, and not nitrate as initially figured, with hydroxylamine and hydrazine as main intermediate products [28].

Strous et al. [29] defined the stoichiometry of the overall synthesis-oxidation reaction (Eq. 2.4) which is to date still widely accepted, basing on mass balances on different microbial cultures enriched in anammox species; however, it was recently revised (Eq. 2.5) using a highly enriched culture of planktonic anammox bacterial cells [30].

$$\mathrm{NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow} \\ \mathrm{\rightarrow 1.02N_2 + 2.03H_2O + 0.066CH_2O_{1.5}N_{0.15} + 0.26NO_3^-} \qquad \text{(Eq. 2.4)}$$

$$\mathrm{NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow} \\ \mathrm{\rightarrow 0.986N_2 + 2.002H_2O + 0.071CH_{1.74}O_{0.31}N_{0.20} + 0.161NO_3^-} \qquad \text{(Eq. 2.5)}$$

Increasing number of studies and applications of anammox-based biological wastewater treatment have been reported since its first appearance [11]. Such processes are characterized by high volumetric nitrogen removal rates (NRR), low operative costs and reduced volume and area requirement [31]. Although the engineering application of anammox based technologies is affected by a limited bacteria growth rate and by a broad spectrum of inhibiting compounds normally present in wastewater [32], it has been successfully applied on a variety of ammonium-rich wastewater, such as anaerobic digestion supernatant, landfill leachate, swine manure, and wastewater from chemical, petrochemical and pharmaceutical industry; within 2014 almost 100 full scale plants were established [1].

2.3.2 Microbiology and kinetic aspects

Anammox bacteria have been found in numerous natural sea and freshwater environments, and it is believed they play a significant role in natural nitrogen cycle: according to Dalsgaard [33], up to 70% of dinitrogen gas produced in marine sediments may be ascribed to anammox activity. They are characterized by a typical reddish pigmentation (Figure 2.1), attributed to the

high content in cytochrome c protein [29]. It is widely accepted that they are also characterized by a low specific growth rate: although different values were reported in literature [11], doubling time of 11 days calculated by Strous et al. [29], corresponding to a μ_{max}=0.065 d^{-1}, still represents a reference value.

Anammox bacteria are affiliated with a monophyletic group in the phylum Planctomycetes [34], and the following five candidate genera have been tentatively proposed based on 16S and 23S rRNA gene sequence similarities: *Candidatus* Kuenenia, *Candidatus* Brocadia, *Candidatus* Anammoxoglobus, *Candidatus* Jettenia and *Candidatus* Scalindua; each bacterial genus contain phylogenetically diverse species of bacteria [35].

All anammox bacteria are strictly anaerobic organisms, characterized by a peculiar cell structure. The central cell structure, bound by a highly curved membrane, is called the anammoxosome, which observations define as a true cell organelle [36]. A variety of heme *c* proteins, such as hydroxylamine oxidoreductase (HAO)-like proteins and the hydrazine synthase (HZS) complex, are involved in anammox catabolism and are hypothesized to reside in the anammoxosome [36]. Another unique feature of anammox bacteria is the nature of the anammoxosome membrane constituents, i.e. the presence of saturated C17-C20 fatty acids and alcohols that are fused by cis-ring junctions to make ladder-like (‘ladderane’) cyclobutane and cyclohexane ring systems, which make the membrane highly packed and impermeable [36].

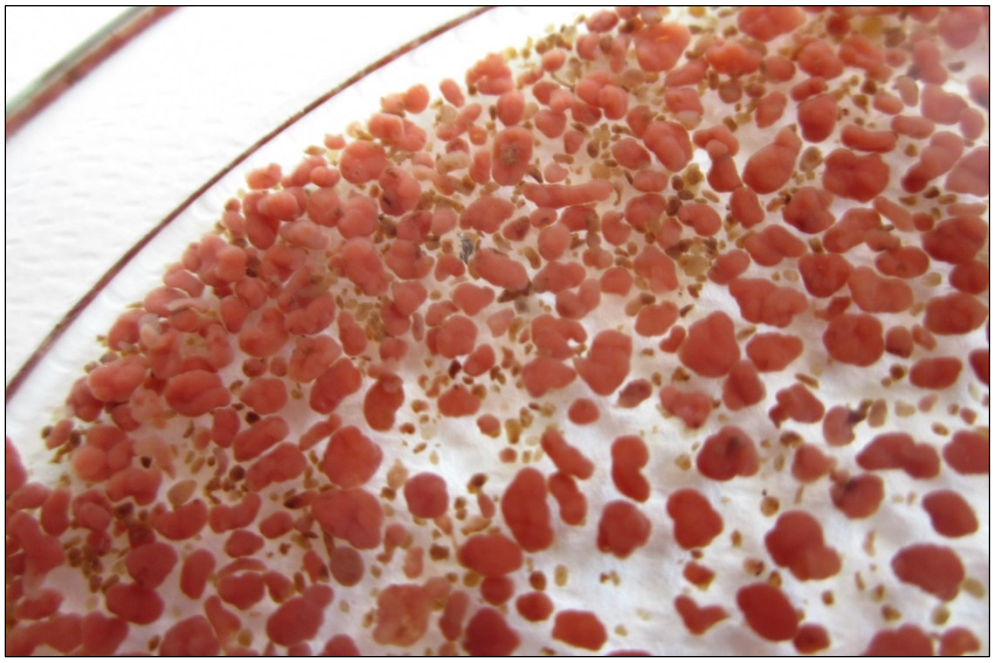

Figure 2.1: Anammox granular aggregates cultivated at DICAAR laboratories by means of a sequencing batch reactor operated at NLR=1.5 gN/L·d.

The detailed description of anammox metabolism is still uncomplete [36]. As reported in recent reviews [35,36], an hypothetical catabolic model taking energy conservation into account was proposed by Strous et al. [37] on the basis of genome analyses and results from physiological experiments, suggesting a three-step process: (i) nitrite reduction to nitric oxide (NO), (ii) hydrazine (N_2H_4) biosynthesis from NO and ammonium and (iii) hydrazine dehydration to N_2 gas. The hydrazine biosynthesis and dehydration are supposed to take place at the anammoxosome.

As reported by Kartal et al. [36], it has been established that anammox organisms are able to convert organic and inorganic compounds to sustain their metabolism, most notably formate, acetate, and propionate; however, organic compounds are fully oxidized to CO_2 rather than being incorporated into cell biomass. Results from dedicated experiments indicated that apparently, ammonium was the preferred electron donor, whereas the organic compounds would be cometabolized, a type of metabolism that might be classified as 'facultative chemo-organotrophy'. The detailed description of such metabolic pathways still remains unclear and is currently investigated.

Anammox bacteria were observed to perform the oxidation of organic (or inorganic) electron donors allowing them to metabolize in the absence of ammonium, thus adopting a 'disguised' denitrifying life-style. In this process, nitrate is first converted to nitrite, half of which is reduced to ammonium. Hereafter, ammonium and nitrite are combined to yield N_2 by the anammox pathway. The six- electron reduction of nitrite to ammonium resembles the dissimilatory nitrite reduction to ammonium (DNRA) mechanism. However, the enzymes catalyzing such reaction still remain elusive. While acting as 'disguised' denitrifiers, both nitrogen atoms in N_2 derive from nitrate, unlike in the standard anammox process. This will make it hard to assign N_2 production through nitrate reduction to anammox or 'true' denitrifiers. This ambiguity may lead to the underestimation of the contribution of anammox bacteria to N_2 production in nature and explain contradictory results [36].

2.3.3 Nitrite, free nitrous acid, ammonium and free ammonia

Nitrite is a toxic compound to many bacteria species, and anammox, although nitrite is directly involved in their metabolism, is not an exception. Thus, nitrite concentration control is a key process control parameter in engineered systems.

Nitrite inhibition has been thoroughly investigated; although it has been widely recognized that anammox inhibition occurs at lower nitrite level compared to ammonium, there is still not a complete agreement on threshold values beyond which inhibition can be observed [11]. Strous

et al. [29] reported a total biomass inhibition at nitrite concentration of 100 mgN/L; later, Dapena-Mora et al. [38] observed 50% activity reduction after the biomass being exposed to concentrations up to 350 mgNO_2-N/L. Such differences may be ascribed to numerous different operating conditions (inoculum, treated wastewater, reactor type, pH, HRT, feeding strategy, biomass aggregation, biomass growth support material, etc.) applied in different studies [39].

Fernandez et al. [40] observed that FNA was the actual inhibiting compound, more than ionized nitrite, while opposite results were later observed by Lotti et al. [41]. In 2012, Jin et al. [32] synthesized many different results regarding nitrite and FNA anammox inhibition, concluding that an influent nitrite concentration of 280 mg/L (~85 mgN/L) can be assumed as 'alarm' threshold, while biomass should not be exposed to concentrations higher than 100 mgN/L (~330 mgNO_2/L) in order to avoid inhibition.

Finally, Puyol et al. [42], assessed anammox activity inhibition through batch tests carried on at different nitrite, FNA and pH conditions. Results were fitted through a non-competitive inhibitory model: such model predicted that anammox inhibition was caused by both ionized nitrite and FNA, and inhibitory constants were predicted to be 561 mgN/L and 0.117 mgN/L for nitrite and FNA, respectively. The authors concluded that pH of the medium strongly affects the behavior of both chemical species, so that at pH values lower than 7.1, FNA is an important contributor to the inhibition; whereas, ionized nitrite is the predominant cause of inhibition at higher pH values.

As to ammonium, first studies did not highlight any relevant inhibiting effect on anammox biomass, up to 1000 mgN/L [29], while Dapena-Mora et al., [38] calculated a 50% activity reducing concentration of 770 mgNH_4-N/L; at the same time, the authors recognized FA as the actual inhibiting compound. Fernandez et al. [40] determined that stable anammox activity required FA concentration below 20-25 mgN/L to be maintained. However, different values were reported in literature, as pointed out by Jin et al. [32].

2.3.4 Inorganic carbon

Inorganic carbon (which is present in water mainly as bicarbonate) plays an important role in anammox process, not only as carbon source for the chemiolitotrophic metabolism, but also as pH regulating factor, thus helping in maintaining FA/ammonium and FNA/nitrite equilibrium.

In 2008, Liao et al. [43] carried on an experiment aimed at assessing effects of different bicarbonate concentration on anammox activity. An increase in process performance and nitrogen removal efficiency was observed as bicarbonate increased from 1.0 to 1.5 and then to

1.75 g/L, while higher (2 g/L) bicarbonate concentrations led to a rapid decrease in process performance, mainly ascribed to FA inhibition caused by pH raise up to 8.1.

2.3.5 Temperature and pH

Temperature and pH influence on anammox bacteria have been studied extensively in recent years. These parameters strongly influence anammox bacteria directly, e.g. when determining the reaction rate and kinetics, as well as indirectly, e.g. when determining the toxicity of ammonium and nitrite, as FA and FNA, respectively [44].

Different authors [11,29,45] reported an optimal temperature range for anammox activity between 30 and 40°C. At temperature >45°C irreversible activity depletion was observed, ascribed to thermal cell lysis [46]. This means that the required temperature is much higher than the average municipal wastewater temperature. An effective low-temperature anammox process seems to be one of the most challenging but profitable processes to be performed in the mainstream of the municipal wastewater treatment plant [44]. Progressive anammox adaptation to low temperature was observed in different studies [46,47]. Several other sources also reported that a stable and reliable anammox process can be performed at room temperatures (<30°C) in single-stage reactors with relatively high nitrogen removal rates; moreover, they suggest that lower temperatures (below 12-13°C) strongly affect the anammox process [44].

Anammox activity is directly affected by pH change [44], as well as indirect effect may be caused by FA or FNA change along with pH. Thus, a broad range of optimal pH values were reported, ranging between 6.5 and 8.3 [44]. In a recent study [44], a meta-analysis of data collected from eight experiments was attempted, using a second-order polynomial equation correlating pH, temperature and relative specific activity with respect to a reference temperature of 30°C. Results indicated that along with the significant temperature influence on anammox activity, higher activity values were observed at higher pH, within a pH range of 7.2-8.1; furthermore, this effect intensified along with a decreasing temperature. Such results were considered consistent with those reported in previous studies.

However, the data confirm that constant pH control is crucial for stable anammox performance. Maintaining the optimal pH in laboratory scale reactors is commonly done [44]. Acid/base dosing systems are also used in full-scale anammox plants [1,44].

2.3.6 Organic matter

Generally, there are two different proposed mechanisms for the non-toxic organic matter inhibition of anammox [32]: (i) anammox bacteria competition with heterotrophic denitrifying

bacteria, which grow faster and can outcompete anammox in nitrite utilization under high concentration of organic matter; (ii) anammox bacteria perform different metabolic pathways, i.e., using organic matter rather than ammonium and nitrite as a substrate, as described in section 2.3.2.

Heterotrophic denitrifiers are characterized, in optimal conditions, by higher growth rates, compared to anammox. However, slowly degradable compounds may represent a limiting substrate to such organisms, thus reducing their growth rate to the same values as anammox, irrespective of the initial COD concentration or COD/N ratio. Coexistence of anammox and denitrifying bacteria may play a significant role for the treatment of industrial wastewater characterized by high concentrations of both nitrogen and COD [48]; such coexistence may be facilitated through efficient controls of dissolved oxygen (DO), pH and temperature [32].

Generally, different authors reported anammox inhibition at COD concentration around 300 mg/L and COD/NO_2-N ratio of 2-3 [49].

Effect of specific organic compounds, both toxic and non-toxic, on anammox bacteria were also reported.

Guven et al. [50] observed that anammox bacteria were irreversibly inhibited when exposed to relatively low concentration (15 mg/L) of ethanol and methanol. Methanol inhibition was also observed by other authors [32]. The mechanism of methanol inhibition of anammox was ultimately found to be formaldehyde inhibition. As reported by Isaka et al. [51], methanol may be converted to formaldehyde intracellularly because of the action of the anammox enzyme hydroxylamine oxidoreductase. Formaldehyde destroys enzyme and protein activity by irreversibly cross-linking the peptide chains, thus causing the irreversible inhibition of anammox. Based on these facts, the inhibition of anammox by alcohols is most likely caused by the anammox enzyme converting the alcohols into their corresponding aldehydes, which directly inhibits the Anammox reaction [32].

Other authors reported that anammox cultures acclimated to toxic compounds such as phenols (300-800 mg/L), cyanides (10-90 mg/L) and tiocyanate (300-500 mg/L) [52].

Short- and long-term inhibition caused by phenols was recently studied in both acclimated and unacclimated biomass in an upflow sludge blanket reactor (UASB), at lab scale, and using synthetic medium [53]. Phenols were observed to exert a remarkable effect on cell membranes, altering their permeability and structural integrity. Short-term effect resulted in a 50% activity reduction at phenol concentration around 680 mg/L; long-term experiments proved that the exposition of an unacclimated biomass to 50 mg/L phenols may significantly, although

reversibly, reduce anammox activity. Acclimation strategy to the same phenol concentration resulted in a limited reduction in nitrogen removal efficiency (-10%) and in specific activity, compared to the initial values.

Few researches focused on assessing anammox inhibition by antibiotics, although high antibiotic concentrations were detected in aquatic environments, such as sewage treatment plant effluents, surface water, and even ground water [32]. To date, ten different antibiotics were tested [54]. Anammox are inhibited by antibiotics; however, results show that resistance of anammox bacteria to antibiotics in mixed-culture systems could be enhanced by the presence of non-anammox biomass [54].

2.3.7 Other influencing and inhibiting factors

Since anammox bacteria were found in anoxic marine environments (mainly ascribed to Scalindua genera), they may represent a solution for the treatment of peculiar industrial wastewater characterized by high salinity. The presence of high-salinity concentrations in anammox systems has been studied deeply in the last decade, and the results confirmed that, although the response depends on the salt concentrations, enriched anammox consortium was sufficiently adapted up to 15 gNaCl/L [54] and there were not effects in specific anammox activity, while reduced activity was observed at 30 gNaCl/L [55]. Recently, Scaglione et al. [56] tested anammox inhibition upon exposure to digestates from biogas plants treating the organic fraction of municipal solid waste, and reported a significant correlation between observed inhibition and wastewater conductivity. The fitting modified non-competitive inhibition model resulted in an IC_{50} (concentration resulting in a 50% inhibition) value of 6.09±1.17 mS/cm (corresponding to ~1.70 gNaCl/L), significantly lower than other values reported in literature. Such low value was explained by several factors, such as using a mixture of different salts rather than using the sole NaCl, different measurement conditions, and inoculum enriched in anammox genera previously reported as more sensitive to salinity changes. The authors concluded that salinity/conductivity may be used as control parameter to infer the short-term inhibition effect of certain wastewaters.

Phosphate is a common inorganic inhibitor of the anammox process, although few specific studies are reported [32]. An IC_{50} value of 650 mgP/L was determined by Dapena Mora in 2007 [38]. More recently, Carvajal-Arroyo et al. [57] investigated anammox inhibition caused by different compounds on both granular and suspended anammox enriched biomass, and observed that exposure to phosphate caused a modest activity decrease in suspended biomass with increasing phosphate concentration (IC_{50}=25.3±5.9 mM, corresponding to ~780 mgP/L);

while phosphate stimulated the activity of the granular biomass by 60% at concentrations ranging 10–50 mM (300-1500 mgP/L). Authors concluded that such results suggested that the impact of phosphate on anammox activity is highly dependent on the aggregation degree of the biomass.

The same authors tested the effect of sulfide, which is commonly found in anaerobic reactors as a product of mineralization of organic matter or sulfate reduction, on both granular and suspended anammox bacteria, in assays supplied with 0.1–10.0 mM Na_2S, i.e. 3.2-321 mgS/L [57]. The toxicity of sulfide has often been associated with its unionized form (H_2S): the authors reported that undissociated H_2S caused serious inhibition of the anammox activity with IC_{50} values of 0.03 and 0.11 mM (0.96 and 3.53 mgS/L) for the suspended and granular biomass, respectively. A concentration of unionized H_2S as low as 0.32 mM (10.3 mgS/L) caused complete inhibition of the suspended biomass, while granular aggregates were able to conserve ~24% of initial activity value at higher concentrations (0.9 mM, i.e. 28.9 mgS/L). No H_2S consumption was observed in either abiotic or biological treatments during the course of the experiment. Authors ascribed the strong sulfide inhibiting effect to the high dependence of anammox process on heme proteins, since sulfide had been reported to interact with heme centers of cytochrome oxidase as well as to cause reduction of the heme iron in cytochrome c [57]. In another experiment, Jin et al. [58] observed that inhibitory effects of sulfide on anammox depended on substrate and sulfide level and exposure time. Long-term exposure to sulfide-S at 32 mg/L damaged the anammox performance, which could restore after a long operation time. The presence of sulfide altered the operational characteristics of the anammox system: specific anammox activity was almost totally depleted within 90 days, and the heme c level decreased by 42.3% after sulfide inhibition. The sulfide in the influent decreased the granule diameter and damaged the cell. The biomass growth rate was inhibited with the apparent doubling time of the nitrogen removal capacity largely extended [58].

Anammox bacteria were proved to be strictly anaerobic and inhibited by the presence of dissolved oxygen. It was reported that anammox process was inhibited reversibly by DO at a low level (<1-2% air saturation) and irreversibly at higher oxygen concentrations (>18% air saturation) [32]. More recently, Carvajal-Arroyo [57] determined IC_{50} value of 3.8 and 2.3 $ppmO_2$ for suspended and granular anammox biomass, respectively. However, DO is normally strictly controlled in anammox systems for avoiding oxygen inhibition, even at full scale [1].

In recent years, several publications have focused on the individual effects of heavy metals on the anammox process; however, the joint effect of heavy metals on the anammox process

remains poorly documented [59]. Heavy metals are not easily biodegradable and can accumulate in organisms, causing biological accumulation toxicity. Some kinds of nitrogen-rich wastewater, such as landfill leachate, often contain high levels of heavy metal ions, such as Cu, Zn, Pb, Cd, Ag, Hg [32,59]. Van de Graaf et al. [27] reported complete anammox inhibition at a $HgCl_2$ concentration of 1 mM. In a recent study [59], concurrent inhibition effect of Cu(II) and Zn(II) ions on anammox activity was evaluated. The most severe inhibition, resulting in a 20.1% residual anammox activity (compared to control value), occurred at Cu(II) and Zn(II) concentrations of 16.3 and 20.0 mg/L, respectively. Notably, the cumulative toxicity was mitigated by intermittent exposure acclimation.

2.4 Anammox-based technologies and applications

Autotrophic nitrogen removal consists of two different processes (i.e., partial nitritation and anammox) which can be carried out either in two different reactors (two-stage PN/anammox) or in a single reactor (single-stage PN/anammox). As recently reported by Lackner et al. [1], early PN/A implementations used two-stage reactor configurations or made use of already existing nitritation systems (e.g. SHARON type reactors). With more lab-, pilot- and full-scale experiences, focus has shifted mainly to single-stage systems, which implied less operational costs; however, two-stage configuration allowed higher process flexibility, e.g. for the treatment of wastewater containing toxic compounds as well as biodegradable organic matter, which were removed in the first stage, avoiding anammox process contamination [60,61].

In single-stage systems, environmental conditions promote simultaneous co-existence of both AOB and anammox bacteria, as well as heterotrophic denitrifying bacteria, while inhibiting NOB activity [62]. Most used strategy in this sense requires microaerated conditions (<0.5-1 ppm) and pH control [1]. Aeration strategy probably represents the most critical operational parameter, as it directly influences DO concentration and gas-liquid oxygen transfer rate: this is of great importance in biofilm or granular systems, where diffusion can be limiting [63]. Different aeration strategies have been proposed to achieve a stable process to speed up the start-up phase and also to mitigate greenhouse gas emissions [64].

A variety of reactors configurations has been applied up to full-scale implementations, including the moving bed biofilm reactor (MBBR), granular sludge processes, up-flow anaerobic sludge blanket (UASB), and SBR, which is the most commonly used [1].

In recent years, major efforts have been made towards the implementation of anammox-based technologies in mainstream municipal wastewater treatment [21,65].

2.5 References

[1] S. Lackner, E.M. Gilbert, S.E. Vlaeminck, A. Joss, H. Horn, M.C.M. van Loosdrecht, Full-scale partial nitritation/anammox experiences - An application survey, Water Res. 55 (2014) 292–303. doi:10.1016/j.watres.2014.02.032.

[2] M.S.M. Jetten, S.J. Horn, M.C.M. van Loosdrecht, Towards a more sustainable municipal wastewater treatment system, Water Sci. Technol. 35 (1997) 171–180. doi:10.1016/S0273-1223(97)00195-9.

[3] A.O. Sliekers, N. Derwort, J.L.C. Gomez, M. Strous, J.G. Kuenen, M.S.M. Jetten, Completely autotrophic nitrogen removal over nitrite in one single reactor, Water Res. 36 (2002) 2475–2482. doi:10.1016/S0043-1354(01)00476-6.

[4] M.C.M. Van Loosdrecht, M.S.M. Jetten, Microbiological conversions in nitrogen removal, Water Sci. Technol. 38 (1998) 1–7. doi:10.1016/S0273-1223(98)00383-7.

[5] M.S.M. Jetten, M. Wagner, J. Fuerst, M. Van Loosdrecht, G. Kuenen, M. Strous, Microbiology and application of the anaerobic ammonium oxidation ('anammox') process, Curr. Opin. Biotechnol. 12 (2001) 283–288. doi:10.1016/S0958-1669(00)00211-1.

[6] M. Soliman, A. Eldyasti, Ammonia-Oxidizing Bacteria (AOB): opportunities and applications—a review, Springer Netherlands, 2018. doi:10.1007/s11157-018-9463-4.

[7] A. Rodriguez-Sanchez, A. Gonzalez-Martinez, M.V. Martinez-Toledo, M.J. Garcia-Ruiz, F. Osorio, J. Gonzalez-Lopez, The effect of influent characteristics and operational conditions over the performance and microbial community structure of partial nitritation reactors, Water (Switzerland). 6 (2014) 1905–1924. doi:10.3390/w6071905.

[8] S. Ge, S. Wang, X. Yang, S. Qiu, B. Li, Y. Peng, Detection of nitrifiers and evaluation of partial nitrification for wastewater treatment: A review, Chemosphere. 140 (2015). doi:10.1016/j.chemosphere.2015.02.004.

[9] B. Sinha, A.P. Annachhatre, Partial nitrification - Operational parameters and microorganisms involved, Rev. Environ. Sci. Biotechnol. 6 (2007) 285–313. doi:10.1007/s11157-006-9116-x.

[10] F.L. Burton, G. Tchobanoglous, R. Tsuchihashi, H.D. Stensel, I. Metcalf & Eddy, Wastewater Engineering: Treatment and Resource Recovery, McGraw-Hill Education, 2013. https://books.google.it/books?id=6KVKMAEACAAJ.

[11] S.W.H. Van Hulle, H.J.P. Vandeweyer, B.D. Meesschaert, P.A. Vanrolleghem, P. Dejans, A. Dumoulin, Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams, Chem. Eng. J. 162 (2010) 1–20. doi:10.1016/j.cej.2010.05.037.

[12] L. Yang, J.E. Alleman, Investigation of Batchwise Nitrite Build-Up by an Enriched Nitrification Culture, Water Sci. Technol. 26 (1992) 997–1005. http://dx.doi.org/10.2166/wst.1992.0541.

[13] G. Sin, K. Villez, P.A. Vanrolleghem, Application of a model-based optimisation methodology for nutrient removing SBRs leads to falsification of the model, Water Sci. Technol. 53 (2006) 95–103. http://dx.doi.org/10.2166/wst.2006.114.

[14] Y. Feng, X. Lu, H. Al-Hazmi, J. Mąkinia, An overview of the strategies for the deammonification process start-up and recovery after accidental operational failures, Rev. Environ. Sci. Biotechnol. 16 (2017) 541–568. doi:10.1007/s11157-017-9441-2.

[15] A.C. Anthonisen, R.C. Loehr, T.B.S. Prakasam, E.G. Srinath, Inhibition of Nitrification by Ammonia and Nitrous Acid, J. (Water Pollut. Control Fed. 48 (1976) 835–852. http://www.jstor.org/stable/25038971.

[16] Y. Ma, S. Sundar, H. Park, K. Chandran, The effect of inorganic carbon on microbial interactions in a biofilm nitritation-anammox process, Water Res. 70 (2015) 246–254. doi:10.1016/j.watres.2014.12.006.

[17] A. Guisasola, S. Petzet, J.A. Baeza, J. Carrera, J. Lafuente, Inorganic carbon limitations on nitrification: Experimental assessment and modelling, Water Res. 41 (2007) 277–286. doi:10.1016/j.watres.2006.10.030.

[18] R. Ganigué, J. Gabarró, A. Sànchez-Melsió, M. Ruscalleda, H. López, X. Vila, J. Colprim, M.D. Balaguer, Long-term operation of a partial nitritation pilot plant treating leachate with extremely high ammonium concentration prior to an anammox process, Bioresour. Technol. 100 (2009) 5624–5632. doi:10.1016/j.biortech.2009.06.023.

[19] D. Scaglione, E. Ficara, V. Corbellini, G. Tornotti, A. Teli, R. Canziani, F. Malpei, Autotrophic nitrogen removal by a two-step SBR process applied to mixed agro-digestate, Bioresour. Technol. 176 (2015) 98–105. doi:10.1016/j.biortech.2014.11.019.

[20] C. Hellinga, A.A.J.C. Schellen, J.W. Mulder, M.C.M. Van Loosdrecht, J.J. Heijnen, The SHARON process: An innovative method for nitrogen removal from ammonium-rich waste water, Water Sci. Technol. 37 (1998) 135–142. doi:10.1016/S0273-

1223(98)00281-9.

[21] G. Xu, Y. Zhou, Q. Yang, Z.M.P. Lee, J. Gu, W. Lay, Y. Cao, Y. Liu, The challenges of mainstream deammonification process for municipal used water treatment, Appl. Microbiol. Biotechnol. 99 (2015). doi:10.1007/s00253-015-6423-6.

[22] A. Mosquera-Corral, F. González, J.L. Campos, R. Méndez, Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds, Process Biochem. 40 (2005) 3109–3118. doi:10.1016/j.procbio.2005.03.042.

[23] A. Mosquera-Corral, F. González, J.L. Campos, R. Méndez, Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds, Process Biochem. 40 (2005) 3109–3118. doi:10.1016/j.procbio.2005.03.042.

[24] A.M. Eilersen, M. Henze, L. Kløft, Effect of volatile fatty acids and trimethylamine on nitrification in activated sludge, Water Res. 28 (1994) 1329–1336. doi:10.1016/0043-1354(94)90298-4.

[25] S.W.H. Van Hulle, S. Van Den Broeck, J. Maertens, K. Villez, B.M.R. Donckels, G. Schelstraete, E.I.P. Volcke, P.A. Vanrolleghem, Construction, start-up and operation of a continuously aerated laboratory-scale SHARON reactor in view of coupling with an Anammox reactor, Water SA. 31 (2005) 327–334. doi:10.4314/wsa.v31i3.5222.

[26] A. Mulder, A.A. van de Graaf, L.A. Robertson, J.G. Kuenen, The anaerobic ammonium oxidation, FEMS Microbiol. Ecol. 16 (1998) 177–183. doi:https://doi.org/10.1016/0168-6496(94)00081-7.

[27] A.A. Van de Graaf, A. Mulder, P. De Bruijn, M.S.M. Jetten, L.A. Robertson, J.G. Kuenen, Anaerobic oxidation of ammonium is a biologically mediated process, Appl. Environ. Microbiol. 61 (1995) 1246–1251. doi:PMC167380.

[28] M.S.M. Jetten, M. Strous, K.T. Pas-Schoonen, J. Schalk, U.G.J.M. Dongen, A.A. Graaf, S. Logemann, G. Muyzer, M.C.M. Loosdrecht, J.G. Kuenen, The anaerobic oxidation of ammonium, FEMS Microbiol. Rev. 22 (1998) 421–437. doi:10.1111/j.1574-6976.1998.tb00379.x.

[29] M. Strous, J.G. Kuenen, M.S.M. Jetten, Key Physiology of Anaerobic Ammonium Oxidation Key Physiology of Anaerobic Ammonium Oxidation, Appl. Environ. Microbiol. 65 (1999) 0–3. doi:papers2://publication/uuid/E9A1573A-6D62-420E-94D0-CA7C84D0FEB9.

[30] T. Lotti, R. Kleerebezem, C. Lubello, M.C.M. van Loosdrecht, Physiological and kinetic characterization of a suspended cell anammox culture, Water Res. 60 (2014) 1–14. doi:10.1016/j.watres.2014.04.017.

[31] W.R.L. van der Star, W.R. Abma, D. Blommers, J.W. Mulder, T. Tokutomi, M. Strous, C. Picioreanu, M.C.M. van Loosdrecht, Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam, Water Res. 41 (2007) 4149–4163. doi:10.1016/j.watres.2007.03.044.

[32] R.C. Jin, G.F. Yang, J.J. Yu, P. Zheng, The inhibition of the Anammox process: A review, Chem. Eng. J. 197 (2012) 67–79. doi:10.1016/j.cej.2012.05.014.

[33] T. Dalsgaard, B. Thamdrup, D.E. Canfield, Anaerobic ammonium oxidation (anammox) in the marine environment, Res. Microbiol. 156 (2005) 457–464. doi:10.1016/j.resmic.2005.01.011.

[34] M. Strous, J.A. Fuerst, E.H. Kramer, S. Logemann, G. Muyzer, K.T. van de Pas-Schoonen, R. Webb, J.G. Kuenen, M.S. Jetten, Missing lithotroph identified as new planctomycete, Nature. 400 (1999) 446–449. doi:10.1038/22749.

[35] M. Oshiki, H. Satoh, S. Okabe, Ecology and physiology of anaerobic ammonium oxidizing bacteria, Environ. Microbiol. 18 (2016) 2784–2796. doi:10.1111/1462-2920.13134.

[36] B. Kartal, N.M. De Almeida, W.J. Maalcke, H.J.M.M. Op den Camp, M.S.M.M. Jetten, J.T. Keltjens, How to make a living from anaerobic ammonium oxidation, FEMS Microbiol. Rev. 37 (2013) 428–461. doi:10.1111/1574-6976.12014.

[37] M. Strous, E. Pelletier, S. Mangenot, T. Rattei, A. Lehner, M.W. Taylor, M. Horn, H. Daims, D. Bartol-Mavel, P. Wincker, V. Barbe, N. Fonknechten, D. Vallenet, B. Segurens, C. Schenowitz-Truong, C. Médigue, A. Collingro, B. Snel, B.E. Dutilh, H.J.M. Op den Camp, C. van der Drift, I. Cirpus, K.T. van de Pas-Schoonen, H.R. Harhangi, L. van Niftrik, M. Schmid, J. Keltjens, J. van de Vossenberg, B. Kartal, H. Meier, D. Frishman, M.A. Huynen, H.-W. Mewes, J. Weissenbach, M.S.M. Jetten, M. Wagner, D. Le Paslier, Deciphering the evolution and metabolism of an anammox bacterium from a community genome, Nature. 440 (2006) 790. https://doi.org/10.1038/nature04647.

[38] A. Dapena-Mora, I. Fernández, J.L. Campos, A. Mosquera-Corral, R. Méndez, M.S.M. Jetten, Evaluation of activity and inhibition effects on Anammox process by batch tests

based on the nitrogen gas production, Enzyme Microb. Technol. 40 (2007) 859–865. doi:10.1016/j.enzmictec.2006.06.018.

[39] S. Cho, Y. Takahashi, N. Fujii, Y. Yamada, H. Satoh, S. Okabe, Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor, Chemosphere. 78 (2010) 1129–1135. doi:10.1016/j.chemosphere.2009.12.034.

[40] I. Fernández, J. Dosta, C. Fajardo, J.L. Campos, A. Mosquera-Corral, R. Méndez, Short- and long-term effects of ammonium and nitrite on the Anammox process, J. Environ. Manage. 95 (2012) 1–5. doi:10.1016/j.jenvman.2010.10.044.

[41] T. Lotti, W.R.L. van der Star, R. Kleerebezem, C. Lubello, M.C.M. van Loosdrecht, The effect of nitrite inhibition on the anammox process, Water Res. 46 (2012) 2559–2569. doi:10.1016/J.WATRES.2012.02.011.

[42] D. Puyol, J.M. Carvajal-Arroyo, R. Sierra-Alvarez, J.A. Field, Nitrite (not free nitrous acid) is the main inhibitor of the anammox process at common pH conditions, Biotechnol. Lett. 36 (2014) 547–551. doi:10.1007/s10529-013-1397-x.

[43] D. Liao, X. Li, Q. Yang, G. Zeng, L. Guo, X. Yue, Effect of inorganic carbon on anaerobic ammonium oxidation enriched in sequencing batch reactor, J. Environ. Sci. 20 (2008) 940–944. doi:10.1016/S1001-0742(08)62190-7.

[44] M. Tomaszewski, G. Cema, A. Ziembińska-Buczyńska, Influence of temperature and pH on the anammox process: A review and meta-analysis, Chemosphere. 182 (2017) 203–214. doi:10.1016/J.CHEMOSPHERE.2017.05.003.

[45] K. Egli, U. Fanger, P.J.J. Alvarez, H. Siegrist, J.R. Van der Meer, A.J.B. Zehnder, Enrichment and characterization of an anammox bacterium from a rotating biological contactor treating ammonium-rich leachate, Arch. Microbiol. 175 (2001) 198–207. doi:10.1007/s002030100255.

[46] J. Dosta, I. Fernández, J.R. Vázquez-Padín, A. Mosquera-Corral, J.L. Campos, J. Mata-Álvarez, R. Méndez, Short- and long-term effects of temperature on the Anammox process, J. Hazard. Mater. 154 (2008) 688–693. doi:10.1016/j.jhazmat.2007.10.082.

[47] T. Lotti, R. Kleerebezem, M.C.M. van Loosdrecht, Effect of temperature change on anammox activity, Biotechnol. Bioeng. 112 (2015). doi:10.1002/bit.25333.

[48] M. Ruscalleda, H. López, R. Ganigué, S. Puig, M.D. Balaguer, J. Colprim, Heterotrophic

denitrification on granular anammox SBR treating urban landfill leachate, Water Sci. Technol. 58 (2008) 1749–1755. doi:10.2166/wst.2008.544.

[49] C. Tang, P. Zheng, C. Wang, Q. Mahmood, Suppression of anaerobic ammonium oxidizers under high organic content in high-rate Anammox UASB reactor, Bioresour. Technol. 101 (2010) 1762–1768. doi:10.1016/J.BIORTECH.2009.10.032.

[50] D. Guven, A. Dapena, B. Kartal, M.C. Schmid, B. Maas, K. van de Pas-Schoonen, S. Sozen, R. Mendez, H.J. Op den Camp, M.S.M. Jetten, M. Strous, I. Schmidt, Propionate Oxidation by and Methanol Inhibition of Anaerobic Ammonium-Oxidizing Bacteria, Appl Env. Microbiol. 71 (2005) 1066–1071. doi:10.1128/AEM.71.2.1066.

[51] K. Isaka, Y. Suwa, Y. Kimura, T. Yamagishi, T. Sumino, S. Tsuneda, Anaerobic ammonium oxidation (anammox) irreversibly inhibited by methanol, Appl. Microbiol. Biotechnol. 81 (2008) 379. doi:10.1007/s00253-008-1739-0.

[52] S. Toh, N. Ashbolt, Adaptation of anaerobic ammonium-oxidising consortium to synthetic coke-ovens wastewater, Appl. Microbiol. Biotechnol. 59 (2002) 344–352. doi:10.1007/s00253-002-1007-7.

[53] G.F. Yang, X.L. Guo, S.X. Chen, J.H. Liu, L.X. Guo, R.C. Jin, The evolution of Anammox performance and granular sludge characteristics under the stress of phenol, Bioresour. Technol. 137 (2013) 332–339. doi:10.1016/j.biortech.2013.03.145.

[54] A. Gonzalez-Martinez, B. Muñoz-Palazon, A. Rodriguez-Sanchez, J. Gonzalez-Lopez, New concepts in anammox processes for wastewater nitrogen removal: recent advances and future prospects, FEMS Microbiol. Lett. 365 (2018) 1–10. doi:10.1093/femsle/fny031.

[55] A. Dapena-Mora, J.R. Vázquez-Padín, J.L. Campos, A. Mosquera-Corral, M.S.M. Jetten, R. Méndez, Monitoring the stability of an Anammox reactor under high salinity conditions, Biochem. Eng. J. 51 (2010) 167–171. doi:10.1016/j.bej.2010.06.014.

[56] D. Scaglione, T. Lotti, E. Ficara, F. Malpei, Inhibition on anammox bacteria upon exposure to digestates from biogas plants treating the organic fraction of municipal solid waste and the role of conductivity, Waste Manag. 61 (2017) 213–219. doi:10.1016/j.wasman.2016.11.014.

[57] J.M. Carvajal-Arroyo, W. Sun, R. Sierra-Alvarez, J.A. Field, Inhibition of anaerobic ammonium oxidizing (anammox) enrichment cultures by substrates, metabolites and common wastewater constituents, Chemosphere. 91 (2013) 22–27.

doi:10.1016/j.chemosphere.2012.11.025.

[58] R.C. Jin, G.F. Yang, Q.Q. Zhang, C. Ma, J.J. Yu, B.S. Xing, The effect of sulfide inhibition on the ANAMMOX process, Water Res. 47 (2013) 1459–1469. doi:10.1016/j.watres.2012.12.018.

[59] Z.Z. Zhang, Q.Q. Zhang, J.J. Xu, R. Deng, Z.Q. Ji, Y.H. Wu, R.C. Jin, Evaluation of the inhibitory effects of heavy metals on anammox activity: A batch test study, Bioresour. Technol. 200 (2016). doi:10.1016/j.biortech.2015.10.035.

[60] J.R. Vázquez-Padín, M. Figueroa, I. Fernández, A. Mosquera-Corral, J.L. Campos, R. Méndez, Post-treatment of effluents from anaerobic digesters by the Anammox process, Water Sci. Technol. 60 (2009) 1135–1143. doi:10.2166/wst.2009.421.

[61] S. Milia, M. Perra, G. Tocco, A. Carucci, The start-up of an anammox reactor as the second step for the treatment of ammonium rich refinery (IGCC) wastewater with high C<inf>org</inf>/N ratio, Ecol. Eng. 106 (2017). doi:10.1016/j.ecoleng.2017.04.068.

[62] C.C. Wang, P.H. Lee, M. Kumar, Y.T. Huang, S. Sung, J.G. Lin, Simultaneous partial nitrification, anaerobic ammonium oxidation and denitrification (SNAD) in a full-scale landfill-leachate treatment plant, J. Hazard. Mater. 175 (2010) 622–628. doi:10.1016/j.jhazmat.2009.10.052.

[63] B. Szatkowska, G. Cema, E. Plaza, J. Trela, B. Hultman, A one-stage system with partial nitritation and Anammox processes in the moving-bed biofilm reactor, Water Sci. Technol. 55 (2007) 19–26. http://dx.doi.org/10.2166/wst.2007.237.

[64] N. Morales, A. Val Del Río, J.R. Vázquez-Padín, R. Gutiérrez, R. Fernández-González, P. Icaran, F. Rogalla, J.L. Campos, R. Méndez, A. Mosquera-Corral, Influence of dissolved oxygen concentration on the start-up of the anammox-based process: ELAN®, Water Sci. Technol. 72 (2015) 520–527. doi:10.2166/wst.2015.233.

[65] T. Lotti, R. Kleerebezem, C. Van Erp Taalman Kip, T.L.G. Hendrickx, J. Kruit, M. Hoekstra, M.C.M. Van Loosdrecht, Anammox growth on pretreated municipal wastewater, Environ. Sci. Technol. 48 (2014) 7874–7880. doi:10.1021/es500632k.

Chapter 3

Preliminary evaluation of PN/anammox process feasibility to treat ammonium-rich effluents produced by double-stage anaerobic digestion of food waste

3.1 Introduction

Due to its increasing production observed in recent years, the eco-sustainable management of food waste (FW) has become a challenging environmental priority. The attractive possibility to modify the anaerobic digestion (AD) process of such wastes in order to achieve the recovery of energy as a mixture of H_2 and CH_4 (biohythane), rather than only CH_4, is currently under investigation [1]. However, since AD has no significant effect on nitrogen, its liquid effluents are characterized by high ammonium concentrations (>1,000 mgNH_4-N/L) and represent, if not properly managed, a threat to the environment. In order to develop a truly eco-sustainable approach, maximization of energy recovery from FW by anaerobic digestion (AD-FW) must be considered as important as the minimization of its potential environmental impacts.

Within this framework, a double-stage system based on partial nitritation (PN) and granular sludge Anammox (ANaerobic AMMonium OXidation) was started up in this study, and fed with a synthetic influent simulating the NH_4-N content and alkalinity of real wastewater produced by an AD-FW system aimed at the recovery of H_2 and CH_4. In order to determine the best operating conditions, different hydraulic retention times (HRT) and nitrogen loading rates (NLR) were tested for both reactors, and the effects of other process parameters and influent characteristics on reactors performance were thoroughly evaluated. Moreover, acute toxicity assessments and prolonged exposure tests were carried out to evaluate the response of unacclimated biomass to the real AD-FW wastewater.

A comprehensive set of information was gathered, which will be helpful for the progressive replacement of the synthetic influent with real AD-FW wastewater.

3.2 Materials and methods

The PN unit consisted of a 2 L continuous flow stirred tank reactor operated as a chemostat (without biomass recirculation). A thermostatic bath was used to control temperature at

35±0.5°C; pH was constantly monitored and kept within the range 6.0-7.5 by dosing acid (H_2SO_4, 1M) or base (NaOH, 1M) solutions. Dissolved oxygen (DO) concentration was continuously monitored, and maintained at the desired level by supplying a variable mixture of air and dinitrogen gas in the bulk liquid at a constant rate (1 L/min).

The reactor was inoculated with activated sludge drawn from the municipal wastewater treatment plant of Cagliari (Italy) and fed with a synthetic medium with a NH_4-N concentration of 1,500 mg/L. Influent flow rate was kept at 1.4 mL/min, resulting in a hydraulic retention time (HRT) and a corresponding sludge retention time (SRT) of 1 d. Total nitrogen loading rate (NLR) was 1.5 gN/L·d. The influent alkalinity to ammonium-nitrogen molar ratio (Alk/N) was increased from 1 to 1.3 by adding bicarbonate (as $NaHCO_3$) to the medium. The overall composition of the synthetic medium was: NH_4HCO_3 8,466 mg/L, KH_2PO_4 1,000 mg/L, $MgSO_4$ 100 mg/L, $NaHCO_3$ 0-2,700 mg/L, and 10 mL/L trace elements according to [2]. The resulting pH was 7.8-8.0.

The plan of the experimental activity is summarized in Table 3.1.

Table 3.1. Plan of the experimental activity PN reactor.

Phase	Duration (d)	NH_4-N inf. (g/L)	NLR (gN/L·d)	HRT (d)	Alk/N (-)	Dissolved Oxygen (DO) (mg/L)
S1	59	1.5	1.0	1.5	1.0*	~sat.
S2	54	1.5	1.2	1.25	1.0	~sat.
S3	60	1.5	1.5	1.0	1.0	~sat.
S4	30	1.5	1.5	1.0	1.0	2.0
S5	21	1.5	1.5	1.0	1.3**	2.0
S6	21	1.5	1.5	1.0	1.3	1.5
S7	6	1.5	1.5	1.0	1.3	1.0

* typical value reported in literature for synthetic influents of partial nitritation reactors.
** average value observed in real AD-FW wastewater.

As to the Anammox unit, a 3 L sequencing batch reactor (SBR) was operated at controlled temperature (35±0.5 °C) and pH (7.0±0.1), and inoculated with granular Anammox biomass originating from a previous experimental campaign. The 6-hour cycle configuration consisted of 200-267 min feeding, 83-150 min reaction, 5 min settling and 5 min effluent withdrawal. Mechanical mixing was provided by a marine impeller (80±5 rpm). At the beginning of each working cycle, inert N_2 gas was flushed for 5 min, in order to assure anaerobic conditions inside the SBR. Temperature was controlled by a water jacket and a thermostatic bath (HAAKE, mod. F3-K); pH was maintained within the chosen range using 1M HCl and 1M NaOH. The vessel

was completely covered with tin foil, in order to avoid any penetration of light which would hinder anammox activity [3]. Temperature, ORP and pH monitoring was performed using InPro 3250i pH/ORP probe (Mettler-Toledo) connected to a digital transmitter (Mettler Toledo, mod. M300). Process timing and control were performed via National Instrument CompactRio system and a custom made LabView (v.10.0) application.

The composition of the synthetic medium was: NH_4HCO_3 3,848-3,938 mg/L, $NaNO_2$ 3,952-4,030 mg/L, $MgSO_4 \cdot 7\ H_2O$ 200 mg/L, KH_2PO_4 6.25 mg/L, $CaCl_2$ 300 mg/L, $FeSO_4 \cdot 7\ H_2O$ 12.5 mg/L and trace elements solution 1.25 mL/L [4]. Total influent nitrogen concentration was kept equal to 1,500 mg/L. Nitrogen loading rate (NLR) was progressively increased during the experiment, and hydraulic retention time (HRT) changed correspondingly, as showed in table 3.2, Accordingly, the influent flow rate and the volumetric exchange ratio varied between 1.0-2.0 mL/min and 0.11-0.17, respectively.

Table 3.2. Plan of the experimental activity and main operating conditions for the Anammox unit.

Phase	Duration	NH_4-N inf.	NO_2-N inf.	Influent NO_2-N/NH_4-N molar ratio	NLR	HRT
	(d)	(g/L)	(g/L)	(-)	(gN/L·d)	(d)
A1	35	0.70	0.80	1.15	1.0	1.5
A2	43	0.70	0.80	1.15	1.2	1.25
A3	176	0.70	0.80	1.15	1.5	1.0
A4	96	0.65	0.85	1.30	1.5	1.0

Influent and effluent NH_4-N concentration was determined according to Standard Methods [5], using a Hitachi U-2000 spectrophotometer at a wavelength of 420 nm. NO_2-N and NO_3-N was determined by ion-chromatography using a DIONEX ICS-90 equipped with an AS14A Ion-PAC 5 µm column. All samples were filtered (0.45 µm) before analyses, which were performed in triplicate. Free Ammonia (FA) and Free Nitrous Acid (FNA) concentrations were estimated according to Anthonisen et al. [6]. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods [5].

Batch assessment of acute toxicity effects of real AD-FW wastewater produced by a two-stage anaerobic digestion process aimed at the recovery of H_2 and CH_4 from food waste on ammonium oxidizing bacteria were carried out as described by Ficara and Rozzi [7] on unacclimated biomass drawn from the PN reactor. Such test is based on the determination of IC_{50} value, i.e. the concentration of the tested wastewater/compound causing a 50% decrease in initial AOB activity. Also, prolonged exposure tests were carried out on the PN reactor by

temporarily replacing the synthetic influent with real AD-FW wastewater, using different exposure times. Specific Anammox activity (SAA) was determined according to [8].

Microbiological characterization was performed by fluorescence in situ hybridization (FISH) on representative biomass samples, according to Amann et al. [9]. Hybridizations with group specific probes for ammonium oxidizing bacteria (NSO1225, NSO190), nitrite oxidizing bacteria (NTSPA and NIT3), and Anammox bacteria (AMX820, AMX368 and PLA46) were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria, and with DAPI staining for quantifying the total number of cells. All probes were purchased from MWG-Biotech (Germany), and synthesized with 5'-FITC (green) and 5'-Cy3 (red) labels. Details on oligonucleotide probes are available at ProbeBase (Loy et al., 2007). Slides were examined with an epifluorescence microscope (Olympus BX51) at different magnifications (100, 400 and 1000x); images were captured with an Olympus XM10 camera using Cell-F software (Olympus, Germany). DAIME software [10] was used for FISH quantification of hybridized cells.

3.3 Results and discussion

Partial nitritation was successfully achieved at each applied HRT (Figure 3.1), and NO_3-N production remained always below 3% of total influent nitrogen, indicating the successful washout of nitrite oxidizing bacteria (NOB). However, a slightly longer period was required to achieve stable NH_4-N removal efficiencies at the lowest HRT tested (it took 6, 5 and 11 days for HRT of 1.5, 1.25 and 1 d, respectively), due to the combined effect of low solids retention time and increased NLR.

According to FISH analysis (Figure 3.2a and 3.2b), ammonium oxidizing bacteria were found to be dominant in the system (74% of total bacteria), and confirmed the almost complete washout of NOB (3% of total bacteria).

Overall process performance did not change substantially, as summarized in Table 3.3. In particular, both NH_4-N removal efficiency and effluent NO_2-N/NH_4-N molar ratio were lower than the stoichiometric values (i.e. 50% and 1.0, respectively), given the influent Alkalinity/NH_4-N molar ratio of 1 and irrespective of the DO concentrations tested. As suggested by Van Hulle et al. [11], the short applied HRT (1 d) may have caused a mild limiting effect on AOB cell growth, as confirmed by the relatively low biomass concentration achieved in the PN reactor (134-198 mgVSS/L). As for other possible inhibiting factors, such as an

excess of free ammonia and free nitrous acid, or a lack of inorganic carbon, they can be reasonably excluded, since they were outside the ranges considered as inhibiting, according to Van Hulle et al. [12] and Guisasola et al. [13], respectively.

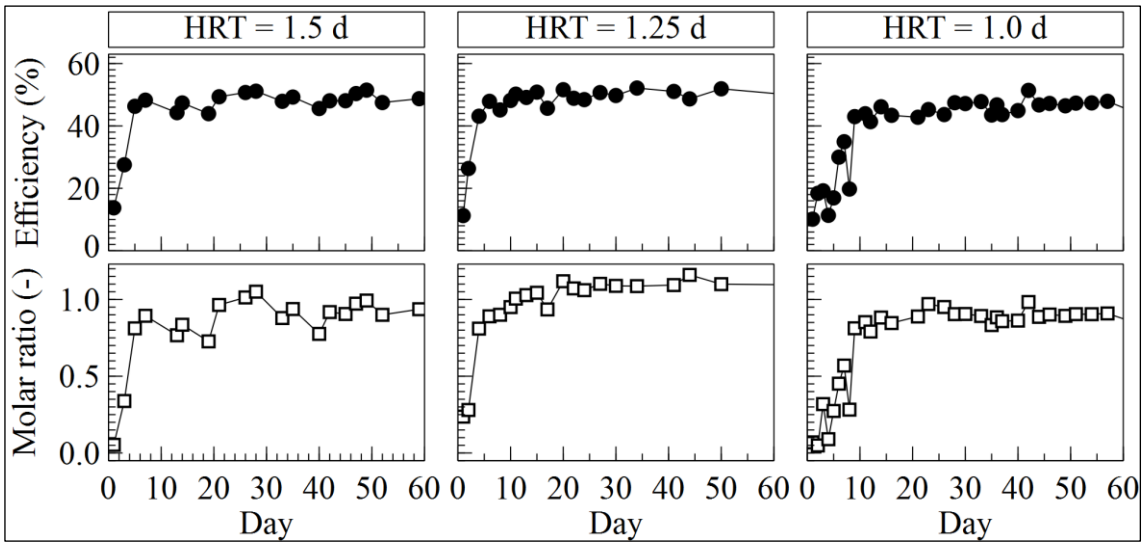

Figure 3.1: Time profiles of ammonium removal efficiency (●) and effluent NO_2-N/NH_4-N molar ratio (□) during Phases S1, S2 and S3 of the Sharon reactor.

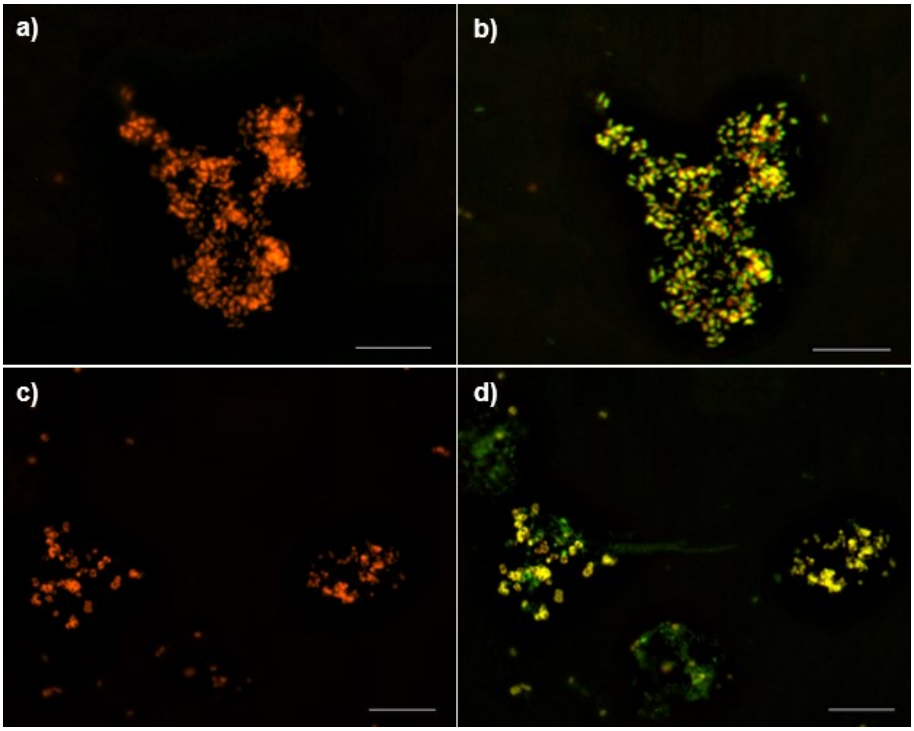

Figure 3.2: FISH micrographs of PN biomass samples drawn at the end of Phase S3 (a, b), and Anammox biomass samples drawn at the end of Phase A4 (c, d). a) Cy3-labeled ammonium oxidizing bacteria (NSO1225 probe); b) overlapping of FITC-labeled EUBmix and Cy3-labeled NSO1225 probes, resulting in yellow AOB cells; c) Cy3-labeled anammox bacteria (AMX820 probe); d) overlapping of FITC-labeled EUBmix and Cy3-labeled AMX820 probes, resulting in yellow anammox cells. Scale bar is 20 µm.

Table 3.3: Average performance values of PN reactor during Phases S1 to S4.

Phase	Effluent NH_4-N (mg L^{-1})	Effluent NO_2-N (mg L^{-1})	Effluent NO_3-N (mg L^{-1})	Effluent NO_2-N/NH_4-N molar ratio (-)	NH_4-N removal efficiency (%)
S1	787±52	627±66	7.7±3.4	0.90±0.09	48.2±2.2
S2	756±33	781±42	19.6±1.9	1.04±0.08	49.2±2.0
S3	799±30	721±20	10.3±3.3	0.90±0.05	46.4±2.2
S4	802±41	714±43	10.8±3.5	0.89±0.09	47.0±2.3

During Phase S5, the increase in Alk/N ratio up to the average value observed in real AD-FW wastewater (i.e., 1.3) led to a corresponding increase in both NH_4-N removal efficiency (from 47.0±2.3 to 60.8±4.5%) and effluent NO_2-N/NH_4-N molar ratio (from 0.89±0.09 to 1.58±0.27), compared to Phase S4. Surprisingly, good process performance was maintained even when DO concentration was reduced to 1.5 ppm (Phase S6), which is lower than DO levels usually adopted in conventional wastewater treatment plants; however, such interesting behavior must be confirmed with real AD-FW wastewater, where organics are also present. Further decrease in DO concentration (Phase S7) led to irreversible worsening of process performance (Figure 3.3).

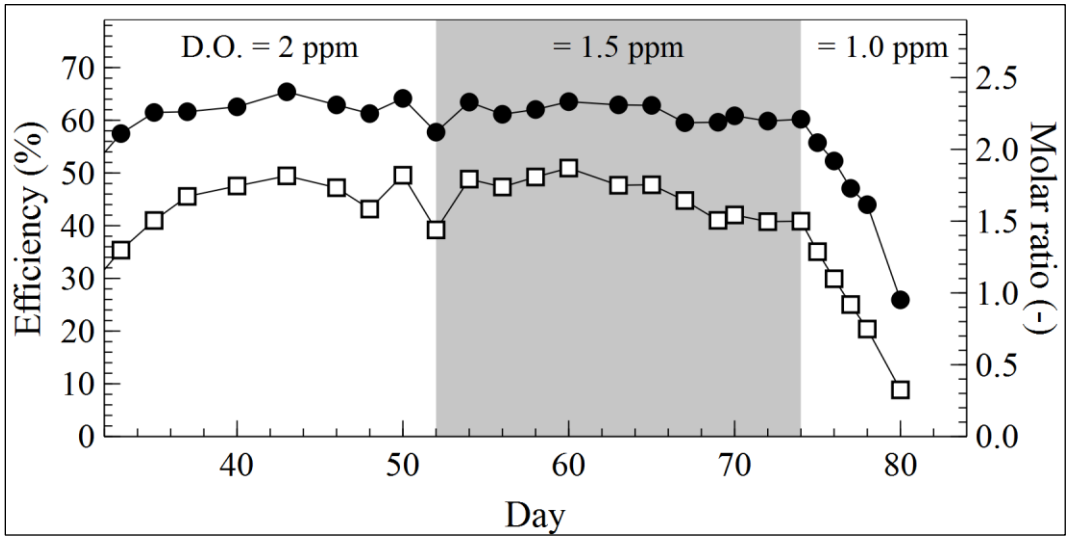

Figure 3.3: Time profiles of ammonium removal efficiency (●) and effluent NO_2-N/NH_4-N molar ratio (□) during Phase S5 (D.O., 2 ppm), S6 (D.O., 1.5 ppm) and S7 (D.O., 1 ppm) of the PN reactor.

Acute toxicity assessments carried out on unacclimated biomass drawn from the PN reactor showed the strong toxicity of real AD-FW even at low concentrations: IC_{50} dosages ranged from 11 to 47 mL/L, with a strong positive correlation with VSS concentration (Figure 3.4). Prolonged exposure tests (exposure times of 1 h, 2 h, 4 h and 8 h) were carried out during S3 experimental phase. Results showed an increase in process performance, which was directly

proportional to exposure time. During the 8 h-long test, NH_4-N removal efficiency and effluent NO_2-N increased from 47% to 56%, and from 726 to 848 mg/L, respectively. Such positive effect progressively decreased after switching back to the synthetic feeding, and it was related to the combination of AD-FW wastewater higher Alk/N ratio compared to S3 synthetic influent (1.3 and 1.0, respectively), and dilution rate in the chemostat, which avoided biomass inhibition.

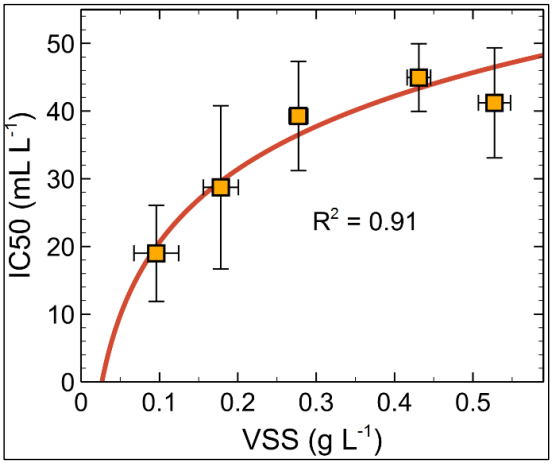

Figure 3.4. Correlation between VSS concentration and measured IC50 value of real AD-FW wastewater on PN biomass.

As to the Anammox reactor, a fairly stable behaviour was observed throughout the whole experiment (Fig. 3.5): nitrite discharge rate (NitDR) was negligible (mostly zero), the NRR (nitrogen removal rate) to NLR ratio (NRR/NLR) was 97±4%, and the total nitrogen removal efficiency (NRE) was 89±4%, indicating good process performance. The observed removed NH_4-N/removed NO_2-N/produced NO_3-N ratio was 1/1.24±0.10/0.19±0.02 during Phases A1 through A3. During Phase A4, a higher influent NO_2-N/NH_4-N ratio was applied, mainly resulting in a slight increase in overall performance (NRR/NLR ratio and NRE increased to 98.5±0.5% and 89.9±0.5%, respectively).

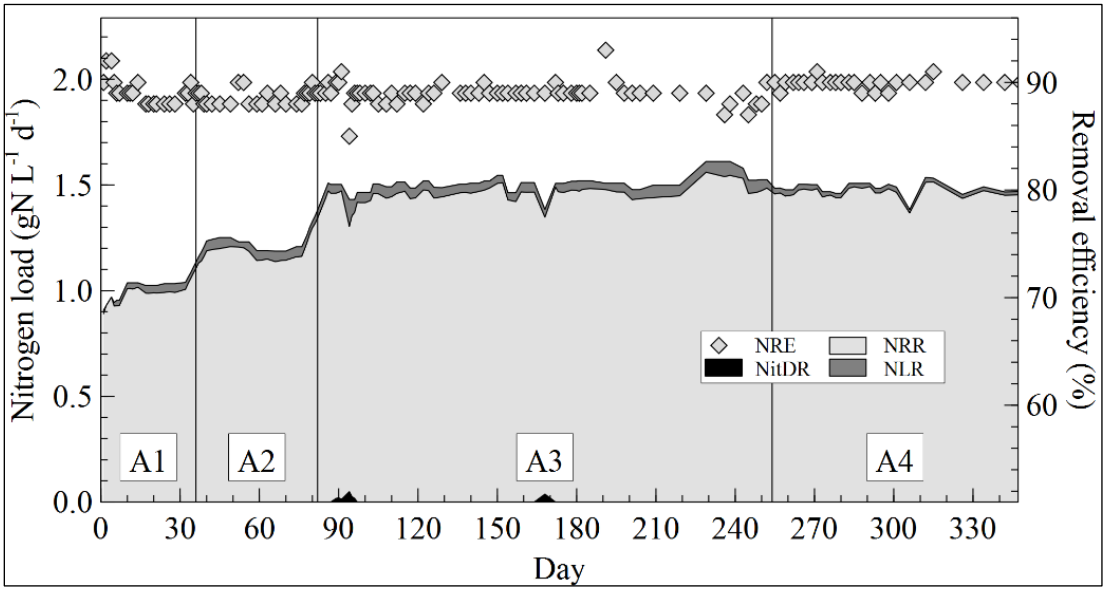

Figure 3.5: Time profiles of NLR, NRR, NitDR and NRE in the Anammox unit.

SAA showed an increasing trend as the applied NLR was increased (Phases A1-A3), while no substantial differences were observed between Phases A3 and A4 (Figure 3.6).

As expected, FISH analysis confirmed the abundance of Anammox bacteria (Figure 3.2.c and 3.2.d), which accounted for 66±1.8% of total bacteria (Phase A4), in agreement with previously reported studies [14].

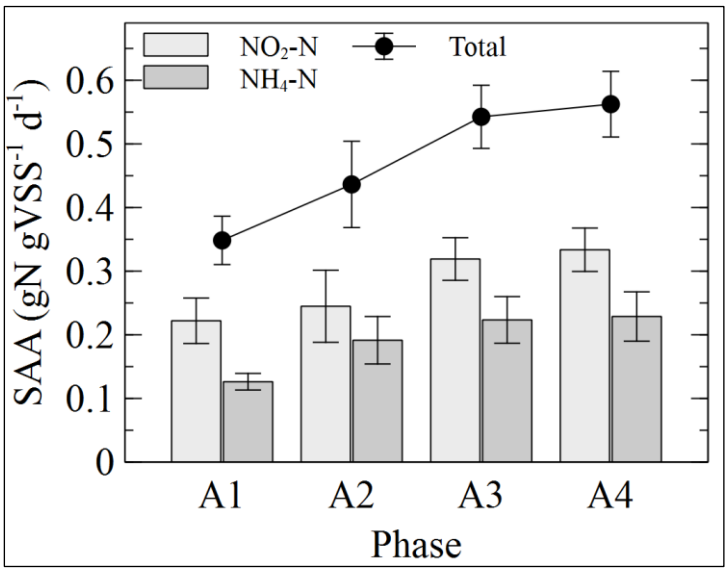

Figure 3.5: Average specific Anammox activity (SAA) observed during Phases A1, A2, A3 and A4.

3.4 Conclusions

Based on the results observed, the following main conclusions can be drawn:

- reducing the HRT in the PN reactor did not cause any significant effect on process performance, although a slightly longer time was required to achieve process stability;
- increasing synthetic influent alkalinity up to typical values of real AD-FW wastewater led to an improvement of PN reactor performance, which produced an effluent suitable to be treated by Anammox even at low DO concentrations (1.5 ppm);
- although acute toxicity batch assessments showed a potential toxicity of real AD-FW wastewater, prolonged exposure tests (continuous operation) showed an improvement of process performance, suggesting the combination of AD-FW wastewater high alkalinity and dilution rate in the chemostat as a possible key factor to avoid inhibition and process failure when switching to real AD-FW wastewater;
- granular Anammox SBR was able to withstand the same NLRs applied to the PN unit, and the increase of influent NO_2-N/NH_4-N molar ratio (corresponding to higher NH_4-N removal rates in the Sharon reactor) led to an increase in NRE.
- The information gathered in this study will be useful for the treatment of real AD-FW wastewater.

3.5 References

[1] S. Roy, D. Das, Biohythane production from organic wastes: present state of art, Environ. Sci. Pollut. Res. 23 (2016) 9391–9410. doi:10.1007/s11356-015-5469-4.

[2] S. Milia, M. Perra, G. Cappai, A. Carucci, SHARON process as preliminary treatment of refinery wastewater with high organic carbon-to-nitrogen ratio, Desalin. Water Treat. 3994 (2015) 1–9. doi:10.1080/19443994.2015.1087341.

[3] W.R.L. Van Der Star, A.I. Miclea, U.G.J.M. Van Dongen, G. Muyzer, C. Picioreanu, M.C.M. Van Loosdrecht, The membrane bioreactor: A novel tool to grow anammox bacteria as free cells, Biotechnol. Bioeng. 101 (2008) 286–294. doi:10.1002/bit.21891.

[4] A.A. Van De Graaf, P. De Bruijn, L.A. Robertson, M.S.M. Jetten, J.G. Kuenen, Autotrophic growth of anaerobic in a fluidized bed reactor, (1996) 2187–2196.

[5] APHA, Standard Methods for the Examination of Water and Wastewater, 21 st, American Public Health Association, 2005.

https://books.google.it/books?id=buTn1rmfSI4C.

[6] A.C. Anthonisen, R.C. Loehr, T.B.S. Prakasam, E.G. Srinath, Inhibition of Nitrification by Ammonia and Nitrous Acid, J. (Water Pollut. Control Fed. 48 (1976) 835–852. http://www.jstor.org/stable/25038971.

[7] E. Ficara, A. Rozzi, pH-Stat Titration to Assess Nitrification Inhibition, J. Environ. Eng. 127 (2001) 698–704. doi:10.1061/(ASCE)0733-9372(2001)127:8(698).

[8] E. Torfs, I. Nopens, M.K.. Winkler, S. Balemans, I. Smets, Experimental Methods In Wastewater Treatment, 2016. doi:10.1017/CBO9781107415324.004.

[9] R.I. Amann, W. Ludwig, K.H. Schleifer, Phylogenetic identification and in situ detection of individual microbial cells without cultivation . Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation, Microbiol. Rev. 59 (1995) 143–169. doi:10.1016/j.jip.2007.09.009.

[10] H. Daims, S. Lücker, M. Wagner, daime, a novel image analysis program for microbial ecology and biofilm research, Environ. Microbiol. 8 (2006) 200–213. doi:10.1111/j.1462-2920.2005.00880.x.

[11] S.W.H. Van Hulle, S. Van Den Broeck, J. Maertens, K. Villez, B.M.R. Donckels, G. Schelstraete, E.I.P. Volcke, P.A. Vanrolleghem, Construction, start-up and operation of a continuously aerated laboratory-scale SHARON reactor in view of coupling with an Anammox reactor, Water SA. 31 (2005) 327–334. doi:10.4314/wsa.v31i3.5222.

[12] S.W. Van Hulle, E.I. Volcke, J.L. Teruel, B. Donckels, M.C. van Loosdrecht, P.A. Vanrolleghem, Influence of temperature and pH on the kinetics of the Sharon nitritation process, J. Chem. Technol. Biotechnol. 82 (2007) 471–480. doi:10.1002/jctb.1692.

[13] A. Guisasola, S. Petzet, J.A. Baeza, J. Carrera, J. Lafuente, Inorganic carbon limitations on nitrification: Experimental assessment and modelling, Water Res. 41 (2007) 277–286. doi:10.1016/j.watres.2006.10.030.

[14] W.R.L. van der Star, W.R. Abma, D. Blommers, J.W. Mulder, T. Tokutomi, M. Strous, C. Picioreanu, M.C.M. van Loosdrecht, Startup of reactors for anoxic ammonium oxidation: Experiences from the first full-scale anammox reactor in Rotterdam, Water Res. 41 (2007) 4149–4163. doi:10.1016/j.watres.2007.03.044.

Chapter 4

Evaluation of nitrous oxide gaseous emissions from a partial nitritation reactor operating under different conditions

4.1 Introduction

According to the intergovernmental panel on climate change [1], nitrous oxide (N_2O) has a strong ozone layer depleting potential, and the third largest radiative forcing (i.e., the capacity of a gas to affect the balance between incoming solar radiation and outgoing infrared radiation, thereby contributing to climate change) among the anthropogenic gases, with an estimated lifetime of 131 years and a global warming potential up to 300 fold higher than CO_2.

Although the emissions of nitrous oxide from wastewater treatment plants (WWTPs) are relatively small (3% of the estimated total anthropogenic N_2O emissions), indeed they represent a significant factor (26%) in the greenhouse gas (GHG) footprint of the total water chain [2]. In recent years, the assessment of N_2O emissions from WWTPs, in particular from biological nitrogen removal processes, has become of great environmental concern. Law et al. [3] reported two key metabolic pathways involved in N_2O production by autotrophic ammonia oxidizing bacteria (AOB): autotrophic ammonia oxidation, where N_2O can be formed as a side product during the conversion of the ammonium-oxidation intermediate hydroxylamine (NH_2OH) to nitrite; and nitrifier denitrification (i.e. the reduction of NO_2 to NO and N_2O by autotrophic AOB, under oxygen limiting conditions).

For the treatment of ammonium-rich liquid streams like, among the others, reject water, landfill leachate, livestock manure, and petrochemical wastewater [4–7], the combination of partial nitritation (PN) and anammox (ANaerobic AMMonium OXidation) has been proved to be an efficient and cost-effective solution, compared with conventional biological processes based on nitrification and denitrification. However, shortcut nitrogen removal via the nitrite pathway is likely a major contributor to overall N_2O emission [8], since nitrite is reported to trigger nitrous oxide production [2].

In this study, a lab-scale PN reactor was fed with an ammonium-rich synthetic medium simulating the NH_4-N content and alkalinity of the liquid effluent produced by the anaerobic digestion of food waste (AD-FW), and gaseous N2O emissions were measured with different

operating conditions, in order to strike the right balance between overall process performance and N2O release in the atmosphere.

4.2 Materials and methods

4.2.1 Reactor setup and operation

The PN unit consisted of a 2 L continuous flow stirred tank reactor operated as a chemostat (without biomass recirculation). A thermostatic bath was used to control temperature at 35±0.5°C; pH was constantly monitored and kept within the range 6.0-7.5 by dosing acid (H_2SO_4, 1M) or base (NaOH, 1M) solutions.

During the experiments, dissolved oxygen (DO) concentration was continuously monitored, and maintained at the desired level by supplying a variable mixture of air and dinitrogen gas in the bulk liquid at a constant rate (1 L/min).

A schematic representation of the experimental apparatus is reported in Figure 4.1.

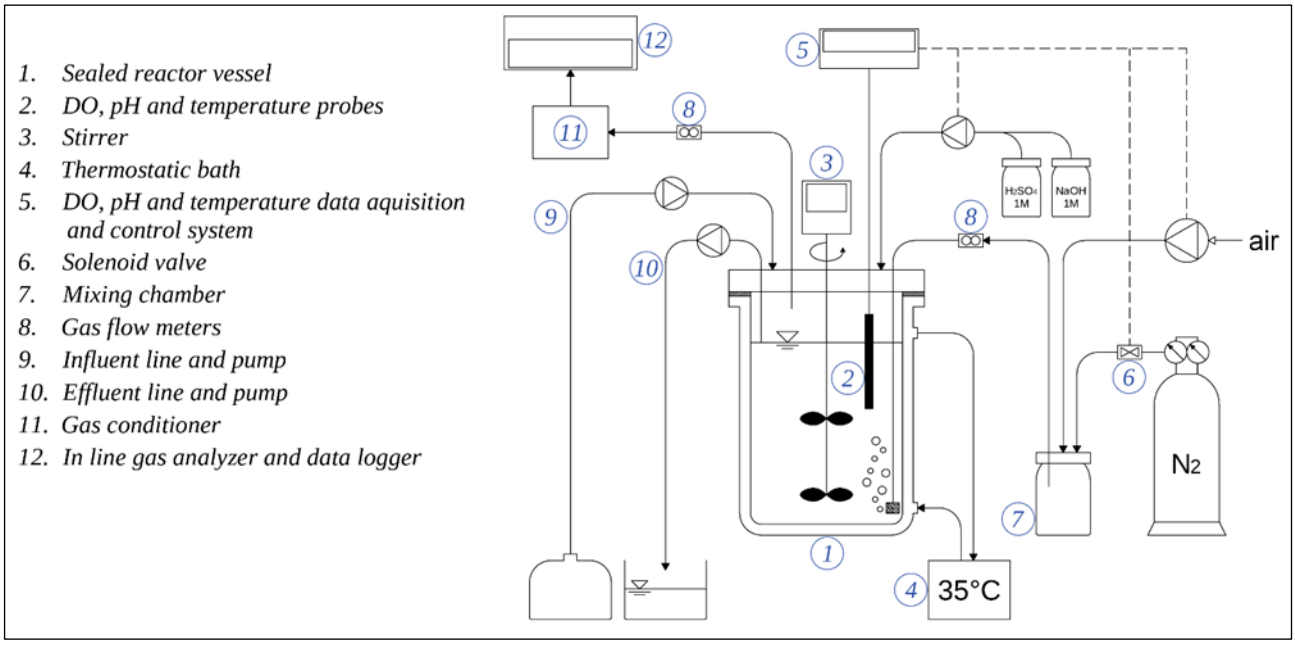

Figure 4.1: Schematic representation of the experimental apparatus.

As reported in Milia et al. [9], the reactor was inoculated with activated sludge drawn from the municipal wastewater treatment plant of Cagliari (Italy), fed with an ammonium-rich synthetic medium (1,500 mgNH_4-N/L), and operated for four months before carrying out the experiments described in this study.

Influent flow rate was kept at 1.4 mL/min, resulting in a hydraulic retention time (HRT) and a corresponding sludge retention time (SRT) of 1 d. Total nitrogen loading rate (NLR) was 1.5 gN/L·d. The influent alkalinity to ammonium-nitrogen molar ratio (Alk/N) was increased from 1 to 1.3 by adding bicarbonate (as $NaHCO_3$) to the medium. The plan of the experimental activity is summarized in Table 1.

Table 4.1: Plan of the experimental activity.

Phase	Duration (d)	DO (mgO_2/L)	Alk/N (-)
1	34	5.0	1.0*
2	19	3.0	1.0
3	17	2.0	1.0
4	21	2.0	1.3**
5	21	1.5	1.3
6	6	1.0	1.3

* typical value reported in literature for synthetic influents fed to PN reactors.
** average value observed in real AD-FW wastewater.

The overall composition of the synthetic medium was: NH_4HCO_3 8,466 mg/L, KH_2PO_4 1,000 mg/L, $MgSO_4$ 100 mg/L, $NaHCO_3$ 0-2,700 mg/L, and 10 mL/L trace elements according to Milia et al. [6]. The resulting pH was 7.8-8.0.

4.2.2 Analytical procedures

Influent and effluent NH_4-N concentration was determined according to Standard Methods [10], using a Hitachi U-2000 spectrophotometer at a wavelength of 420 nm. The concentration of NO_2-N and NO_3-N was determined by ion-chromatography using a DIONEX ICS-90 equipped with an AS14A Ion-PAC 5 µm column. All samples were filtered (0.45 µm) before analyses, which were performed in triplicate. Free Ammonia (FA) and Free Nitrous Acid (FNA) concentrations were estimated according to Anthonisen et al. (1976) [11]. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods [10].

Nitrous oxide measurement campaigns were carried out during each experimental Phase, as steady state conditions were achieved (the only exception was Phase 6, due to the lack of process stability): headspace gas was collected from the reactor at constant flow rate (1 L/min), sent to a gas conditioning system (Bühler, mod. TGAK 3) and then to a gas analyzer (Servomex, mod. 4100), where continuous measurement of N_2O concentration (as ppmv) was performed via infra-red gas-filter correlation. Continuous mixing and aeration avoided N2O accumulation in the bulk liquid. Each measurement campaign lasted from 13 to 24 hours, and data acquisition

rate was set at 1 sample/min. The N_2O-N emission rate (ER) was calculated according to Lv et al. [12], with some modifications (Eq. 1):

$$ER = c \cdot Q \cdot p \cdot M_N \cdot 2/(1000 \cdot R \cdot T \cdot V_L) \quad \text{(Eq. 1)}$$

Where: ER is the N_2O-N emission rate (mgN/L·d), c is the N_2O level in the gas sample (ppmv), Q is the volumetric flow rate of the off-gas (L/d), p is the atmospheric pressure (1 atm), M_N is the molar mass of nitrogen (g/mol), R is the gas constant (0.082056 L·atm/mol·K), T is the temperature (K) and V_L is the working volume of the reactor (L).

The N_2O-N emission factor (EF) was then calculated by dividing ER by the corresponding average ammonium-nitrogen oxidation rate (AOR, mgN/L·d).

Liquid samples were also collected from the mixed liquor at the beginning/end of each N_2O measurement campaign, and analyzed in order to determine NH_4-N, NO_2-N and NO_3-N concentrations. The dissolved N_2O concentration could not be measured in the present experiment; by the way, continuous blowing of a variable mixture of air and dinitrogen gas at a constant rate allowed continuous stripping of dissolved nitrous oxide from the liquid phase. Moreover, given the operational conditions applied, gas solubility could be considered as a constant, thus, the evaluation of nitrous oxide gaseous emissions could be assumed as representative of overall N_2O production.

4.3 Results and discussion

4.3.1 Reactor performance

During the whole experiment, error in nitrogen balances averaged out at less than 1%. As shown in Figure 4.2, the decrease in DO concentration from 5 to 2 mgO_2/L (Phases 1-3) did not cause any significant change in NH_4-N, NO_2-N and NO_3-N effluent concentrations; coherently, the average ammonium removal efficiency and effluent NO_2/NH_4 molar ratio measured through Phases 1 to 3 were 47±3% and 0.9±0.1, respectively, indicating very stable process performance. Despite the high process stability, such values were found to be slightly lower than expected (i.e., 50% and 1.0, respectively), considering the applied Alk/N molar ratio [13]. As suggested by Van Hulle et al. [13], the short applied HRT (1 d) may have caused a mild limiting effect on AOB cell growth, as confirmed by the relatively low biomass concentration achieved in the PN reactor (Table 4.2). As for other possible inhibiting factors, such as an excess of free ammonia and free nitrous acid, or a lack of inorganic carbon, they can be reasonably

excluded, since they were outside the ranges considered as inhibiting, according to Van Hulle et al. [14] and Guisasola et al. [15], respectively.

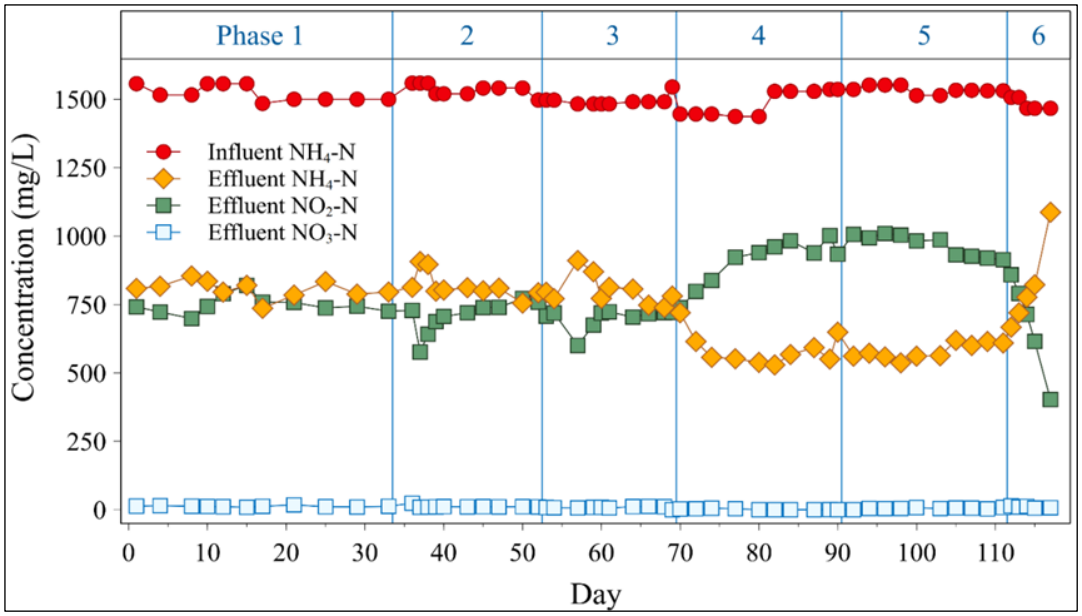

Figure 4.2. Trends of NH_4-N (influent and effluent), NO2-N (effluent) and NO3-N (effluent) concentrations observed during the whole experimental study.

Starting from day 70 (Phase 4), the increase in Alk/N molar ratio to 1.3 led to a corresponding increase in both NH_4-N removal efficiency (from 47±3 to 61±5%) and effluent NO_2/NH_4 molar ratio (from 0.9±0.1 to 1.6±0.3), compared to previous Phases. Decreasing DO concentration to 1.5 mgO_2/L (Phase 5) did not cause any significant change in overall process performance, suggesting that limiting conditions did not occurred even below the DO concentration usually adopted in conventional WWTPs.

Table 4.2. Average process performance observed during each experimental Phase, under steady state conditions (Phase 6 is not considered, due to process instability).

Phase	Influent NH_4-N (mg/L)	Effluent NH_4-N (mg/L)	Effluent NO_2-N (mg/L)	Effluent NO_3-N (mg/L)	NH_4 removal efficiency (%)	Effluent NO_2/NH_4 ratio (-)	Biomass concentration (mgVSS/L)	Maximum FA concentration (mgNH3/L)
1	1522±29	806±32	749±33	12±2	47±2	0.9±0.1	157±47	13.8
2	1536±21	819±47	707±59	12±4	47±3	0.9±0.1	134±26	14.0
3	1490±6	789±42	709±16	9±2	47±3	0.9±0.1	135±25	13.5
4	1482±47	580±59	902±90	2±2	61±5	1.6±0.3	177±16	9.9
5	1535±13	598±59	953±64	4±2	61±4	1.6±0.2	198±25	10.2

However, the further decrease in DO concentration from 1.5 to 1.0 mgO_2/L (Phase 6) led to the irreversible worsening of process performance (Figure 4.2): ammonia started to accumulate and pH raised up to 7.5 due to the lower alkalinity removal. After few days an almost complete washout of biomass was observed. Maximum FA concentration in the reactor was estimated

around 50 mgNH_3/L, much lower than those indicated as inhibiting by Van Hulle et al. [14]; therefore, the worsening of process performance was ascribed to the occurrence of oxygen limiting conditions. The threshold level observed in this study for DO concentration (1.5 mgO_2/L) may depend on several factors (e.g., reactor configuration, low HRT, etc.), and is consistent with the broad range of DO threshold concentrations reported in previous studies: Guisasola et al. [16] indicated an oxygen affinity constant for autotrophic ammonium oxidation within the range 0.16-2.0 mgO_2/L; Van Hulle et al. [14] observed the worsening of process performance in a Sharon reactor operating at DO concentration lower than 3 mgO_2/L; more recently, partial nitritation SBRs were successfully operated at DO concentration even lower than 1 mgO_2/L [12,17]. Although it cannot be excluded that fine tuning of process parameters would allow to reduce the DO threshold concentration, such investigation was out of the scope of this study.

4.3.2 N_2O emissions

As long as process performance was good and stable in terms of AOR and ammonium conversion to nitrite (Phases 1-5), N_2O emissions in the off-gas remained very low (average N_2O concentration in the off-gas and ER ranged between 2.5-3.2 ppmv and 2.1-2.7 mgN/L·d, respectively), regardless of the applied DO concentration (Figure 4.3a). Coherently, the increase in AOR due to the higher Alk/N molar ratio applied during Phases 4 and 5 was accompanied by a significant reduction of N2O-N emission factor, i.e. from 0.35% (Phases 1-3) to 0.23% (Phases 4-5) of AOR (Figure 4.3b). The low N_2O-N emission factors observed during Phases 1 to 5 can be likely ascribed to the positive effect of continuous mixing and aeration, which minimized the occurrence of anoxic conditions in the lab-scale reactor even at relatively low DO concentrations (1.5 mgO_2/L, Phase 5), thus reducing the occurrence of nitrifier denitrification. Indeed, such results look promising, compared with those previously reported in literature, although a direct comparison may be difficult, since the broad range of reported N_2O emission rates refer to very different system configurations (i.e. chemostat, continuous flow stirred tank reactor, sequencing batch reactor), size (i.e. lab-scale, pilot plant, full-scale plant) and operating conditions [2,18,19].

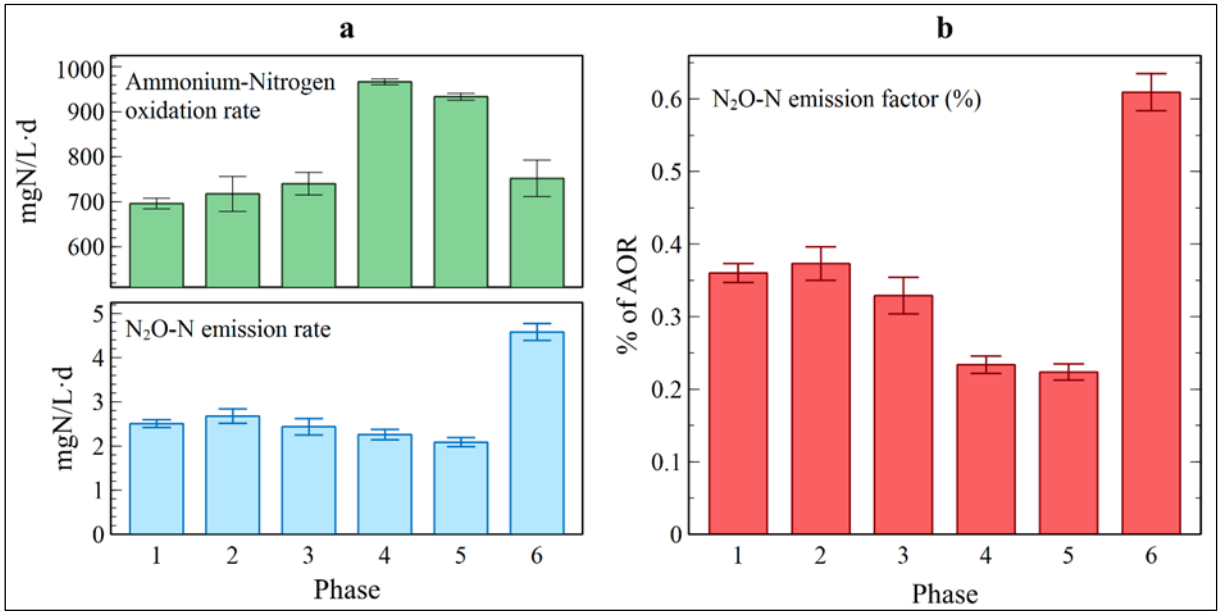

Figure 4.3. Process performance in terms of AOR and N2O-N emission rate (a), and N2O-N emission factor (b), observed during the experimental campaign.

De Graaff et al. [20] treated the liquid effluent of a UASB reactor using a continuous flow reactor without biomass retention, with a DO concentration above 2 mgO_2/L, and detected a N_2O emission factor ranging between 0.6 and 2.6% of total nitrogen load (0.14-0.30% in this study); Law et al. [21] determined an average N_2O emission factor of 1.0±0.1% of total ammonium converted in a PN-SBR; lab-scale PN-SBR systems, fed with synthetic influents and operated at different DO levels, were also studied by Rathnayake et al. [22] (DO = 2 ppm), who reported quite variable nitrous oxide emission factors, averaged out at 1.5±0.8% of the converted ammonium, and Kinh et al. [8], who observed a N_2O emission factor of 0.11-0.90% of oxidized ammonium, depending on pH, with DO kept in the range of 0.5-1.0 ppm: in both cases hydroxylamine oxidation, which is proportional to ammonia oxidation rate, was identified as the major N_2O production contributor. On the other hand, a different behavior was observed in our study, since nitrous oxide emission factor decreased as AOR increased (Phases 4-5), thus indicating the minimization of hydroxylamine oxidation-driven N2O production at DO levels of 2 ppm or lower. Pijuan et al. [23] studied the effect of different DO concentrations on N_2O emissions from a continuous pilot-scale granular airlift reactor performing both full and partial nitritation: the lowest emission factor (2.2% of total converted ammonium) was measured at DO concentrations above 4.5 mgO_2/L (when DO was reduced, a proportional increase of N_2O emission factor was observed, up to 6% of converted ammonium); a similar behavior was reported by Lv et al. [12] who achieved stable partial nitritation in a SBR operating at oxygen-limiting conditions (DO = 0.35-0.85 mgO_2/L), and measured N_2O emissions ranging from 0.57 to 2.35% of total influent nitrogen; conversely, in this study the emission factor decreased as

dissolved oxygen decreased (Phases 1-5), as long as limiting conditions did not occur. A schematic comparison among results reported in literature and those achieved in this study is reported in Table 4.3.

As DO concentration was further reduced to 1 mgO_2/L (Phase 6), the sudden increase in both N_2O emission rate (> 4.5 mgN/L·d) and factor (up to 0.61% of AOR) was observed, consistently with the worsening of the overall process performance described previously. At DO concentrations below 1.5 mgO_2/L, the shortage of available oxygen likely caused the increase in the anoxic formation of N_2O due to nitrifier denitrification, which has been recognized as the main pathway contributing to N_2O production [3]. Law et al. [24] observed an opposite behavior (i.e., the decrease in N_2O production with decreasing DO concentration) in AOB cultures previously adapted to low DO concentrations (0.5-0.8 mgO_2/L) and exposed to relatively high NH_4-N and NO_2-N concentrations (500 mgN/L); however, influent NH_4-N concentration was much higher in our study (1,500 mgN/L), so that complete AOB acclimation to low DO levels may not be enough to achieve stable partial nitritation.

4.4 Conclusions

In this study, a PN reactor was fed with a synthetic medium at a constant NLR of 1.5 gN/L·d, and N_2O gaseous emissions were measured with different applied Alk/N molar ratios (1.0-1.3) and DO concentrations (5.0-1.0 mgO_2/L). As DO concentration was not limiting, stable process performance was achieved in terms of AOR and ammonium conversion to nitrite, and N_2O emissions in the off-gas were lower than most of the values reported in previous studies. The increase in influent Alk/N was accompanied by the corresponding increase in AOR, while N_2O emission rates did not change significantly, thus resulting in the reduction of the N_2O-N emission factor. Unlike many of the results previously reported in literature, where increase in AOR or decrease in DO level appeared to trigger nitrous oxide production, reactor configuration adopted in this study (i.e., non-aerated settling and discharge phases were avoided) coupled with a continuous aeration strategy led to minimization of anoxic conditions. As a consequence, this contributed to the reduction of N_2O emissions even at low, as long as not process-limiting, dissolved oxygen concentrations. As DO was set at 1.0 mgO_2/L, overall process performance was irreversibly compromised: beside the drop in AOR, a corresponding increase in N_2O-N emission factor was observed. Nitrifier denitrification, more than hydroxylamine oxidation, was suggested as the main pathway contributing to N_2O formation.

Results showed that the environmental footprint of partial nitritation process can potentially be reduced by applying proper aeration strategy at relatively low DO concentrations; moreover, chemostat reactor configuration can represent a suitable choice even at high N load. Results are promising, and further investigation in this sense will be carried out treating real wastewater.

Table 4.3. Schematic comparison among results reported in literature.

Reference	PN-reactor type	Influent type	Influent NH_4-N (mg/L)	HRT (d)	NLR (gN/L d)	D.O. (mg/L)	N_2O-N emission factor (per influent NH_4-N)	N_2O-N emission factor (per oxidized NH_4-N)
This study*	Chemostat, lab-scale	Synthetic	1,535±13	1.0	1.5	1.5	0.14%	0.23%
[8]	SBR, lab-scale	Synthetic	300-1,000	1.0	0.3-1.0	0.5-1.0	-	0.11-0.90%
[12]	SBR, lab-scale	Synthetic	600	0.5	1.2	0.35-0.85	0.57-2.35%	-
[18]	Chemostat, full scale	Reject water	1,200-1,600	2.0-3.0	0.4-0.8	2.5	1.7%	3.4%
[20]	Air-lift continuous reactor, lab-scale	Anaerob. digested black water	1,500±0.19	1.3-1.7	0.88-1.15	> 2	0.6-2.6%	-
[21]	SBR, lab-scale	Synthetic	1,000	1.0	8.0	0.5-0.8	-	1.0%
[22]	Granular SBR, lab-scale	Synthetic	350	0.3	1.0	2.0	0.8±0.4%	1.5±0.8%
[23]	Granular airlift continuous reactor, pilot scale	Reject water	726±50	0.4-0.6	0.85	4.4-6.7	-	2.2±0.4 %
[23]	Granular airlift SBR, pilot scale	Reject water	450±78	0.4-0.6	approx. 0.9	5.7-7.2	-	19.3±7.5 %

* Values observed during Phase 5 (best performance)

.

4.5 References

[1] Intergovernmental Panel on Climate Change, Climate Change 2013 – The Physical Science Basis: Working Group I Contribution to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change, Cambridge University Press, Cambridge, 2014. doi:DOI: 10.1017/CBO9781107415324.

[2] M.J. Kampschreur, H. Temmink, R. Kleerebezem, M.S.M. Jetten, M.C.M. van Loosdrecht, Nitrous oxide emission during wastewater treatment, Water Res. 43 (2009) 4093–4103. doi:10.1016/j.watres.2009.03.001.

[3] Y. Law, L. Ye, Y. Pan, Z. Yuan, Nitrous oxide emissions from wastewater treatment processes, Philos. Trans. R. Soc. Lond. B. Biol. Sci. 367 (2012) 1265–77. doi:10.1098/rstb.2011.0317.

[4] S.W.H. Van Hulle, H.J.P. Vandeweyer, B.D. Meesschaert, P.A. Vanrolleghem, P. Dejans, A. Dumoulin, Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams, Chem. Eng. J. 162 (2010) 1–20. doi:10.1016/j.cej.2010.05.037.

[5] S. Milia, A. Carucci, G. Cappai, G. De Gioannis, A. Muntoni, M. Perra, M. Piredda, Sharon-anammox process for treatment of ammonium rich wastewater and Aerobic Granular Sludge technology for treatment of wastewater and groundwater contaminated by chlorinated organic pollutants. A state-of-the-art on recent and past research activities, in: K. Kuchta, M. Ritzkowsky, J. Heerenklage (Eds.), 1st Ger. Waste Dialog - Hamburg. Berichte, Verlag Abfall aktuell, Stuttgart, 2013: pp. 179–195.

[6] S. Milia, M. Perra, G. Cappai, A. Carucci, SHARON process as preliminary treatment of refinery wastewater with high organic carbon-to-nitrogen ratio, Desalin. Water Treat. 3994 (2015) 1–9. doi:10.1080/19443994.2015.1087341.

[7] S. Milia, M. Perra, G. Tocco, A. Carucci, The start-up of an anammox reactor as the second step for the treatment of ammonium rich refinery (IGCC) wastewater with high Corg/N ratio, Ecol. Eng. 106 (2017) 358–368. doi:10.1016/j.ecoleng.2017.04.068.

[8] C.T. Kinh, J. Ahn, T. Suenaga, N. Sittivorakulpong, P. Noophan, T. Hori, S. Riya, M. Hosomi, A. Terada, Free nitrous acid and pH determine the predominant ammonia-oxidizing bacteria and amount of N_2O in a partial nitrifying reactor, Appl. Microbiol. Biotechnol. (2016) 1–11. doi:10.1007/s00253-016-7961-2.

[9] S. Milia, G. Tocco, G. Erby, G. De Gioannis, A. Carucci, Preliminary Evaluation of Sharon-Anammox Process Feasibility to Treat Ammonium-Rich Effluents Produced by Double-Stage Anaerobic Digestion of Food Waste, in: G. Mannina (Ed.), Front. Wastewater Treat. Model. FICWTM 2017, Springer International Publishing, Cham, 2017: pp. 536–543. doi:10.1007/978-3-319-58421-8_84.

[10] APHA, Standard Methods for the Examination of Water and Wastewater, 21st, American Public Health Association, 2005.

[11] A.C. Anthonisen, R.C. Loehr, T.B.S. Prakasam, E.G. Srinath, Inhibition of Nitrification by Ammonia and Nitrous Acid, J. (Water Pollut. Control Fed. 48 (1976) 835–852. http://www.jstor.org/stable/25038971.

[12] Y. Lv, K. Ju, T. Sun, L. Wang, R. Miao, T. Liu, X. Wang, Effect of the dissolved oxygen concentration on the N2O emission from an autotrophic partial nitritation reactor treating high-ammonium wastewater, Int. Biodeterior. Biodegrad. 114 (2016) 209–215. doi:10.1016/j.ibiod.2016.01.022.

[13] S.W.H. Van Hulle, S. Van Den Broeck, J. Maertens, K. Villez, B.M.R. Donckels, G. Schelstraete, E.I.P. Volcke, P.A. Vanrolleghem, Construction, start-up and operation of a continuously aerated laboratory-scale SHARON reactor in view of coupling with an Anammox reactor, Water SA. 31 (2005) 327–334. doi:10.4314/wsa.v31i3.5222.

[14] S.W.H. Van Hulle, E.I. Volcke, J.L. Teruel, B. Donckels, M.C. van Loosdrecht, P.A. Vanrolleghem, Influence of temperature and pH on the kinetics of the Sharon nitritation process, J. Chem. Technol. Biotechnol. 82 (2007) 471–480. doi:10.1002/jctb.1692.

[15] A. Guisasola, S. Petzet, J.A. Baeza, J. Carrera, J. Lafuente, Inorganic carbon limitations on nitrification: Experimental assessment and modelling, Water Res. 41 (2007) 277–286. doi:10.1016/j.watres.2006.10.030.

[16] A. Guisasola, I. Jubany, J.A. Baeza, J. Carrera, J. Lafuente, Respirometric estimation of the oxygen affinity constants for biological ammonium and nitrite oxidation, J. Chem. Technol. Biotechnol. 80 (2005) 388–396. doi:10.1002/jctb.1202.

[17] K. Ju, L. Wang, Y. Lv, X. Zhang, R. Miao, X. Wang, Nitrous oxide emission in autotrophic partial nitritation system: Macro- and microanalyses, J. Biosci. Bioeng. 120 (2015) 419–425. doi:10.1016/j.jbiosc.2015.02.005.

[18] M.J. Kampschreur, W.R.L. van der Star, H.A. Wielders, J.W. Mulder, M.S.M. Jetten, M.C.M. van Loosdrecht, Dynamics of nitric oxide and nitrous oxide emission during

full-scale reject water treatment, Water Res. 42 (2008) 812–826. doi:10.1016/j.watres.2007.08.022.

[19] M. Ali, R.M.L.D. Rathnayake, L. Zhang, S. Ishii, T. Kindaichi, H. Satoh, S. Toyoda, N. Yoshida, S. Okabe, Source identification of nitrous oxide emission pathways from a single-stage nitritation-anammox granular reactor, Water Res. 102 (2016) 147–157. doi:10.1016/j.watres.2016.06.034.

[20] M.S. de Graaff, G. Zeeman, H. Temmink, M.C.M. van Loosdrecht, C.J.N. Buisman, Long term partial nitritation of anaerobically treated black water and the emission of nitrous oxide, Water Res. 44 (2010) 2171–2178. doi:10.1016/j.watres.2009.12.039.

[21] Y. Law, P. Lant, Z. Yuan, The effect of pH on N2O production under aerobic conditions in a partial nitritation system, Water Res. 45 (2011) 5934–5944. doi:10.1016/j.watres.2011.08.055.

[22] R.M.L.D. Rathnayake, Y. Song, A. Tumendelger, M. Oshiki, S. Ishii, H. Satoh, S. Toyoda, N. Yoshida, S. Okabe, Source identification of nitrous oxide on autotrophic partial nitrification in a granular sludge reactor, Water Res. 47 (2013) 7078–7086. doi:10.1016/j.watres.2013.07.055.

[23] M. Pijuan, J. Torà, A. Rodríguez-Caballero, E. César, J. Carrera, J. Pérez, Effect of process parameters and operational mode on nitrous oxide emissions from a nitritation reactor treating reject wastewater, Water Res. 49 (2014) 23–33. doi:10.1016/j.watres.2013.11.009.

[24] Y. Law, B.J. Ni, P. Lant, Z. Yuan, N2O production rate of an enriched ammonia-oxidising bacteria culture exponentially correlates to its ammonia oxidation rate, Water Res. 46 (2012) 3409–3419. doi:10.1016/j.watres.2012.03.043.

Chapter 5

Application of the two-step PN/Anammox process to the treatment of liquid residues produced by double-stage anaerobic digestion of food waste

5.1 Introduction

In the present chapter, the experimental activity carried out using the real target wastewater was described and results reported. The liquid fraction originating from a scale two-step anaerobic digestion of food waste system was characterized and fed in the PN reactor. PN reactor was seeded with conventional active sludge, and operated in a semi-continuous batch mode in order to promote the biomass AOB enrichment. Stable nitritation process was achieved within 42 days, using the real wastewater as the only feeding. Subsequently, reactor operation was switched to continuous flux without biomass retention (chemostat). Different operating conditions were applied in order to achieve stable partial nitritation; fine tuning of operational parameters was also performed in order to achieve an effluent nitrite/ammonium molar ratio that matched the range considered suitable for subsequent anammox process.

The effluent from PN unit progressively replaced the synthetic influent to the anammox reactor, and anammox was fed using 100% real wastewater for 74 days overall. Influent nitrite/ammonium molar ratio was at first regulated using chemicals, and then by mixing the effluent from PN unit with the target wastewater as such, i.e. not previously treated by PN unit. Such solution, although proposed by many authors, to the best of our knowledge was reported to be tested only once.

A novel approach to anammox sludge characterization was also attempted in this experiment, based on the determination of the color of the biomass. To the best of our knowledge, a similar approach was proposed only once. Anammox bacteria are well-known to show a clear reddish color due to high content in heme c proteins: in this experiment, color of the biomass was univocally measured and related to increasing share of real wastewater on the influent, as well as to SAA and microbiological composition of the sludge, in order to assess whether a significant correspondence among those parameters could support such characterization as a potential quick, simple and cost-effective indirect measurement of process performance, metabolic activity and biomass enrichment

5.2 Materials and methods

5.2.1 Reactors

The Sharon unit consisted of a 2 L continuous flow stirred tank reactor operated as a chemostat (without biomass recirculation), at controlled temperature, pH and dissolved oxygen (DO) concentration. In particular, pH and DO were monitored using InPro 4280i and 6850i probes (Mettler-Toledo), respectively, which were connected to a digital transmitter (Mettler-Toledo, mod. M300). pH was kept below 7.6 by dosage of 1M H_2SO_4, while no low setpoint value was adopted. Different DO levels (2.5 mg/L to saturation) were provided by intermittent air supply. Air flow rate was adjusted to 1 NL/min. Temperature was kept at 35±0.5°C by a water jacket and a thermostatic bath (HAAKE, mod. F3-K).

Influent flow rate was changed during the experiment, resulting in a variable HRT ranging from 1.5 to 0.5 days. Reactor was initially inoculated with activated sludge drawn from the municipal wastewater treatment plant of Is Arenas, Cagliari (Italy), and operated under different conditions in order to achieve selection of ammonia oxidizing bacteria (AOB) and stable partial nitritation (see section 5.2.7).

The Anammox unit consisted of a 3 L sequencing batch reactor (SBR), with a working volume of 2.13 L, operated at controlled temperature (35±0.5 °C) and pH (7.0±0.1), inoculated with granular Anammox biomass originating from a previous experimental campaign. The reactor was operated in fed-batch mode with a 6-hour cycle configuration (267 min feeding, 83 min reaction, 5 min settling and 5 min effluent withdrawal). Mechanical mixing was provided by a marine impeller (80±5 rpm). At the beginning of each working cycle, inert N_2 gas was flushed for 5 min, in order to assure anaerobic conditions inside the SBR. The influent flowrate was set at 2.0 mL/min; the volumetric exchange ratio was kept equal to 0.17, corresponding to a hydraulic retention time (HRT) of 1 d. Temperature was controlled by a water jacket and a thermostatic bath (HAAKE, mod. F3-K); pH was maintained within the chosen range using 1M HCl and 1M NaOH. The vessel was completely covered with tin foil, in order to avoid any penetration of light which would hinder anammox activity [3]. Temperature, ORP and pH monitoring was performed using InPro 3250i pH/ORP probe (Mettler-Toledo) connected to a digital transmitter (Mettler Toledo, mod. M300). Process timing and control were performed via National Instrument CompactRio system and a custom made LabView (v.10.0) application. A schematic representation of the whole Anammox apparatus is reported in Figure 5.1.

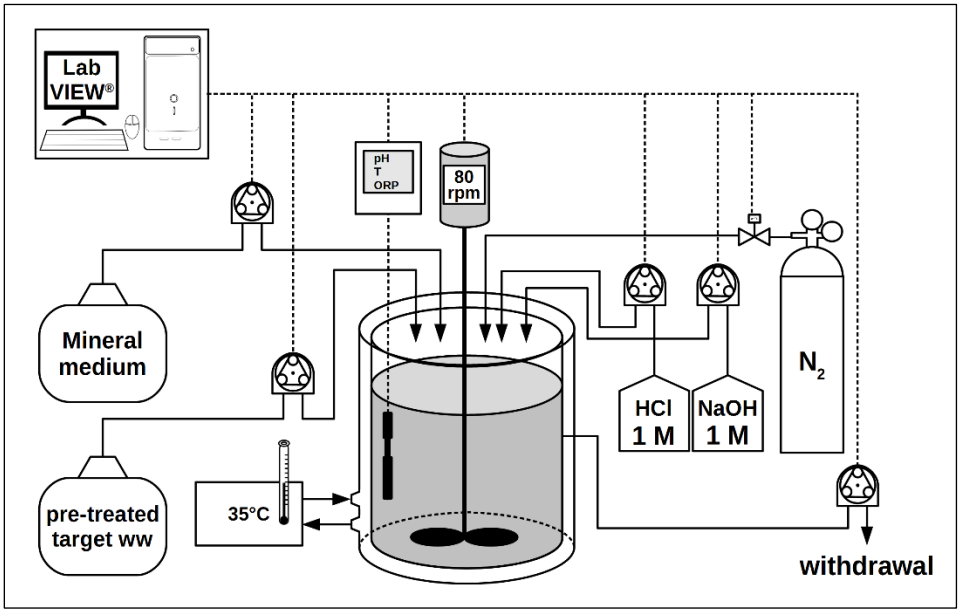

Figure 5.1: Schematic representation of the whole Anammox apparatus.

5.2.2 Treated wastewater

According to Hy.Me.C.A. project goals, the *target* real wastewater, which was subsequently fed to the PN reactor, originated from the outcomes of a lab-scale system developed at DICAAR laboratories, and aimed at the production of hydrogen and methane by double stage anaerobic digestion of food waste (AD-FW). The liquid fraction was obtained through coagulation-flocculation treatment, using a 3% v/v dosage of a cationic polyelectrolyte (Tillmanns Tillflock 1460, 5 g/L emulsion). Average characterization is provided in Table 5.1.

Main VFA detected were acetic acid (50%), propionic acid (13%) and butyric acid (13.5%).

Table 5.1: Average composition of the target wastewater.

Parameter	Value	u.m.
pH	8.1±0.2	-
NH_4-N	1,507±98	mg/L
TN_b	1,611±105	mg/L
Alkalinity	6,950±201	mg$CaCO_3$/L
TOC, soluble	480±60	mg/L
COD, total	1,530±77	mg/L
COD, soluble	1,121±56	mg/L
BOD_5/COD_{tot}	0.19±0.02	-
VFA	50±2	mg/L
TSS	0.51±0.10	g/L
VSS	0.38±0.10	g/L

5.2.3 Analytical methods

NH_4-N concentration was determined according to Standard Methods [4] using a Hitachi U-2000 spectrophotometer at a wavelength of 420 nm. Concentrations of NO_2-N and NO_3-N were determined by ion-chromatography using a DIONEX ICS-90 equipped with an AS14A Ion-PAC 5 µm column. All samples were filtered (0.45 µm) before analyses, which were performed in triplicate. Free Ammonia (FA) and Free Nitrous Acid (FNA) concentrations were estimated according to Anthonisen et al. (1976) [5]. Total nitrogen bound (TN_b) was determined according to standard method EN ISO 11905-1 (digestion with peroxodisulphate and subsequent photometric detection) using Hach LCK338 cuvette test and Hach DR6800 spectrophotometer. Alkalinity was measured by potentiometric titration to preselected end-point pH, using an automatic titrator (AT-510, KEM electronics).

Content in organic matter was routinely measured as dissolved total organic carbon (DOC) by means of a Shimadzu TOC-V analyzer. All samples were filtered (0.45 µm) before analysis, which was performed in triplicate. Total COD and soluble COD (i.e., after filtration of samples through a 0.45 µm membrane) were measured according to Standard Methods [4] 5220 B (open reflux method, digestion with potassium dichromate). The remarkable amount of nitrite (up to 1,000 mgN/L) in the effluent of the Sharon unit / influent to the anammox unit, represented an unavoidable interference [4] that hindered the possibility of using COD assessment for reliable mass balance of organic matter. BOD was measured using a respirometry set based on a LSS (liquid phase, static gas, static liquid) principle [6] (Velp BOD Sensor System 6). It consisted of a set of dark glass airtight bottles equipped with pressure sensors for continuous measurement of the oxygen uptake. VFAs were measured by static headspace gas chromatography (Agilent, mod. 6890-N; headspace auto-sampler, Agilent, mod. 7694) equipped with a DB-FFAP column (30 m × 0.25 mm × 0.25 µm) and a flame ionization detector (FID). 1 g NaCl, 0.2 mL H_2SO_4 2M and 20 µL crotonic acid 10 mM were added to samples (2 mL) before analysis.

Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were determined according to Standard Methods [4].

5.2.4 Nitrogen mass balances

During the experiment, influent and effluent total nitrogen was calculated as the sum of NH_4-N, NO_2-N and NO_3-N. Nitrogen uptake by heterotrophs in aerobic and anaerobic conditions was estimated, while nitrogen loss due to the growth of autotrophic bacteria (AOB, NOB and anammox) was generally neglected.

Supposedly, only aerobic metabolism took place inside the Sharon reactor. Apart from AOB and NOB activity, a contribution to ammonium removal may have been given by aerobic heterotrophic bacteria, since organic substrates were available in the influent. Ammonia uptake due to carbon removal by heterotrophs was then approximated according to stoichiometry with acetate as carbon source, as reported in Burton et al. [7], eq. 7-87:

$$0.125\ CH_3COO^- + 0.0295\ NH_4^+ + 0.103\ O_2 \rightarrow$$
$$\rightarrow 0.0295 C_5H_7O_2N + 0.0955 H_2O + 0.0955 HCO_3^- + 0.007 CO_2$$

Resulting in 0.118 moles of NH_4 consumed per each mole of oxidized organic carbon, or 0.1376 g of ammonia nitrogen per each gram of carbon.

In Anammox unit, since complete anaerobic conditions were provided inside the reactor, consumption of organic carbon was only ascribed to denitrification activity. During feeding and reaction phases, both nitrate and nitrite were available in the bulk liquid as potential electron acceptors for denitrification. Previous studies on competition between nitrite and nitrate as electron acceptors in denitrification process showed that nitrate is preferred to nitrite, since the first is consumed and the latter accumulated at the same time [8], and that shortage of organic substrate leads to nitrite accumulation that can be removed only by the further addition of a carbon source [9]. Consequently, the observed DOC removal was related to a correspondent nitrate reduction to N_2, and ammonium uptake for biomass synthesis was calculated according to the stoichiometry of full denitrification reported in Burton et al. [7], eq. 7-114, with acetate as electron donor:

$$NO_3^- + H^+ + 0.33\ NH_4^+ + 1.45\ CH_3COO^- \rightarrow$$
$$\rightarrow 0.5\ N_2 + 0.33\ C_5H_7O_2N + 1.60\ H_2O + 1.12\ HCO_3^- + 0.12\ CO_2$$

Resulting in 0.345 moles of NO_3 reduced and 0.1138 moles of NH_4 consumed per each mole of oxidized organic carbon, or 0.402 g of nitric nitrogen and 0.1327 g of ammonia nitrogen per each gram of carbon.

Studies on denitrification potential of different carbon sources (both simple compounds and complex wastewater) reported different values. Generally, higher values of denitrifying biomass yield (YDN, gVSS/gCOD) lead to lower denitrification potential values (gN/gCOD) [7]. Lee and Welander [10] reported denitrification potential values of 0.25-0.28 gN/gCOD (0.67-0.75 gN/gTOC) for acetate. Sage et al. [11] also reported denitrification potentials of different and complex carbon sources ranging between 0.14 and 0.24 gN/gCOD (lactic acid, lactate, and others). The estimation of nitrate and ammonia consumption based on acetic acid-based denitrification stoichiometry could thus lead to the underestimation of heterotrophic

denitrification impact on overall performance. Nonetheless, calculations lead to consistent nitrogen mass balances where denitrification accounted for 0-5% of total nitrogen removal.

5.2.5 Characterization of anammox granular sludge

Specific anammox activity (SAA) was performed according to chemical tracking method described by Van Loosdrecht et al. [6]. Test was performed *in situ*: at the end of feeding phase, a solution containing both NO_2^- and NH_4^+ (2,000 mgN/L each, as $NaNO_2$ and NH_4Cl) was spiked directly into the reactor in order to achieve an initial concentration of 40 mgN/L for both ammonium and nitrite. Samples were collected at fixed time intervals and analyzed in order to track nitrite, nitrate and ammonium profiles. Linear regression of the data resulted in volumetric ammonium and nitrite removal rates (r_{NH4} and r_{NO2}, respectively) and nitrate production rate (r_{NO3}), expressed as mgN/L·min. Finally, SAA (gN_2-N/gVSS·d) was calculated as follows:

$$\text{SAA} = \frac{r_{NH4} + r_{NO2} - r_{NO3}}{\text{VSS}} \cdot \frac{60 \cdot 24}{1000}$$

Also, stoichiometric ratios were calculated using the following expressions:

$$\text{Y}_{NO2\_NH4} = \frac{|r_{NO2}|}{|r_{NH4}|}$$

$$\text{Y}_{NO3\_NH4} = \frac{|r_{NO3}|}{|r_{NH4}|}$$

Density of granules was assessed according to dextran blue method described by Beun et al. [12]. Granular aggregates were morphologically characterized in terms of size (particle size distribution, PSD) and aspect (roundness, aspect ratio) through image analysis (IA) technique. Biomass samples were collected from the reactor, sieve-drained, washed and resuspended in clean deoxygenated water, and put in 10 mm diameter Petri dishes. High resolution digital images of dark granules contrasted to a white background were acquired via a HP ScanJet 5590 scanner. Identification of granules outlines and measure of morphological parameters was performed using Image-Pro Plus 6 (Cybermedia) software. Measured parameters were: mean diameter, aspect (i.e., the ratio between the minor and the major axis of the ellipse equivalent to the object) and roundness (i.e., an index ranging from 0 to 1 based on the ratio between area and perimeter of the object and of its equivalent circle – perfect circular shape giving a value of 1).

Digital color analysis of Anammox granules was performed using a Konica Minolta CM 3610 spectrophotometer. Data were represented using Konica Minolta SpectraMagic NX software. Colour was represented according to CIE Lab color space [13] as three numeric coordinates,

labeled as L*, a* and b* color components, ranging from 0 to 100 (L*) and from -128 to +127 (a* and b*). L* is a measure of luminance; a* and b* are related to Hering's color opponent process theory, i.e. a* represents the green-red opposition (negative and positive values, respectively), while b* the blue-yellow opposition (negative and positive values, respectively). (a*, b*) cartesian coordinate duplet was converted to corresponding polar coordinates, labelled as C* (chroma, which can also be intended as saturation), and h (hue, or hue angle, expressed as degrees), according to the following equations:

$$C^* = \sqrt{(a^*)^2 + (b^*)^2}$$

$$h = \arctan\left(\frac{b^*}{a^*}\right)$$

Collected data were subsequently processed using spreadsheet.

Granular biomass was prepared according to the following procedure: a mixed liquor sample (~10 mL) was collected from the reactor; granules were sieve-drained and then carefully disrupted using a glass-made mortar and pestle, in order to get a homogeneous suspension expressing not only the color of the surface of the granules, but also of their inner part. The suspension was then filtered through a glass fiber membrane with a porosity of 1.2 µm, and the resulting filter cake was analyzed to determine color composition. Since treated wastewater showed its own color (due to dissolved compounds and suspended particles), its contribution to granules color was also assessed; in particular, the filtrate from preliminary 1.2 µm filtration step was further filtered through a clean membrane which was analyzed as a blank sample. Color difference between samples and blanks was calculated according to CIE dE_{00} standard [14], which is a non linear expression involving L*, a* and b* and resulting in a positive number proportional to color "distance" in CIE Lab vector space.

5.2.6 Microbiological characterization

Microbiological characterization was performed by fluorescence in situ hybridization (FISH) on representative biomass samples, according to Amann *et al*. [15]. Hybridizations with group specific probes for ammonium oxidizing bacteria (NSO1225, NSO190), nitrite oxidizing bacteria (NTSPA and NIT3), and Anammox bacteria (AMX820, AMX368 and PLA46) were carried out simultaneously with probes EUB338, EUB338-II and EUB338-III combined in a mixture (EUB338mix) for the detection of most bacteria, and with DAPI staining for quantifying the total number of cells. All probes were purchased from MWG-Biotech (Germany), and synthesized with 5'-FITC (green) and 5'-Cy3 (red) labels. Details on oligonucleotide probes are available at ProbeBase [16]. Slides were examined with an

epifluorescence microscope (Olympus BX51) at different magnifications (100, 400 and 1000x); images were captured with an Olympus XM10 camera using Cell-F software (Olympus, Germany). DAIME software [17] was used for FISH quantification of hybridized cells.

5.2.7 Plan of experimental activity

Experimental activity on Sharon unit can be divided into two main phases: a startup phase and an operative phase.

Startup phase lasted for 42 days and provided the selection of AOB over NOB and the achievement of stable partial nitritation in a reactor seeded with ~4 gTSS/L of conventional activated sludge from a municipal wastewater treatment plant located in Cagliari (Sardinia, Italy), and fed with the target wastewater.

As summarized in Table 5.2, the selection reactor was operated in a semi-continuous batch mode and its activity was structured in cycles, each cycle entailing a reaction phase, followed by biomass settling, removing 1.4 L of supernatant and replacing it with an equal amount of untreated wastewater, and restart of reaction. Seven cycles were carried out, with different duration and DO and pH control settings, while temperature was kept at the setup value of 35°C. To control pH, acid (H_2SO_4 1M) and/or alkaline ($NaHCO_3$ 1M) solutions were dosed.

Table 5.2: Structure of startup phase of the Sharon unit.

Cycle	Duration [d]	DO [ppm]	pH control
1	7	~ sat	No
2	7	”	”
3	7	”	Yes (7.0 – 7.3)
4	8	3.0	Yes (≤7.5)
5	4	”	”
6	2	”	”
7	5	”	”

Once satisfactory PN performance was achieved, reactor was suddenly converted to CFSTR mode (operative phase), operating as described in section 5.2.1. The HRT was progressively decreased in order to further exert the selective pressure based on low biomass retention time.

During the operative phase, target wastewater was fed to Sharon unit operated in CFSTR mode, under different DO and HRT setup, in order to assess the feasibility of the treatment and to find the best operating conditions to produce a suitable effluent for subsequent treatment by anammox.

The whole operative phase lasted 248 days, and can be divided into three main Runs. Each run can be further divided into different steps, according to different HRT and DO setup values. Activity plan is summarized in Table 5.3.

As to the Anammox unit, the whole experimental activity lasted 178 days and can be divided into three different phases, as summarized in Table 5.4.

Table 5.3: Structure of operative phase of Sharon unit.

Run	Step	Duration [d]	HRT [d]	DO [ppm]
1	A	8	3.00	3.0
	B	7	2.50	3.0
	C	7	2.00	3.0
	D	12	1.50	3.0
	E	51	1.00	3.0
	F	36	1.00	2.5
	G	9	1.25	2.5
2	A	6	1.25	~sat
	B	9	1.00	~sat
	C	20	1.00	3.0
	D	10	1.00	2.5
	E	7	1.00	2.75
3	A	2	1.50	3.0
	B	4	1.25	3.0
	C	7	1.00	3.0
	D	7	0.80	3.0
	E	8	0.70	3.0
	F	8	0.60	3.0
	G	7	0.50	3.0
	H	5	0.55	3.0
	I	18	0.525	3.0

The first phase was an acclimation phase: mineral influent used in previous experimental campaign (see Chapter 3) was progressively substituted by pre-treated target wastewater (also labelled as “real ww”, i.e. the supernatant of Sharon unit effluent), following a conservative exponential law described by Lopez et al. [18]:

$$\mathrm{NLR}(t) = A\, e^{f \cdot \mu_{max} \cdot t}$$

Where: *A*, pre-exponential factor, corresponding to the initial NLR (gN/L d); *f*, safety factor (0.3); μ_{max}, maximum anammox specific growth rate (0.065 d^{-1}), as proposed by Strous et al. (1998) [19]; *t*, time (d). Total nitrogen concentration of real ww was assumed to be equal to the

target value of 1,500 mgN/L (actual concentration slightly fluctuated), also used for mineral medium: under this assumption, NLR was proportional to influent volume only. An initial share of 10% of real ww was applied, corresponding to an initial *A* value of 0.15 gN/L d; resulting time course of substitution is graphically represented in Figure 5.2.

Table 5.4: Structure of Anammox unit experimental activity.

Phase	Duration [d]	Description
1	105	Progressive increase of pre-treated target wastewater (i.e., effluent from PN treating the target real ww) on total influent volume
2	18	100% pre-treated target wastewater feeding; manual correction of NO_2/NH_4 influent ratio by NH_4Cl dosage
3	56	Mixed pre-treated/untreated target wastewater influent providing an acceptable NO_2/NH_4 influent ratio (no manual correction)

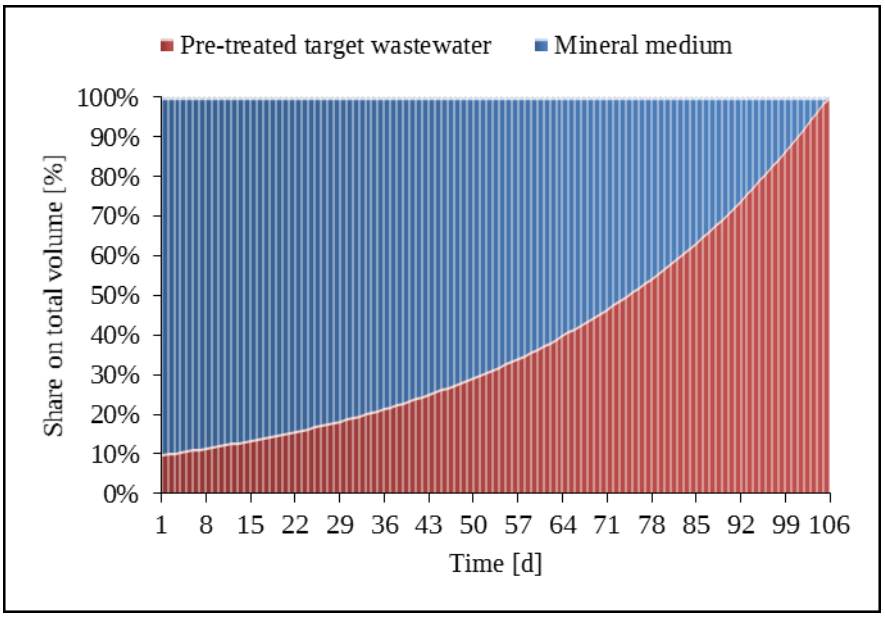

Figure 5.2: Progressive substitution of influent mineral medium with pre-treated target wastewater during Phase 1 of Anammox unit.

Such feeding strategy, which was successfully applied in a previous study [20], was chosen in order to minimize the risk of anammox bacteria inhibition.

From day 105 onward, reactor was only fed with the real ww. As discussed below, stable PN treatment of target wastewater often produced an effluent with a higher NO_2/NH_4 molar ratio than the optimal range values for Anammox process (1.0-1.3). During Phase 2 manual correction of nitrite to ammonium ratio was applied by appropriate NH_4Cl dosage.

A different solution for correcting influent NO_2/NH_4 molar ratio was then tested during Phase 3. Pre-treated target wastewater was mixed with untreated target wastewater; such mix

consisted of 15% untreated and 85% pre-treated on average, and resulted in a NO_2/NH_4 molar ratio of 1.0-1.3, which was considered acceptable for Anammox operation.

5.3 Results

5.3.1 PN unit

Startup phase was aimed at AOB selection over NOB, and establishment of a stable partial nitritation process using target wastewater as the only substrate. Experiment with mineral medium showed that successful startup could be achieved under the following conditions (see Chapter 3 for details): continuous flux stirred reactor without biomass retention, temperature equal to 35°C, pH≤7.6, HRT=1.0-1.5 days. Same conditions were initially applied to startup the Sharon unit with the target wastewater, but no ammonium removal was observed, while biomass was completely washed out in 3-5 days (data not shown). Such results led to pin down a different startup strategy. A selector system, aimed at the sludge enrichment in acclimated nitrifying biomass, was then proposed. Such selector system was designed to operate at low dilution rate and to allow biomass retention and acclimation to wastewater; moreover, variable biomass/wastewater contact times were applied, long enough to allow complete COD removal before nitrification occurred, thus avoiding competition between nitrifying bacteria and ordinary heterotrophic organisms for dissolved oxygen utilization. The simplest solution found was then the semi-continuous batch mode, described in section 5.2.7.

During first two cycles neither oxygen or pH were controlled: average values of 3.6±0.8 ppm and 9.1±0.2 were measured, respectively. As depicted in Figure 5.3, in both cycles DOC rapidly decreased in first 1-2 days until stable values were observed, while ammonium and alkalinity were progressively consumed. By the way, no nitrite or nitrate production was detected. Such behavior could likely be ascribed to volatilization of ammonia and CO_2 promoted by the combination of stable high pH and continuous aeration. pH control was then operated in cycle 3 and kept within the range 7.0-7.3 by addition of acid (1M H_2SO_4) and alkaline (1M $NaHCO_3$) solutions. Volatilization of free ammonia was avoided at first, but acid addition caused alkalinity consumption down to limiting concentration for nitrifiers activity. Eventually, a malfunction occurred during the weekend, causing an uncontrolled dosage of alkaline solution into the reactor, resulting in a pH raise to 11 and the almost total volatilization of ammonia.

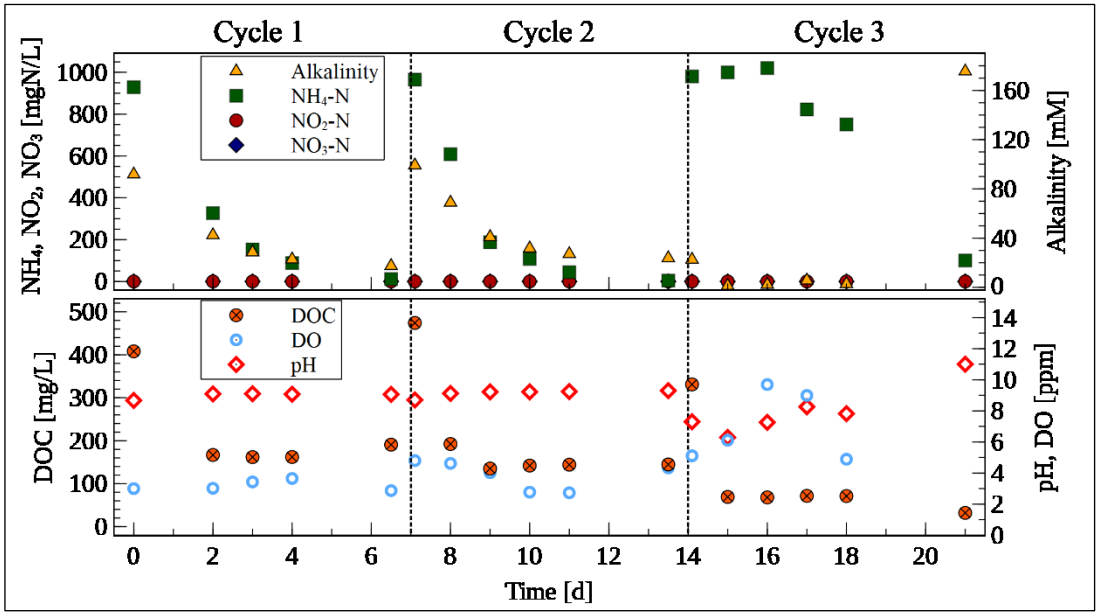

Figure 5.3: Performance of Sharon unit during startup phase, cycles 1-3.

Reactor was then inoculated again and restarted on day 23. Aeration mode was turned from continuous to intermittent, in order to avoid continuous stripping of ammonia and CO_2. Air pump was automatically switched on (off) when measured DO was lower (higher) than the setpoint value of 3 ppm. Moreover, pH was still controlled and kept lower than 7.5.

During cycle 4 (days 23-31), ammonium initially remained stable, while alkalinity rapidly dropped to very low values, due to its consumption to neutralize acid added for pH control. To provide enough alkalinity to sustain ammonium oxidation by AOB, manual dosage of $NaHCO_3$ was operated on day 26. On the following days, ammonium removal started to occur, while increasing nitrite levels were observed (Figure 5.4). More bicarbonate was dosed again on day 29 and day 30, in order to promote total NH_4 oxidation. On day 31 removed NH_4 accounted for 95% of initial value, then another cycle was initiated. On cycle 5 a similar but enhanced behavior was observed: ammonium started to decrease and nitrite started to increase from day 32; acid dosage occurred on day 32 only, as well as the need for manual dosage of alkalinity. On day 35, 98% of initial ammonium was oxidized; final nitrite and nitrate concentrations were 1,074 and 18 mgN/L, respectively.

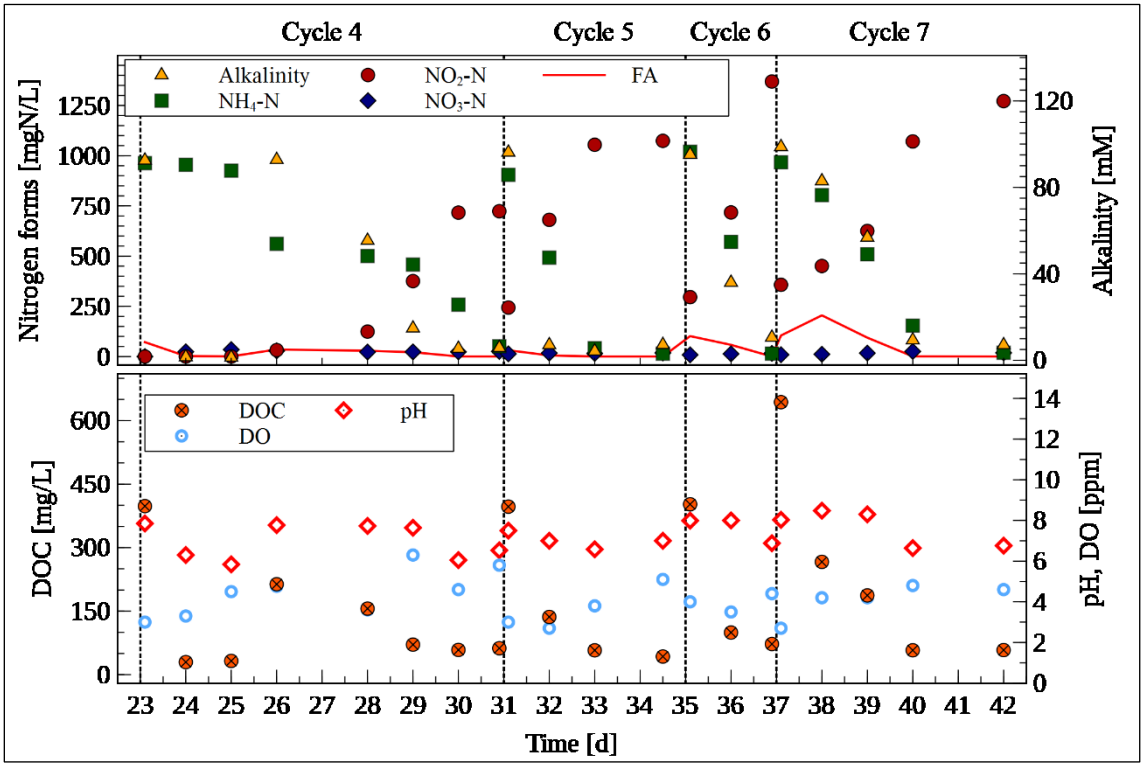

Figure 5.4: Performance of Sharon unit during startup phase, cycles 4-7.

Cycle 6 showed the best performance: a quasi-linear ammonium oxidation and corresponding nitrite production were observed. No acid dosage was needed since pH remained lower than setpoint value. Almost complete (98%) ammonium removal was detected after two days only. On cycle 7 a slightly worse performance was observed, since ammonium oxidation rate appeared lower than that on previous cycle (10 and 21 mgNH_4-N/L·h, respectively). pH tended to increase, and a manual dosage of alkalinity was needed on day 39. Anyway, total conversion of ammonium to nitrite was observed in 5 days.

On cycles 4-7, organic matter was quickly removed within the first 1-2 days of every cycle, consistently with the higher oxygen consumption rate ascribed to heterotrophs compared to autotrophs, and final DOC removal efficiency ranged between 82 and 91%. Free ammonia concentration >30-50 mgNH_3-N/L was observed at the beginning of each cycle (up to 200 mgN/L on day 38), and likely accumulated in the liquid fraction due to limited gas stripping. As nitrite accumulation and pH decrease occurred (last days of each cycle), free nitrous acid concentration up to 0.5-1.4 mgN/L were measured. Despite the high solids retention time and the availability of nitrite and dissolved oxygen, NOB activity was never relevant, and nitrate production always accounted for ~1% of oxidized ammonium. Likely, NOB were not washed out of the reactor, even though a decrease in biomass concentration was observed (Figure 5.5), but their activity was inhibited.

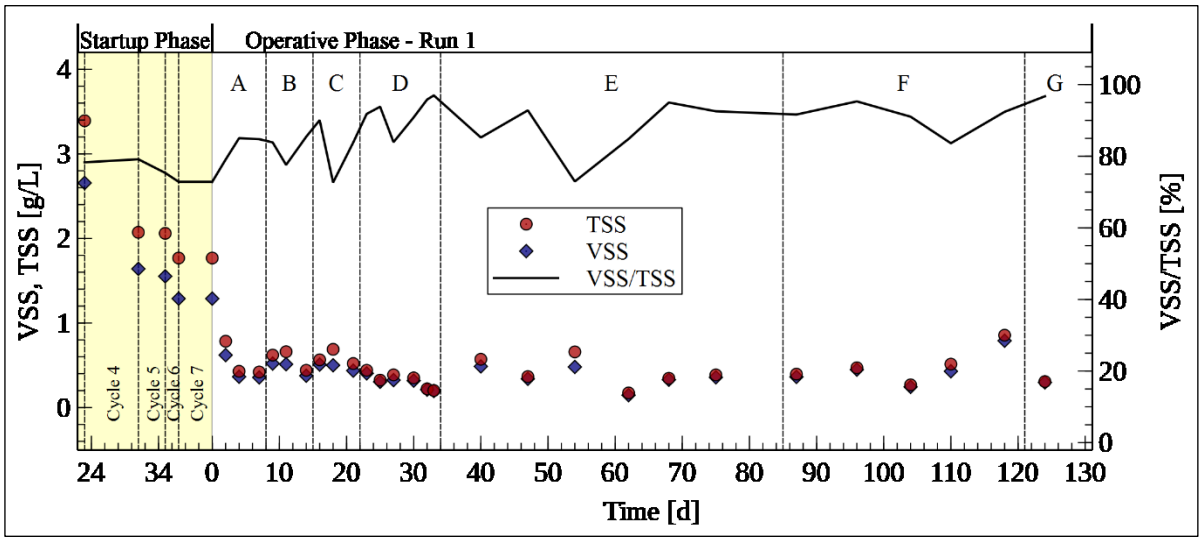

Figure 5.5: Biomass concentration in Sharon unit during startup phase, days 23-42, and subsequent operative phase, Run 1.

Run 1. Change from semi-continuous batch to CFSTR (with HRT and SRT of 3 days) operating condition caused, at first, a decrease in MLTSS and MLVSS concentration (from 1.77 and 1.28 g/L, day 0, to 0.42 and 0.36, day 7, respectively).

Initially, the HRT was decreased from 3.0 to 1.5 d (steps A-D). As depicted in Figure 5.6, during this period ammonium oxidation rate (AOR) progressively increased as well as nitrite accumulation rate (NAR), resulting in increasing values of effluent nitrite/ammonium molar ratio, up to 1.92 (end of step D). Removal efficiency of organic matter progressively decreased, from average values of 80±5% (step A) to 55±11% (step D), even though DOC effluent concentration decreased as well. This behavior may likely be ascribed to lower influent organic content and biodegradable fraction, which then allowed AOB not to be outcompeted by heterotrophic bacteria.

During step E, target HRT of 1 d, corresponding to the highest theoretical planned NLR (1.5 kgN/L·d), was successfully reached. Anyway, a series of malfunctions to stirring, aeration and pH control caused the biomass to suffer prolonged lack of oxygen (day 43 and 47) and exposure to high free ammonia concentrations (day 54, up to 400 mgNH_3/L), which hindered both autotrophic and heterotrophic performance. Progressive recovery of both partial nitritation and organic matter removal performance was subsequently achieved from day 55 onward. On day 83 an ammonia oxidation rate and effluent nitrite/ammonia molar ratio of 66% and 2.1, respectively, were reached. Although such values closely matched the expected stoichiometric

values (given the influent alkalinity/ammonium ratio), they were too high in view of subsequent treatment by anammox.

During step F, then, DO setup level was decreased to 2.5 ppm, while HRT was maintained at 1 d, in order to reduce the effluent NO_2-N/NH_4-N ratio, thus providing a more suitable influent to subsequent anammox unit.

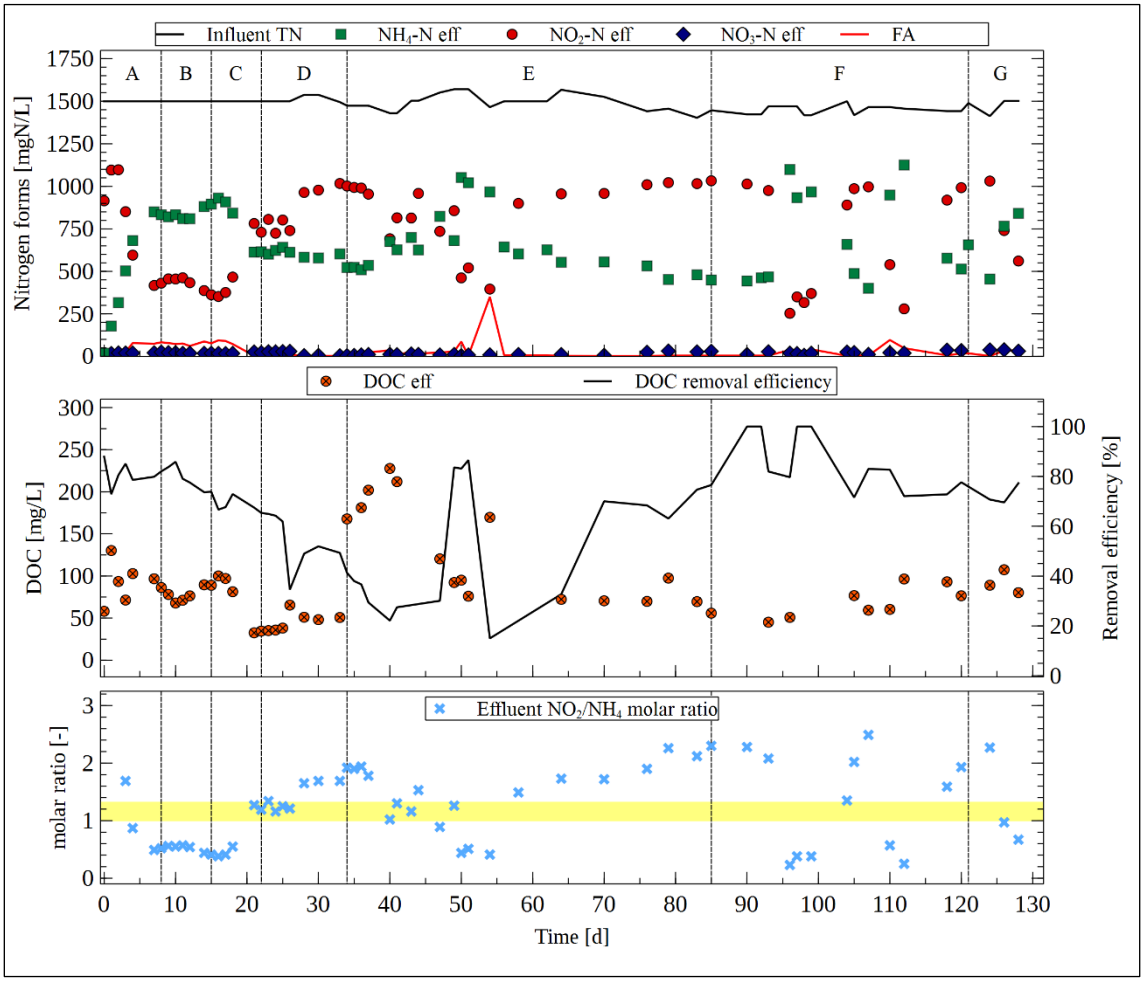

Figure 5.6: Performance of Sharon unit during the operative phase, Run 1. On bottom plot, yellow horizontal belt indicates Anammox influent optimal NO_2/NH_4 molar ratio values (1.0 – 1.3).

While heterotrophic activity appeared not to be affected by oxygen decrease (86±12% average DOC removal efficiency), a certain instability of nitritation process was observed. In order to match the bacterial oxygen demand with DO provided by new aeration level, and thus achieve again a stable process, both nitrogen and organic loads were reduced by increasing the HRT (from 1 to 1.25 d, step G), still resulting in unstable process performance. However, reactor broke on day 129 and experiment was interrupted. Biomass was collected from reactor and temporarily stored until a new vessel was set up.

Remarkably, no significant nitrate production was observed throughout the whole experimental Run: average effluent concentration was 19±9 mgNO_3-N/L, accounting for 2±1% of AOR. Also, estimation of ammonium uptake due to heterotrophic bacterial growth resulted in 4±3% of total ammonia removed on average.

Experimental Run 2 was set off by seeding the reactor with ~2 gTSS/L of selected biomass previously collected from experimental Run 1 settled effluents and subsequently stored at +4°C. Reactor was operated in CFSTR mode immediately. In order to speed up the recovery of process performance, continuous aeration was initially provided, while HRT was set to 1.25 d and then decreased to 1 d (step A and B, respectively). Nitritation process was successfully established again (Figure 5.7), and its stability under intermittent aeration with a DO setup level of 3 ppm was confirmed (Run 1 Step E, and Run 2 Step C). Differently, a quite variable efficiency in organic matter removal was observed, ranging between 40-80%).

During step D, again a decrease in DO setup level (from 3.0 to 2.5 ppm) was attempted. A quick worsening in overall process performance was observed: AOR/NLR ratio dropped from 68% (day 35) to 20% (day 45), as well as effluent nitrite concentration and NO_2-N/NH_4-N ratio (from 1068 mgN/L and 2.30 to 220 mgN/L and 0.18, respectively) and DOC removal efficiency (from 80% to 43%, day 35 and 46, respectively).

Operating conditions applied in Run 2 (steps C and D) replicated those of Run 1 (steps E and F), and their results were also comparable. Anyway, a different strategy was attempted to restore process stability. While in Run 1, HRT was increased in order to reduce oxygen demand (step G), on Run 2 (step E) DO level was increased from 2.5 to 2.75 ppm, while keeping nitrogen and organic loads unchanged. Eventually, further deterioration of process performance was observed, despite a recirculation of washed out biomass carried out on day 48, and reactor was stopped on day 52.

Results from Runs 1 and 2 confirmed the feasibility to achieve a stable partial nitritation process using a CFSTR reactor seeded with conventional active sludge and fed with the target wastewater only, and suggested the application of intermittent aeration with a threshold DO value of 3 ppm as optimal operating condition at HRT values down to 1 d, corresponding to a NLR up to 1.5 gN/L·d. Stable process performance, by the way, produced an effluent with an excess of nitrite compared to residual ammonium, resulting in a not completely acceptable stream for the subsequent anammox process.

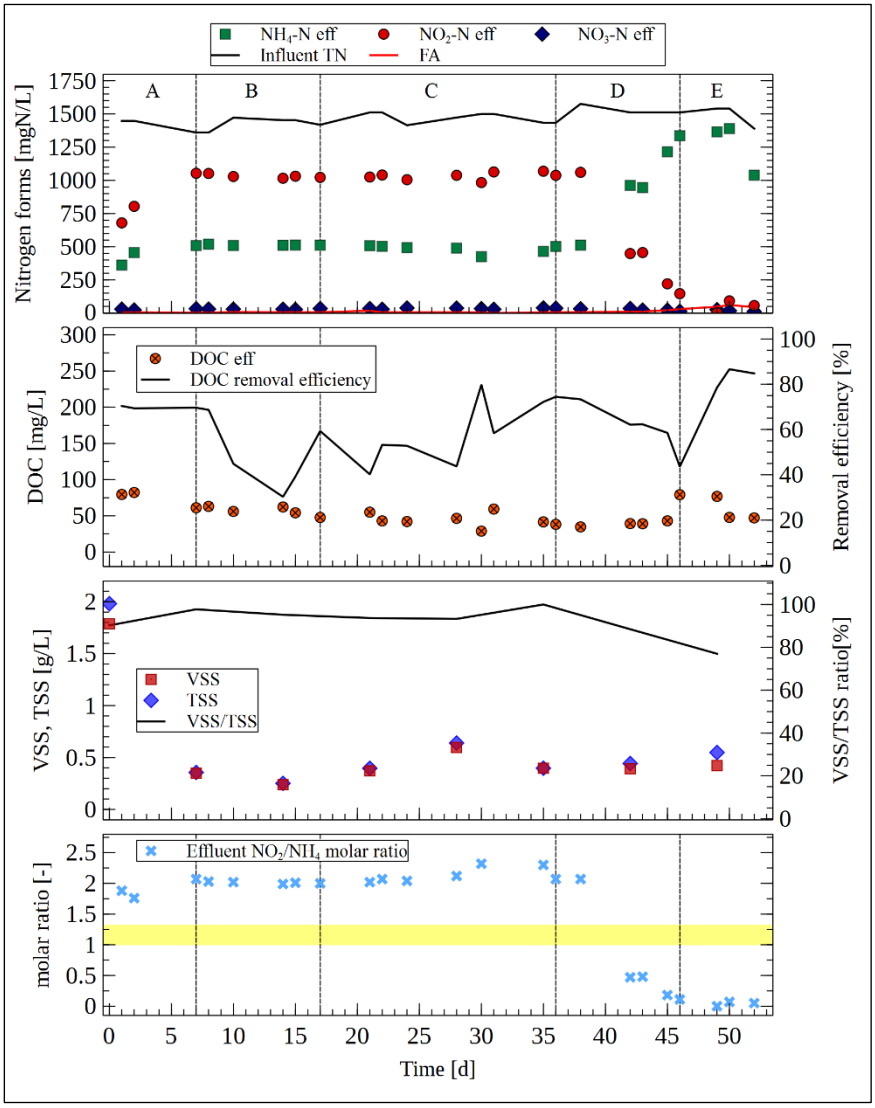

Figure 5.7: Performance of Sharon unit during the operative phase, Run 2. On bottom plot, yellow horizontal belt indicates Anammox influent optimal range of NO_2/NH_4 molar ratio (1.0 – 1.3).

A third experimental run was then carried out in order to apply a strategy aimed at creating limiting conditions to nitritation activity, based on progressive increase of nitrogen and organic loads by reducing HRT, under constant aeration conditions. Results are depicted in Figure 5.8.

Reactor was seeded again with residual biomass from the previous experimental run, and initially operated with a HRT of 1.5 d and intermittent aeration (DO setpoint equal to 3 ppm). Under these conditions, optimal process performance was rapidly achieved. HRT was then progressively reduced down to 0.6 d (step F), corresponding to an average NLR of 2.5 gN/L·d. Both partial nitritation and heterotrophic activity consistently occurred throughout steps A-F, but while the latter was not significantly affected by the increasing load (68±4% and 66±9% DOC removal efficiency, steps C and F, respectively), the former showed slightly decreasing performance, which led to a decrease in effluent NO_2/NH_4 molar ratio from 1.9±0.1 to 1.6±0.1.

Further HRT decrease to 0.5 d (step G), however, resulted in performance worsening, causing a moderate instability to both heterotrophic and AOB activity; HRT was then immediately raised to 0.55 d (step H) and, after performance recovery, subsequently decreased to 0.525 d (step I).

Despite increasing TSS and VSS values had been observed throughout steps D-H, likely due to bacterial growth enhanced by increasing organic load, biomass washout started to occur during step I, which eventually resulted in irreversible worsening of nitritation activity.

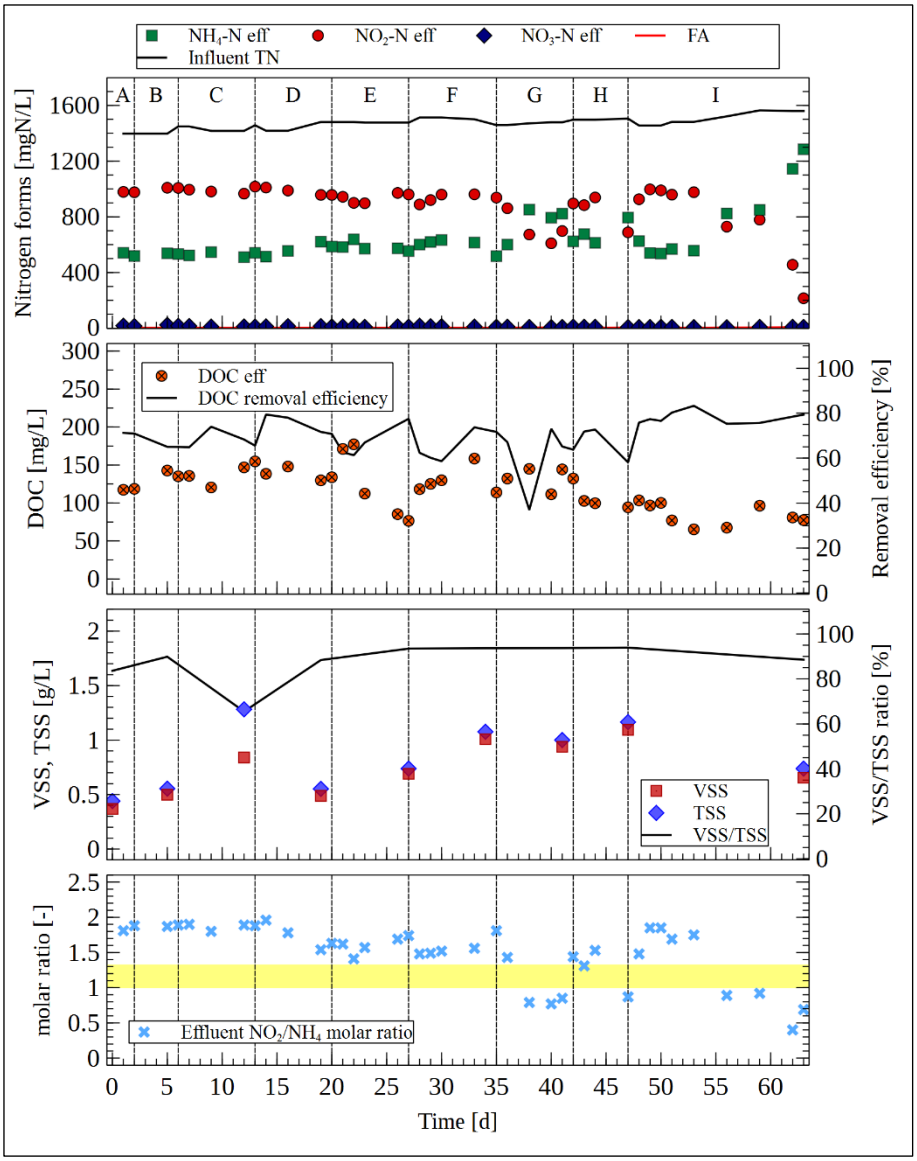

Figure 5.8: Performance of Sharon unit during the operative phase, Run 3. On bottom plot, yellow horizontal belt indicates Anammox influent optimal NO_2/NH_4 molar ratio values (1.0 – 1.3).

Throughout the whole Run 3, DOC removal efficiency averaged out at 70±9%; nonetheless, estimated nitrogen uptake due to biomass growth peaked at 5% of total oxidized ammonium, thus not significantly undermining ammonium availability to AOB.

Throughout the whole experiment, when stable process was observed, ammonia oxidation proceeded up to stoichiometric limit, i.e. until inorganic carbon availability was not limiting (see Chapter 3), as far as oxygen was not also limiting, and resulted in an effluent nitrite/ammonium molar ratio of 1.9-2.1, quite higher than the optimum for the subsequent anammox treatment. While reducing the oxygen level repeatedly led to overall process failure, increasing load strategy initially showed to exert the required limiting effect to ammonia oxidation efficiency. However, once process equilibrium was altered, performance recovery was not rapidly achieved despite of different strategies proposed, indicating the process lacked in robustness.

5.3.2 Anammox unit

As to the Anammox unit, main results concerning nitrogen and organic matter removal are synthesized in Table 5.5 and Table 5.6, respectively, and expressed as average values on each experimental phase.

Table 5.5: Average performance of Anammox unit during each experimental phase (nitrogen removal).

	Effluent TN	NRE	NRR/NLR ratio	Removed NO_2/Removed NH_4 ratio	Produced NO_3/Removed NH_4 ratio
	[mgN/L]	[%]	[%]	[mol/mol]	[mol/mol]
Phase 1	152±11	90±1	98±1	1.26±0.08	0.18±0.02
Phase 2	172±27	89±2	97±2	1.26±0.23	0.17±0.04
Phase 3	199±81	87±5	94±6	1.34±0.17	0.23±0.03

Table 5.6: Average performance of Anammox unit during each experimental phase (carbon removal).

	Effluent DOC	DOC removal efficiency	N removed by denitrification
	[mgN/L]	[%]	[% on total N removed]
Phase 1	45±10	35±13	0.9±0.4
Phase 2	40±6	33±13	0.8±0.4
Phase 3	54±9	58±7	3.2±0.8

During phase 1, the increasing share of pre-treated real ww did not affect the process performance in terms of nitrogen removal efficiency (Figure 5.9). NRE and NRR/NLR ratio averaged out at 90±1% and 98±1%, respectively, and nitrite was almost always completely removed from the system. Observed stoichiometric removed NH_4/removed NO_2/produced NO_3 molar ratio mostly matched the range of values between those indicated by Strous et al. (1999) [19] and Lotti et al. (2014) [21] of 1:1.32:0.26 and 1:1.15:0.16, respectively. On the other hand,

as depicted in Figure 5.10 variable carbon removal efficiency values were observed (9-58%), in any case corresponding to actually limited mass consumption of organic matter: resulting estimated contribution of denitrification activity to overall N removal was in fact less than 1% on average.

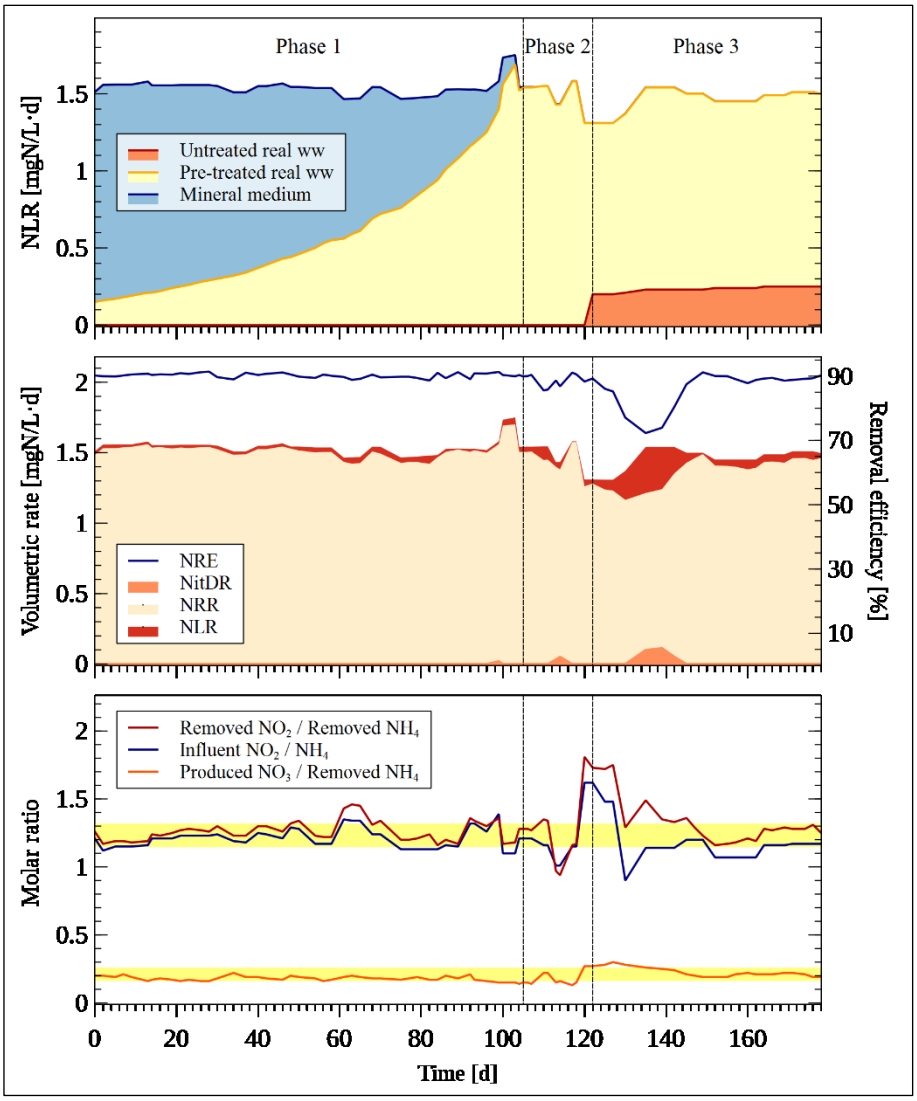

Figure 5.9: Anammox unit performance throughout the whole experimental period. (Top) Composition of NLR; (middle) NRE, NLR, NRR and NitDR; (bottom) measured influent and effluent stoichiometric ratios. Yellow horizontal belts indicate a range of stoichiometric values, considering those proposed by Strous et al. (1999) [19] and Lotti et al. (2014) [21].

During Phase 2, no significant changes in overall process performance indicators were observed, despite of a malfunction causing a small biomass loss on day 118; the addition of untreated real ww in the influent (Phase 3) initially caused the worsening in nitrogen removal. NRE decreased from day 122 and peaked to its lowest on day 135 (72%); coherently, nitrite accumulated up to 113 mgNO_2-N/L (day 139). In order to prevent irreversible inhibition of anammox metabolism due to very prolonged exposure to high nitrite concentration (NO_2-

N≥100 mg/L), biomass was washed and resuspended three times in tap water on day 140. Performance recovery was then observed, and process stabilized by day 145.

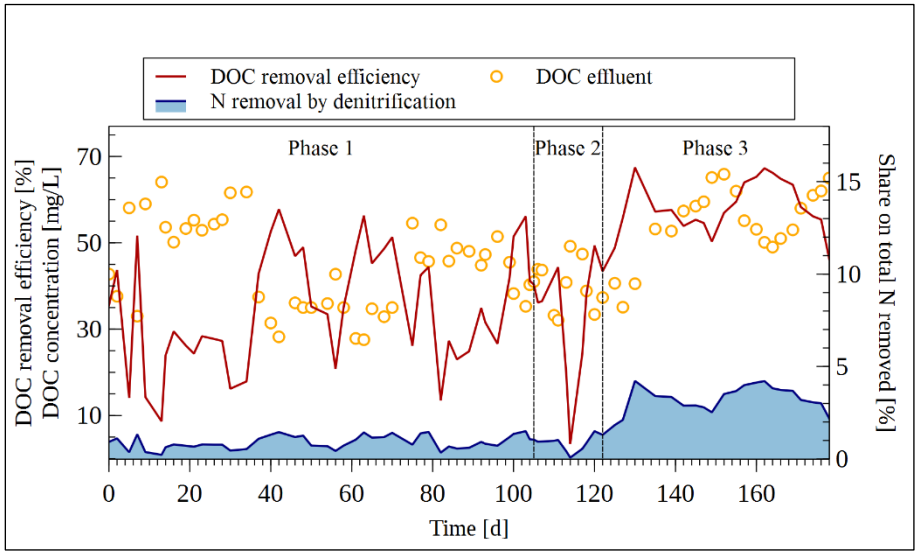

Figure 5.10: DOC effluent concentration and removal efficiency, and estimated contribution of denitrification on total nitrogen removal in Anammox unit.

Addition of untreated real wastewater caused the presence of readily biodegradable substrate which enhanced the occurrence of denitrification: DOC removal efficiency increased up to 67% and denitrification contribution to nitrogen uptake significantly rose, peaking at ~5% of total removed nitrogen.

Both TSS and VSS concentration inside the reactor showed an increasing trend during phase 1 (Figure 5.11), likely due to residual solid content of pre-treated real wastewater which accumulated in the mixed liquor and also contributed to increasing TSS and VSS effluent concentration. Because of a biomass loss caused by a malfunction on day 118, a sudden drop (from 6.2 to 4.0 gVSS/L) in solids concentration inside the reactor was observed at the beginning of Phase 3, subsequently followed by a decrease in effluent TSS and VSS.

With regard to specific anammox activity, as depicted in Figure 5.11 an increasing trend was observed throughout phase 1, with a maximum value of 0.71 gN_2-N/gVSS·d on day 60. As the share of pre-treated real ww increased, SAA progressively decreased, reaching its lowest at the beginning of phase 3 (0.39 gN_2-N/gVSS·d). Such decrease in SAA, combined with inhibitory effect of accumulated nitrite, may have led to process worsening observed on days 122-140. Even though optimal process performance was achieved again during the second half of phase 3, SAA stabilized around 0.4 gN_2-N/gVSS·d (Figure 5.12).

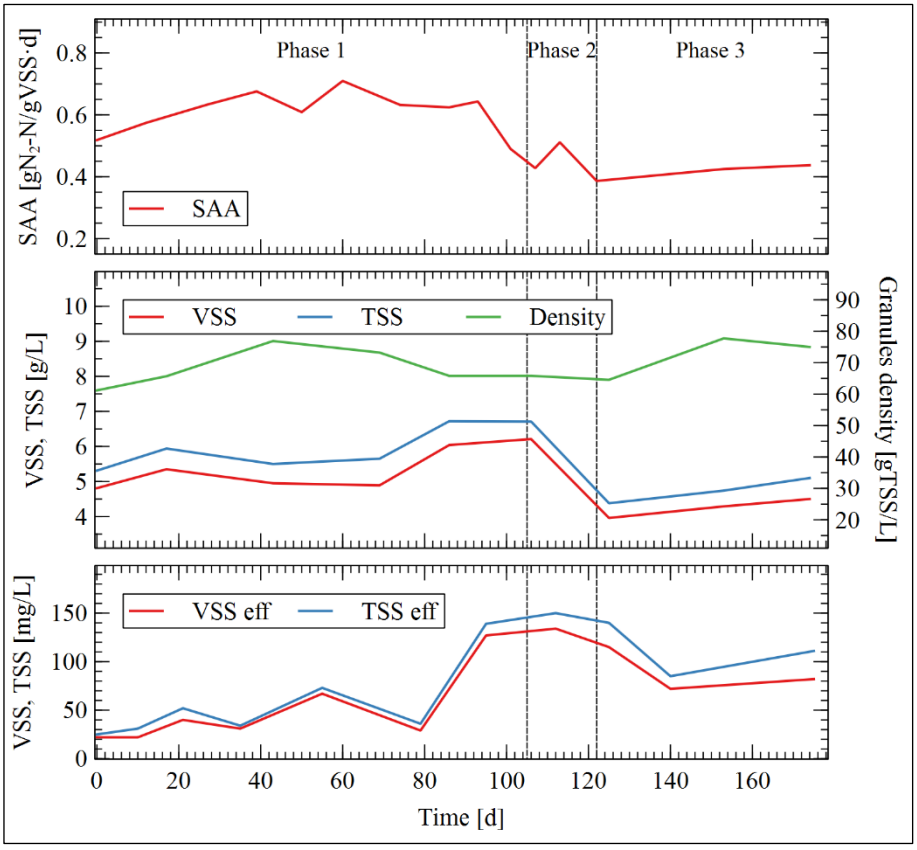

Figure 5.11: (Top) Specific Anammox Activity; (middle) reactor suspended solids and granules density; (bottom) effluent suspended solids, respectively, in Anammox unit.

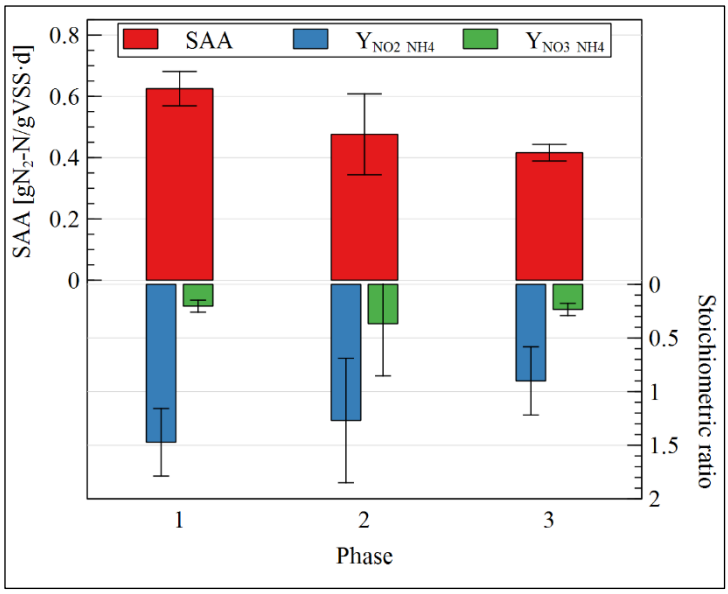

Figure 5.12: SAA and molar ratios average values for each experimental Phase.

As to granular sludge characterization, granules density did not vary significantly throughout the whole experimentation (ranging between 61 and 78 gTSS/L, Figure 5.11); however, small changes in size distribution were measured, as represented in Figure 5.13 and Table 5.7

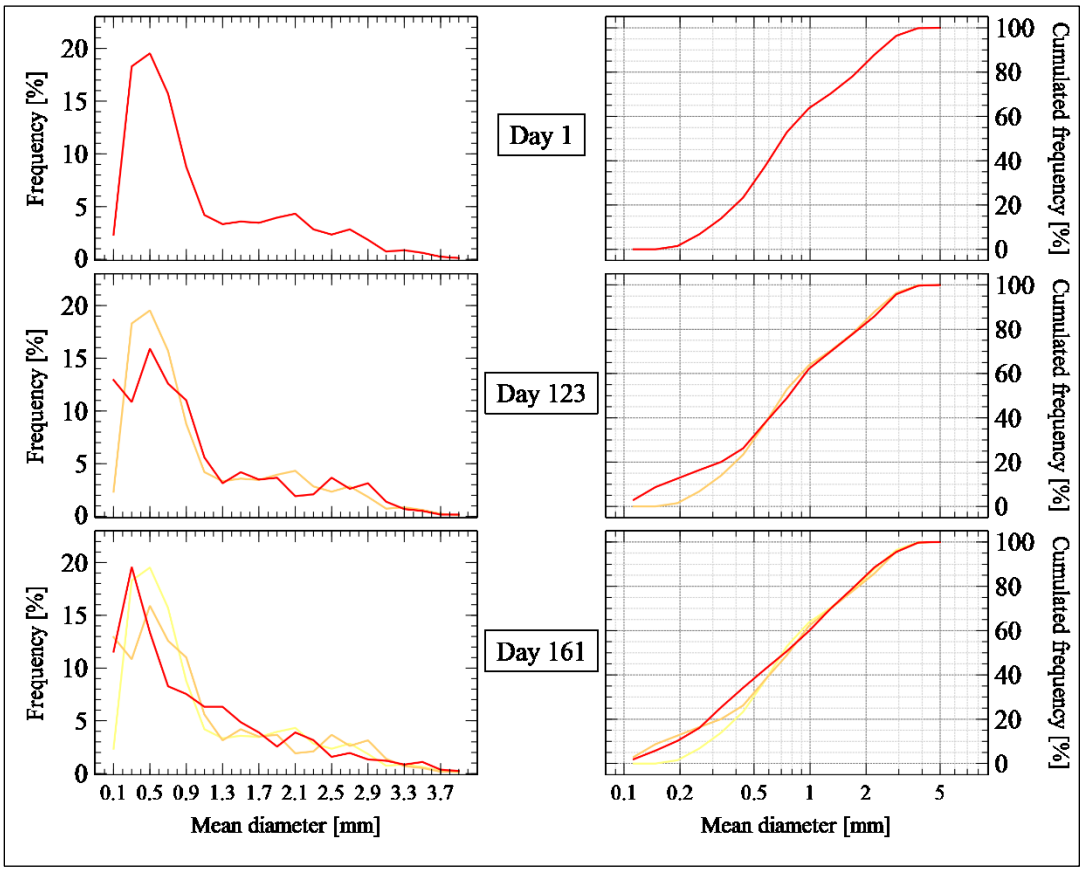

Figure 5.13: Granules size distribution during the experimental activity. Left, absolute frequency distribution (number of classes, 20; minimum and maximum diameter, 0.0 and 4.0 mm, respectively; size class width, 0.2 mm); right, cumulative particle size distribution.

At a global scale, no significant differences were detected among main morphological indicators (mean diameter, aspect and roundness), with the exception of a slight decrease in shape regularity (mainly expressed by roundness) as the experiment proceeded, as further confirmed by the decreasing rate of granules with a roundness value ≥0.8: 62%, 51% and 39% for day 1, 123 and 161, respectively.

Table 5.7: Average values of main morphological indicators of granular sludge.

Sample	Mean diameter [mm]	Aspect [-]	Roundness [-]
Day 1	1.05±0.82	0.67±0.16	0.82±0.13
Day 123	1.06±0.87	0.68±0.15	0.80±0.11
Day 161	1.02±0.86	0.66±0.17	0.75±0.14

Particle size distribution assessment showed relevant changes in smaller size frequency (≤1 mm): from initial state, at the end of phase 2 a significant decrease in 0.3-0.7 mm classes abundance was observed, together with the increase in their close classes (0.1 mm and 0.9-1.1 mm), suggesting the creation of new granules coupled with either the enlargement or the disruption of part of existing granules. On day 163, increase in 0.3 mm and 1.1-1.3 mm classes combined with the corresponding decrease of 0.7-0.9 mm classes seemed to confirm this behavior.

Color analysis was performed on samples – and corresponding blanks – collected on day 50, 76, 91, 111, corresponding to a share of real ww on total influent of 30%, 50%, 70% (phase 1) and 100% (phase 2), respectively, and then on day 130 (phase 3). Data were expressed as three numerical coordinates in CIE Lab color space, i.e. L*, a* and b*. Software automatically provided the conversion of a* and b* into the corresponding polar coordinates, C* and h, which is also proportional to a*/b*ratio. Results of measurement are reported on average in Table 5.8; a representation on Lab color space is also provided on Figure 5.14.

Table 5.8: Average values of measured color parameters. Standard deviation was always below 0.1.

Sample	L*	a*	b*	C*	h	a*/b*
30%	48.1	26.0	32.6	41.7	51.4	0.80
50%	40.8	25.7	26.5	36.9	45.9	0.97
70%	36.1	21.5	20.1	29.5	43.2	1.07
100%	36.1	21.5	20.1	29.4	43.0	1.07
Phase 3	38.7	23.0	23.1	32.5	45.1	1.00

Blank	L*	a*	b*	C*	h	a*/b*
30%	95.4	0.1	-1.0	1.0	274.5	-0.08
50%	94.5	-0.1	0.6	0.6	95.5	-0.09
70%	93.2	-0.3	2.7	2.7	96.4	-0.11
100%	91.3	-0.1	1.5	1.5	92.9	-0.05
Phase 3	85.9	-0.1	2.0	2.0	93.1	-0.05

Biomass color changed throughout the experiment. In particular, as share of pre-treated ww increased up to 100%, wider variations were observed in L* (lightness) and C* sample values, while hue (and a*/b*ratio) value showed limited variations. With regard to blank values, a progressive decrease in L* value was observed, while negligible variations were detected for a* and b* values.

Progressive darkening of both biomass and liquid fraction were observed, likely due to the higher content in suspended solids of influent wastewater.

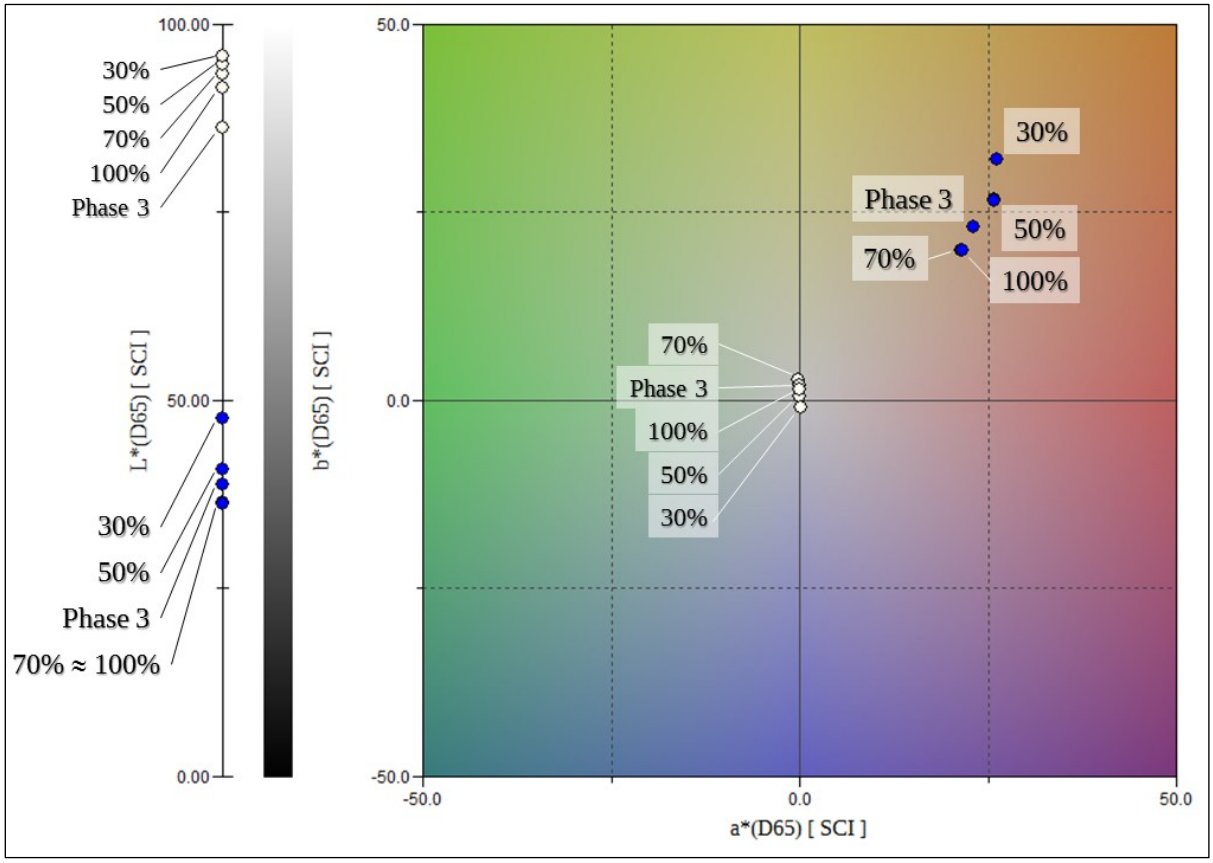

Figure 5.14: Representation of color samples (blue circles) and blanks (white circles) in the CIE Lab color space.

CIE dE_{00} color difference indicator was calculated for each "sample/blank" couple, in order to assess whether samples color changed not only because of the effect of increasing real wastewater share on total influent. Also, corresponding biomass samples were microbiologically characterized through FISH analysis, and relative abundance of anammox bacteria was determined (Table 5.9).

Table 5.9: Calculated dE_{00} color difference and anammox bacteria relative abundance measurement.

Sample	dE_{00} [-]	Anammox bacteria abundance [%]
30%	42.79	54.2
50%	48.01	53.1
70%	51.23	59.5
100%	50.26	48.8
Phase 3	45.16	n.a.

Finally, dE_{00} color difference between each "sample-blank" couple was chosen as color main indicator, together with absolute C* value (which showed wider variations, and depended by a* and b* only) and a*/b*ratio (as indicator for hue changes, according to [1]), and then related

to corresponding share of real ww on total influent, SAA (as performance indicator) and anammox bacteria relative abundance. Linear fitting was operated in order to highlight potential positive correlations. Results are depicted on Figure 5.15.

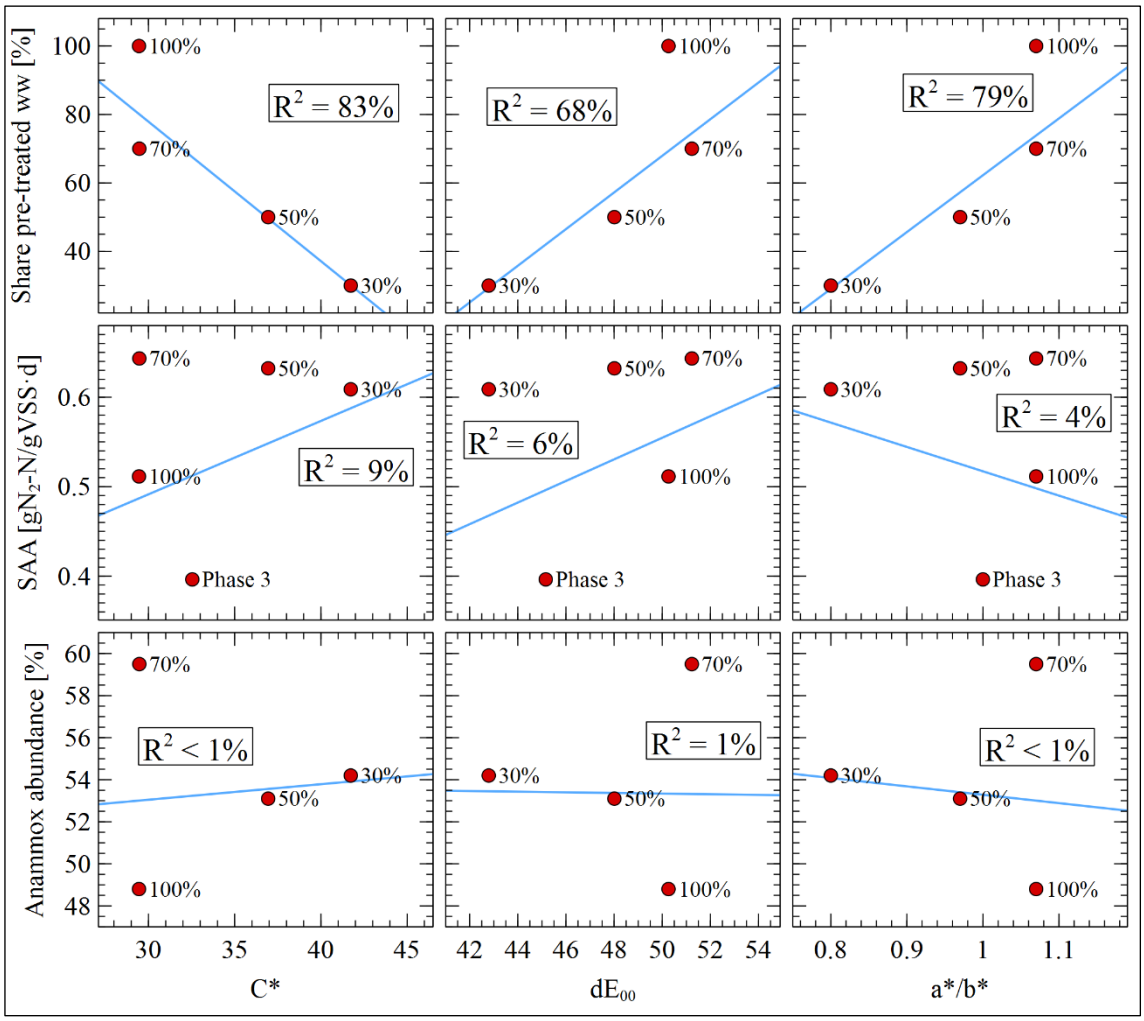

Figure 5.15: Scatter plots correlating color parameters with increasing share of pre-treated real ww on total influent, anammox biomass activity and bacteria abundance.

Results confirmed the good correlation between color change and the increasing share of real ww on total influent, clearly suggesting that a dyed influent may alter the biomass observed color, likely because of adsorption of dyed compounds and/or particles on granules. No other significant correlation was found between color and biomass activity and composition indicators.

5.4 Discussion

5.4.1 Startup of PN reactor

Many different configurations and approaches were proposed for the startup and operation of (partial) nitritation reactors, either aimed at one- or two-stage (autotrophic) deammonification processes [22]; they all can be referred to three different strategies [23]:

1. the combination of a relatively high temperature and short solids retention time (SRT) to promote the growth of AOB and the wash-out of NOB;
2. process operation under high concentrations of FA and/or FNA to take advantage of NOB inhibition in these conditions;
3. process operation under low concentrations of DO to take advantage of the lower NOB affinity for DO.

The first strategy was at the base of the first described coupled Sharon-anammox process [24]: it allowed the achieving of stable partial nitritation within 2 weeks in a CSTR reactor fed with supernatant from AD of waste sludge, and operating at 35°C, HRT = 2 d and continuous aeration. In our study, a similar approach did not lead to stable PN, despite wastewaters showed a comparable composition (i.e., a COD/NH_4-N ratio ≈1.02). Such result could be ascribed to (a) occurrence of acute inhibiting effect of the target wastewater over nitrifying bacteria, as reported on Chapter 3, and (b) higher organic matter content. Such factors did not allow ammonium oxidizing bacteria initially present in the inoculum to grow fast enough to avoid to be washed out of the system. The semi-continuous batch selector system, described in sections 5.2.7 and 5.3.1, was then successfully applied and allowed to achieve satisfactory nitritation rates within 42 days.

Systems based on decoupling of HRT and SRT, and thus allowing high SRT values (>6 d), such as SBR and MBR, have been widely used to achieve partial nitritation by means of different selection strategies and operational conditions, with startup duration varying from 40 to 300 days [25]. At room temperature, strategies based on FA and low DO control aimed at NOB inhibition were proved to be efficient. Katsou et al. [26] took 100 d to operate a stable pilot-scale nitritation-denitritation SBR at room temperature, fed with low strength UASB effluent and fermented biowaste and sewage, using both FA and low DO level to promote NOB inhibition; Gu et al. [27] tested a combined real-time control strategy, based on blower frequency and pH, achieving successful PN in a pilot-scale SBR at low temperature (11-16°C) and fed with low strength sewage, within 40 days. More recently, Liu et al. [28] achieved stable PN in a lab-scale SBR, operating at 29°C and fed with domestic wastewater, by submitting the

biomass (collected from a conventional activated sludge system and used as inoculum) to a long aerated starvation period (21 d) which irreversibly hindered the recovery of NOB activity. Moreover, a recent study [25] suggested that startup of PN SBR can be shortened by appropriate selection of seed inoculum, i.e. by avoiding the use of NOB-containing inoculum such as nitrifying conventional active sludge.

In the present experiment, startup configuration was not specifically designed to suppress NOB; nonetheless, the occurrence of remarkable concentrations of both FA (up to 150-200 mgNH_3/L, at the beginning of each cycle) and FNA (up to 2-4 mgHNO_2/L, at the end of each cycle) likely contributed in strong inhibition of NOB activity. Similar results were reported by Caffaz et al. [29], who observed stable nitrite accumulation after 22 d of operation in a lab-scale MBBR seeded with CAS, fed with a real anaerobic supernatant after phosphate removal via struvite precipitation, and operating at 30°C with not limiting oxygen conditions; although the applied HRT was relatively short 0.5-2.0 d, the presence of biofilm carriers likely allowed both AOB and NOB to avoid hydraulic washout; nonetheless, only AOB activity was observed, while NOB inhibition was ascribed to FNA concentration (0.3-0.8 mg/L).

Such results are consistent with the quite wide range of FA/FNA inhibiting values of AOB/NOB activity reported in literature. According to Feng et al. [22], such variability can be explained by differences in microbial communities; however, NOB always showed either FA or FNA inhibition at much lower concentrations compared to AOB.

In conclusion, successful selection of AOB was achieved within 42 days by operating a semi-continuous batch reactor seeded with conventional activated sludge and fed with the supernatant produced by a two-stage AD of OFMSW, under the following conditions: (a) prolonged biomass/wastewater contact time, aimed at achieving the complete oxidation of organic substrate before nitrification, thus avoiding competition between heterotrophic bacteria and AOB; (b) pH control, which reduced free ammonia concentration; (c) intermittent aeration, which allowed limited stripping of FA and CO_2; (d) supplementary bicarbonate addition, which compensated alkalinity consumed by acid and promoted ammonium oxidation once organic substrate had been removed.

5.4.2 PN reactor performance and effluent NO_2/NH_4 molar ratio regulation

Main results from PN experimentation can be synthesized as follows:

- Successful stable PN of target ww was achieved in a CSTR operated at T=35°C, DO=3 ppm, HRT≥0.6 d and NLR≤2.5 kgN/m^3·d;

- when stable process proceeded, effluent nitrite/ammonium molar ratio was higher than the optimum for the subsequent anammox treatment;
- oxygen regulation was proved not to be a suitable strategy to control ammonium oxidation rate and thus effluent NO_2/NH_4 ratio; process performance rapidly and irreversibly worsened when DO was set below 3 ppm, and neither DO increase nor NLR reduction led to its recovery;
- progressive shortening of HRT, with the corresponding increase in NLR (1.5-2.5 gN/L·d) initially led to a decrease in effluent NO_2/NH_4 ratio; however, excessive load (>2.5 gN/L·d) could not be managed by the system.

Nitritation reactors have been previously operated for the treatment of effluents from AD of OFMSW mixed with other organic substrates, as waste sludge, piggery manure, vegetable and fruit waste [26,30,31]. In those studies, however, full (not partial) nitritation was requested, in order to realize a nitritation/denitritation process. In this sense, applied selective pressure aimed at the suppression of NOB only, without any limitation to nitrite accumulation. A recent overview on biological nutrient removal from supernatant originating by AD of OFMSW reported only one application of PN-anammox process for the treatment of such wastewater [30]; no literature references were found on the application of two-stage PN/anammox process for treating the liquid fraction produced by two-step, biohydrogen producing, AD systems of OFMSW.

When PN is coupled with the anammox process, the control of nitritation rate in order to produce an effluent with a suitable nitrite/ammonium molar ratio is of crucial importance [32]. It has been theoretically and experimentally recognized that nitritation performance is heavily dependent on the available alkalinity (i.e., bicarbonate, inorganic carbon, IC) [33].

Stoichiometric alkalinity requirements for the production of a suitable anammox influent are 1.14 molC/molN, or 4.07 $gCaCO_3/gNH_4$-N [23,33]. When alkalinity is too low, the effluent NO_2/NH_4 ratio is lower than the optimal value, thus potentially leading to ammonium accumulation and subsequent inhibition in the anammox unit; a control strategy based on addition of chemicals (bicarbonate) and manual influent Alk/N ratio adjusting was proposed by Ganiguè et al. [34].

When alkalinity is too high, more nitrite than needed may be produced by PN. In this situation, several approaches were proposed. Scaglione et al. [35] treated centrifuge supernatant coming from a full scale anaerobic digester fed with a mixture of piggery manure, poultry manure, and agro-wastes in a pilot scale PN SBR; ammonium oxidation efficiency was controlled by

regulating the influent alkalinity with HCl dosage to obtain the optimal NH_4-N to alkalinity molar ratio of 1:1. Other solutions did not implicate addition of chemicals. According to Magrì et al. [23], three main options are possible based on the organic load availability: (i) take advantage of heterotrophic denitrification, (ii) appropriate fitting of the N loading rate (NLR), or (iii) bypass part of the influent and mix it with the effluent from the PN system. In addition, other strategies are based on aeration/DO control and on real time control through continuous monitoring of different parameters ([36–38]).

Most recent studies on PN/anammox applied to anaerobic digester effluents, where PN process was carried on with continuous flow reactors, were Caffaz et al. [29], Yamamoto et al. [39], and Mosquera Corral et al. [40]. Caffaz et al. [29] used a continuous flux MBBR to treat the effluent produced by a full scale anaerobic co-digestion plant of OFMSW, olive mill wastewater and septage, operating at 30°C, and achieved a 1:1 nitrite/ammonium effluent ratio during 1 year of operation at a NLR of 1.2 gN/L·d; however, no information were provided regarding alkalinity content of treated wastewater or about any specific strategy aimed at achieving such results. Similarly, lack in such information were observed for both Yamamoto et al. [39] and Mosquera-Corral et al. [40]; moreover, the latter reported quite unstable process when real wastewater, i.e. the effluent from anaerobic reactor treating the wastewater from a fish cannery, was fed into the Sharon reactor at a NLR of 1.0 gN/L·d.

In the present study, influent alkalinity/ammonium molar ratio averaged out at 1.3, which led to an effluent NO_2/NH_4 molar ratio higher than the optimal one. Decreasing dissolved oxygen concentration (i.e., from 3.0 to 2.5) and increasing NLR (1.5-2.5 gN/L·d) were tested during phase 1-2 and 3, respectively, to regulate ammonium oxidation rate: both of them proved not to be suitable strategies. Differently, Magrì et al. (2012) [41] achieved significant corrections of effluent NO_2/NH_4 molar ratio testing both strategies on a pilot scale PN SBR operating at 30°C and treating swine wastewater with 1026-1385 mgNH_4-N/L and alkalinity/ammonium 1.18-1.35 fold the stoichiometric ratio. In a first phase, aeration was fixed, while NLR changed (0.8-2.4 gN/L·d) apparently on a daily basis, achieving an average effluent NO_2/NH_4 molar ratio of 1.38, i.e. slightly higher than optimum. In a second phase, NLR was kept at 1.2 gN/L·d and continuous aeration flow rate was progressively improved, causing DO values vary in 0.0-3.5 ppm range. Increasing air supply resulted in the corresponding increase in effluent nitrite to ammonium ratio (0.37-1.76 mol/mol). Even though clear regulating effects of those parameters were proved, their fine tuning resulting in the achievement of stable correct effluent NO_2/NH_4 molar ratio was not reported. More recently, Li et al. [37] operated a continuous flux MBR to achieve partial nitritation treating mature (>5 years) landfill leachate with high

alkalinity/ammonium ratio, testing both HRT (consequently, NLR) and DO as key factors regulating effluent nitrite/ammonium ratio. An effluent NO_2/NH_4 molar ratio ranging between 1.38-1.42 was achieved during 20 days operating at HRT=13.9 h (0.58 d; corresponding to a NLR=1.7 gN/L·d); however, subsequent decrease in nitrite build-up was observed, suggesting that half-nitritation could be quickly and easily realized by HRT control, but it may be difficult to maintain in the long-term. Such conclusion is in agreement with the results observed in the present study (Run 3). In a second phase of the same study, while NLR was kept at 1.1 gN/L·d, DO was slowly reduced from 3.0 to 1.0 ppm resulting in the system stably operated for 29 d with an effluent NO_2/NH_4 molar ratio of 1.14–1.57 (1.35 on average). Such results are in contrast with the ones from the present experiment. An explanation could be found in the difference in sludge retention time. In the present study, SRT was coupled with HRT and resulted in a fragile equilibrium between biomass growth and drainage, especially regarding autotrophic biomass: when the equilibrium was altered by oxygen decrease (Run 1-2; HRT=1 d) growth rate likely decreased to insufficient values to counteract continuous drainage, eventually resulting in biomass washout and unsuccessful process recovery, while such washout was avoided in biomass retaining reactors such as MBR and SBR.

Nonetheless, stable PN operation of CSTR fed with a real wastewater without any influent correction was achieved in the present study.

5.4.3 Performance of Anammox unit

It has to be noticed that despite PN-anammox process has been applied to the treatment of effluent from anaerobic digestion of a great variety of organic matter, even at full scale [42,43], very few references could be found regarding anammox applied to digestate of OFMSW [30].

Anammox unit showed a good acclimation to pre-treated target ww (phase 1), and overall performance was not affected by progressive substitution of mineral medium. Only SAA showed significant variability along phase 1, first increasing from 0.52 to 0.71 gN_2-N/gVSS·d (day 60, 36% real ww on total influent) and then decreasing and stabilizing around 0.44 gN_2-N/gVSS·d during phase 2. A similar behavior was reported by Qiao et al. [45] whose preliminary assessment of possible inhibitory effects of pretreated wastewater on Anammox biomass resulted in an improved SAA (maximum value: 0.27 gN_2-N/gVSS·d) compared to that measured in reference mineral medium. SAA values observed throughout the whole present experiment are higher than those reported by Caffaz et al. [29] (i.e., 0.102 gN_2-N/gVSS·L) for a pilot scale Anammox SBR operated at 35°C and fed with a pre-treated supernatant originating

from AD of OFMSW. During phase 2, stable performance and no biomass inhibition due to prolonged exposure to real wastewater were observed.

As discussed in section 5.4.2, effluent from PN reactor showed a higher NO_2/NH_4 molar ratio than optimum for anammox unit. During phase 2, manual correction of such ratio was operated by means of chemicals addition: this solution is not substantially different than the correction of initial alkalinity/ammonium ratio of raw wastewater, which was previously discussed.

Different authors [23,34] proposed the mixing of pretreated and untreated wastewater as the simplest solution for influent nitrite/ammonium ratio correction. However, only one study was found to having applied such solution. In their experiment, Qiao et al. [45] treated a livestock manure digester liquor with a two stage PN-anammox system. Anammox was fed with a mix of pretreated and raw wastewater (relative share not reported); authors observed a nitrite accumulation after the first week of operation, ascribed to unbalanced effluent with accumulated nitrite from PN reactor, which resulted in severe inhibition of anammox activity, followed by 100 days of unstable performance; eventually the resumption of anammox activity was achieved just after the pH was adjusted from 7.9 to 7.5. However, no specific positive or negative effect was ascribed to the presence of untreated wastewater in the influent.

In the present experiment, addition of untreated wastewater (phase 3) resulted in a temporary decrease in process performance, which was subsequently recovered within 20 days. In a recent study, Scaglione et al. [46] tested for the first time the treatability of the liquid fraction of digested OFMSW with the anammox process by means of batch tests, and conductivity was identified as the most reliable aggregate parameter to evaluate potential short-term inhibitory effect of such wastewater. Mid term or long term inhibition was never specifically assessed. Results from the present experiment indicated that addition of influent untreated liquid fraction of digested OFMSW to an unacclimated Anammox reactor resulted in a temporary inhibition, which was spontaneously neutralized within 20 days.

However, given the complex composition of such AD effluents [30,47], it was not possible to identify any specific compound or parameter at the origin of the temporary decrease in removal efficiency observed at the beginning of phase 3. A hypothesis may involve interference of organic matter to anammox metabolism, whose mechanisms are still unclear and depend on concentration and organic compound [48]; moreover, drastic lack in specific studies regarding application of PN-anammox to effluent from AD of OFMSW did not allow to find reliable comparisons.

5.4.4 Color analysis

To the best of our knowledge, color characterization of granular sludge through specific measurement, equipment and CIE Lab (or any other) color space representation was previously tested by Kang et al. [1] only. In that study, three reactors fed with synthetic influent and operated at different biomass loading rate (BLR, i.e. the ratio between NLR and biomass concentration) led to detect three different surface-colored ("black", "brown" and "red") granular biomass characterized by different SAA and anaerobic ammonia oxidizing bacteria (AnAOB) enrichment grade. As main color indicator, authors indicated a*/b* ratio (which is proportional to h value), which was positively correlated through linear fitting both to SAA and BLR, but not with AnOB abundance neither cytochrome-c concentration.

In the present study, differently, such approach was tested for the first time on a reactor fed with real wastewater, which showed its own color that may have contributed to overall biomass color. Such potential interference was estimated by introducing the blank measurement and the relative color difference assessment (dE_{00}) as a prospective color indicator. Moreover, biomass was already enriched in metabolically active anammox bacteria at the beginning of the experiment, and always showed the typical reddish coloration. This resulted in a limited variability of a*/b* ratio, as a consequence of the quite stable hue observed throughout the experiment; therefore, a*/b* ratio did not appear as the best color indicator, especially when compared to C* parameter.

Results of color/SAA and color/anammox abundance correlation did not allow to draw unambiguous conclusions; nevertheless, they may suggest the following observations:

a) chroma C* can be considered as a more reliable index for color changes assessment in anammox-enriched biomass, compared to hue and other related parameters, such as a*/b* ratio;
b) the occurrence of a dyed and/or a high SS containing influent wastewater may affect granules color, likely due to adsorption of colored particles on granules surface: an evaluation of this potential interference must be assessed or estimated, in order to evaluate color changes not depending by the influent wastewater. In this study, preparation and measurement of a blank sample was proposed, together with the evaluation of "net" color change of each "sample-blank" couple, expressed as color difference, dE_{00}.

Moreover, it can be reasonably stated that in a stable conditions-long term running anammox reactor, biomass may show a pretty stable color. Changes in operating conditions can result in

small biomass color alterations: in this case, it may be useful to identify a color reference (e.g., biomass color under unaltered conditions) and to evaluate relative color differences from reference sample. The CIE dE_{00} index would supposedly work better in this sense, since it was indeed designed to spot small color differences.

Digital assessment of biomass color in anammox process may represent a quick, simple and cost-effective indirect measurement of process performance, metabolic activity and biomass enrichment; more studies are needed in this direction, with special regard to real wastewater treatment and/or to pilot/full scale plant application.

5.5 Conclusions

In this study, two-stage PN-anammox process was applied for the first time to the treatment of the liquid fraction produced by two-stage anaerobic digestion of municipal solid waste (target ww).

Nitritation was first achieved within 42 days in a semi-continuous batch reactor seeded with conventional activated sludge and fed with target ww only. Startup strategy involved intermittent aeration and pH control to avoid NH_3 production and subsequent stripping, and occasional $NaHCO_3$ addition to regulate alkalinity; semi-continuous batch mode allowed prolonged biomass/wastewater contact and solid retention, promoting biomass acclimation and AOB growth; NOB inhibition was mainly ascribed to both free ammonia and free nitrous acid inhibition.

The semi-continuous batch reactor was then converted into a CFSTR system. Stable PN was achieved treating the target ww only, under the following operating conditions: T=35°C, DO=3 ppm, HRT≥0.6 d and NLR≤2.5 gN/L·d. Because of high influent alkalinity/ammonium ratio (1.3 mol/mol), PN effluent mostly resulted in a NO_2/NH_4 molar ratio higher than optimum range requested by subsequent anammox unit (1.6-2.1). Decreasing DO and increasing NLR were tested as control parameters for the adjustment of the effluent nitrite/ammonium molar ratio. While DO regulation led to process failure, NLR regulation initially seemed to be effective; however, biomass was not able to withstand NLR≥2.5 gN/L·d.

Anammox SBR unit was operated in a fed batch mode at an average NLR of 1.5 gN/L·d. Synthetic influent was progressively replaced by PN effluent according to an exponential law (phase 1), in order to promote a gradual acclimation of the biomass. 100% pre-treated target ww was eventually fed (phase 2), and chemical addition was used to correct the influent

NO_2/NH_4 molar ratio. Stable good process performance was observed throughout phase 1 and 2. Subsequently, influent NO_2/NH_4 molar ratio was corrected by mixing pre-treated and untreated target ww (phase 3). Such solution was tested for the first time using effluent from AD of OFMSW. A decrease in anammox performance was observed at first, but process recovery was achieved within 20 days. Average NRE ranged between 87 and 90% in each phase. Maximum SAA (0.71 gN_2-N/gVSS·d) was observed during Phase 1 when share of pre-treated target ww on total influent was 36%; during phase 2-3, SAA averaged out at 0.44 gN_2-N/gVSS·d.

Digital color characterization of anammox granular biomass and subsequent representation through CIE Lab color space was applied for the first time on a system fed with real wastewater. Although results were not statistically well correlated to other performance indicator or to anammox bacteria abundance, some issues were pointed out about the need for measurement protocols considering possible interference by mixed liquor/influent wastewater color and suspended solids content. In this sense, a protocol introducing a blank assessment was proposed. Moreover, chroma C* was identified as a more representative color parameter.

5.6 References

[1] D. Kang, Q. Lin, D. Xu, Q. Hu, Y. Li, A. Ding, M. Zhang, P. Zheng, Color characterization of anammox granular sludge: Chromogenic substance, microbial succession and state indication, Sci. Total Environ. 642 (2018) 1320–1327. doi:10.1016/J.SCITOTENV.2018.06.172.

[2] B. Kartal, W.J. Maalcke, N.M. de Almeida, I. Cirpus, J. Gloerich, W. Geerts, H.J.M. Op den Camp, H.R. Harhangi, E.M. Janssen-Megens, K.-J. Francoijs, H.G. Stunnenberg, J.T. Keltjens, M.S.M. Jetten, M. Strous, Molecular mechanism of anaerobic ammonium oxidation, Nature. 479 (2011) 127. http://dx.doi.org/10.1038/nature10453.

[3] W.R.L. Van Der Star, A.I. Miclea, U.G.J.M. Van Dongen, G. Muyzer, C. Picioreanu, M.C.M. Van Loosdrecht, The membrane bioreactor: A novel tool to grow anammox bacteria as free cells, Biotechnol. Bioeng. 101 (2008) 286–294. doi:10.1002/bit.21891.

[4] APHA, Standard Methods for the Examination of Water and Wastewater, 21 st, American Public Health Association, 2005.

[5] A.C. Anthonisen, R.C. Loehr, T.B.S. Prakasam, E.G. Srinath, Inhibition of Nitrification by Ammonia and Nitrous Acid, J. (Water Pollut. Control Fed. 48 (1976) 835–852.

http://www.jstor.org/stable/25038971.

[6] M.C.M. van Loosdrecht, P.H. Nielsen, C.M. Lopez-Vazquez, D. Brdjanovic, Experimental Methods in Wastewater Treatment, 2016. doi:10.1017/CBO9781107415324.004.

[7] F.L. Burton, G. Tchobanoglous, R. Tsuchihashi, H.D. Stensel, I. Metcalf & Eddy, Wastewater Engineering: Treatment and Resource Recovery, McGraw-Hill Education, 2013. https://books.google.it/books?id=6KVKMAEACAAJ.

[8] J.S. Almeida, M.A.M. Reis, M.J.T. Carrondo, Competition between nitrate and nitrite reduction in denitrification byPseudomonas fluorescens, Biotechnol. Bioeng. 46 (1995) 476–484. doi:10.1002/bit.260460512.

[9] S.S. Adav, D.J. Lee, J.Y. Lai, Enhanced biological denitrification of high concentration of nitrite with supplementary carbon source, Appl. Microbiol. Biotechnol. 85 (2010) 773–778. doi:10.1007/s00253-009-2265-4.

[10] N.M. Lee, T. Welander, The effect of different carbon sources on respiratory denitrification in biological wastewater treatment, J. Ferment. Bioeng. 82 (1996) 277–285. doi:10.1016/0922-338X(96)88820-9.

[11] M. Sage, G. Daufin, G. Gésan-Guiziou, Denitrification potential and rates of complex carbon source from dairy effluents in activated sludge system, Water Res. 40 (2006) 2747–2755. doi:10.1016/j.watres.2006.04.005.

[12] J.J. Beun, A. Hendriks, M.C.M. van Loosdrecht, E. Morgenroth, P.A. Wilderer, J.J. Heijnen, Aerobic granulation in a sequencing batch reactor, Water Res. 33 (1999) 2283–2290. doi:https://doi.org/10.1016/S0043-1354(98)00463-1.

[13] Commission Internationale de l'Eclairage, Colorimetry, 3rd ed., CIE publications, 2004. doi:ISBN 3 901 906 33 9.

[14] M.R. Luo, G. Cui, B. Rigg, The development of the CIE 2000 colour-difference formula: CIEDE2000, Color Res. Appl. 26 (n.d.) 340–350. doi:10.1002/col.1049.

[15] R.I. Amann, W. Ludwig, K.H. Schleifer, Phylogenetic identification and in situ detection of individual microbial cells without cultivation . Phylogenetic Identification and In Situ Detection of Individual Microbial Cells without Cultivation, Microbiol. Rev. 59 (1995) 143–169. doi:10.1016/j.jip.2007.09.009.

[16] A. Loy, F. Maixner, M. Wagner, M. Horn, probeBase--an online resource for rRNA-

targeted oligonucleotide probes: new features 2007, Nucleic Acids Res. 35 (2007) D800–D804. doi:10.1093/nar/gkl856.

[17] H. Daims, S. Lücker, M. Wagner, daime, a novel image analysis program for microbial ecology and biofilm research, Environ. Microbiol. 8 (2006) 200–213. doi:10.1111/j.1462-2920.2005.00880.x.

[18] H. López, S. Puig, R. Ganigué, M. Ruscalleda, M.D. Balaguer, J. Colprim, Start-up and enrichment of a granular anammox SBR to treat high nitrogen load wastewaters, J. Chem. Technol. Biotechnol. 83 (2008) 233–241. doi:10.1002/jctb.1796.

[19] M. Strous, J.G. Kuenen, M.S.M. Jetten, Key Physiology of Anaerobic Ammonium Oxidation Key Physiology of Anaerobic Ammonium Oxidation, Appl. Environ. Microbiol. 65 (1999) 0–3. doi:papers2://publication/uuid/E9A1573A-6D62-420E-94D0-CA7C84D0FEB9.

[20] S. Milia, M. Perra, G. Tocco, A. Carucci, The start-up of an anammox reactor as the second step for the treatment of ammonium rich refinery (IGCC) wastewater with high C<inf>org</inf>/N ratio, Ecol. Eng. 106 (2017). doi:10.1016/j.ecoleng.2017.04.068.

[21] T. Lotti, R. Kleerebezem, C. Lubello, M.C.M. van Loosdrecht, Physiological and kinetic characterization of a suspended cell anammox culture, Water Res. 60 (2014) 1–14. doi:10.1016/j.watres.2014.04.017.

[22] Y. Feng, X. Lu, H. Al-Hazmi, J. Mąkinia, An overview of the strategies for the deammonification process start-up and recovery after accidental operational failures, Rev. Environ. Sci. Biotechnol. 16 (2017) 541–568. doi:10.1007/s11157-017-9441-2.

[23] A. Magrí, F. Béline, P. Dabert, Feasibility and interest of the anammox process as treatment alternative for anaerobic digester supernatants in manure processing - An overview, J. Environ. Manage. 131 (2013) 170–184. doi:10.1016/j.jenvman.2013.09.021.

[24] L.G.J.M. van Dongen, M.C.M. Van Loosdrecht, M.S.M. Jetten, Combined Sharon / Anammox Process, Utrecht, 2001.

[25] T. Dobbeleers, M. Caluwé, D. Daens, L. Geuens, J. Dries, Evaluation of two start-up strategies to obtain nitrogen removal via nitrite and examination of the nitrous oxide emissions for different nitritation levels during the treatment of slaughterhouse wastewater, J. Chem. Technol. Biotechnol. 93 (2018) 569–576. doi:10.1002/jctb.5403.

[26] E. Katsou, S. Malamis, N. Frison, F. Fatone, Coupling the treatment of low strength anaerobic effluent with fermented biowaste for nutrient removal via nitrite, J. Environ. Manage. 149 (2015) 108–117. doi:10.1016/j.jenvman.2014.09.008.

[27] S. Gu, S. Wang, Q. Yang, P. Yang, Y. Peng, Start up partial nitrification at low temperature with a real-time control strategy based on blower frequency and pH, Bioresour. Technol. 112 (2012) 34–41. doi:10.1016/j.biortech.2011.12.028.

[28] W. Liu, Q. Yang, B. Ma, J. Li, L. Ma, S. Wang, Y. Peng, Rapid Achievement of Nitritation Using Aerobic Starvation, Environ. Sci. Technol. 51 (2017) 4001–4008. doi:10.1021/acs.est.6b04598.

[29] S. Caffaz, E. Bettazzi, D. Scaglione, C. Lubello, An integrated approach in a municipal WWTP: Anaerobic codigestion of sludge with organic waste and nutrient removal from supernatant, Water Sci. Technol. 58 (2008) 669–676. doi:10.2166/wst.2008.683.

[30] S. Malamis, E. Katsou, S. Di Fabio, D. Bolzonella, F. Fatone, Biological nutrients removal from the supernatant originating from the anaerobic digestion of the organic fraction of municipal solid waste., Crit. Rev. Biotechnol. 8551 (2013) 1–14. doi:10.3109/07388551.2013.791246.

[31] N. Frison, S. Lampis, D. Bolzonella, P. Pavan, F. Fatone, Two-stage start-up to achieve the stable via-nitrite pathway in a demonstration SBR for anaerobic codigestate treatment, Ind. Eng. Chem. Res. 51 (2012) 15423–15430. doi:10.1021/ie3009742.

[32] S.W.H. Van Hulle, H.J.P. Vandeweyer, B.D. Meesschaert, P.A. Vanrolleghem, P. Dejans, A. Dumoulin, Engineering aspects and practical application of autotrophic nitrogen removal from nitrogen rich streams, Chem. Eng. J. 162 (2010) 1–20. doi:10.1016/j.cej.2010.05.037.

[33] R. Ganigué, H. López, M. Ruscalleda, M.D. Balaguer, J. Colprim, Operational strategy for a partial nitritation–sequencing batch reactor treating urban landfill leachate to achieve a stable influent for an anammox reactor, J. Chem. Technol. Biotechnol. 83 (n.d.) 365–371. doi:10.1002/jctb.1822.

[34] R. Ganigué, J. Gabarró, A. Sànchez-Melsió, M. Ruscalleda, H. López, X. Vila, J. Colprim, M.D. Balaguer, Long-term operation of a partial nitritation pilot plant treating leachate with extremely high ammonium concentration prior to an anammox process, Bioresour. Technol. 100 (2009) 5624–5632. doi:10.1016/j.biortech.2009.06.023.

[35] D. Scaglione, E. Ficara, V. Corbellini, G. Tornotti, A. Teli, R. Canziani, F. Malpei,

Autotrophic nitrogen removal by a two-step SBR process applied to mixed agro-digestate, Bioresour. Technol. 176 (2015) 98–105. doi:10.1016/j.biortech.2014.11.019.

[36] H. Li, S. Zhou, G. Huang, B. Xu, Achieving stable partial nitritation using endpoint pH control in an SBR treating landfill leachate, Process Saf. Environ. Prot. 92 (2014) 199–205. doi:10.1016/j.psep.2013.01.005.

[37] Y. Li, J. Li, B. Zhao, X. Wang, Y. Zhang, J. Wei, W. Bian, A coupled system of half-nitritation and ANAMMOX for mature landfill leachate nitrogen removal, Environ. Technol. (United Kingdom). 38 (2017) 2335–2343. doi:10.1080/09593330.2016.1259356.

[38] S. Wang, L. Deng, D. Zheng, L. Wang, Y. Zhang, H. Yang, Y. Jiang, F. Huang, Control of partial nitrification using pulse aeration for treating digested effluent of swine wastewater, Bioresour. Technol. 262 (2018) 271–277. doi:10.1016/j.biortech.2018.04.084.

[39] T. Yamamoto, K. Takaki, T. Koyama, K. Furukawa, Long-term stability of partial nitritation of swine wastewater digester liquor and its subsequent treatment by Anammox, Bioresour. Technol. 99 (2008) 6419–6425. doi:10.1016/j.biortech.2007.11.052.

[40] A. Mosquera-Corral, F. González, J.L. Campos, R. Méndez, Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds, Process Biochem. 40 (2005) 3109–3118. doi:10.1016/j.procbio.2005.03.042.

[41] A. Mosquera-Corral, F. González, J.L. Campos, R. Méndez, Partial nitrification in a SHARON reactor in the presence of salts and organic carbon compounds, Process Biochem. 40 (2005) 3109–3118. doi:10.1016/j.procbio.2005.03.042.

[42] A. Magrí, M.B. Vanotti, A.A. Szögi, K.B. Cantrell, Partial Nitritation of Swine Wastewater in View of Its Coupling with the Anammox Process, J. Environ. Qual. 41 (2012) 1989. doi:10.2134/jeq2012.0092.

[43] A. Joss, D. Salzgeber, J. Eugster, R. König, K. Rottermann, S. Burger, P. Fabijan, S. Leumann, J. Mohn, H.R. Siegrist, Full-scale nitrogen removal from digester liquid with partial nitritation and anammox in one SBR, Environ. Sci. Technol. 43 (2009) 5301–5306. doi:10.1021/es900107w.

[44] S. Lackner, E.M. Gilbert, S.E. Vlaeminck, A. Joss, H. Horn, M.C.M. van Loosdrecht, Full-scale partial nitritation/anammox experiences - An application survey, Water Res.

55 (2014) 292–303. doi:10.1016/j.watres.2014.02.032.

[45] S. Malamis, E. Katsou, S. Di Fabio, D. Bolzonella, F. Fatone, Biological nutrients removal from the supernatant originating from the anaerobic digestion of the organic fraction of municipal solid waste, Crit. Rev. Biotechnol. 34 (2014) 244–257. doi:10.3109/07388551.2013.791246.

[46] S. Qiao, T. Yamamoto, M. Misaka, K. Isaka, T. Sumino, Z. Bhatti, K. Furukawa, High-rate nitrogen removal from livestock manure digester liquor by combined partial nitritation-anammox process, Biodegradation. 21 (2009) 11–20. doi:10.1007/s10532-009-9277-8.

[47] D. Scaglione, T. Lotti, E. Ficara, F. Malpei, Inhibition on anammox bacteria upon exposure to digestates from biogas plants treating the organic fraction of municipal solid waste and the role of conductivity, Waste Manag. 61 (2017) 213–219. doi:10.1016/j.wasman.2016.11.014.

[48] A. Schievano, A. Tenca, B. Scaglia, G. Merlino, A. Rizzi, D. Da, R. Oberti, F. Adani, Two-Stage vs Single-Stage Thermophilic Anaerobic Digestion: Comparison of Energy Production and Biodegradation Efficiencies, Environ. Sci. Technol. 46 (2012) 8502–8510. doi:10.1021/es301376n.

[49] R.C. Jin, G.F. Yang, J.J. Yu, P. Zheng, The inhibition of the Anammox process: A review, Chem. Eng. J. 197 (2012) 67–79. doi:10.1016/j.cej.2012.05.014.

Chapter 6

Single-stage partial nitritation/anammox/denitrification SBR reactors operated in continuous and discontinuous mode: assessment of process performance and biomass characterization

6.1 Introduction

The present chapter synthesizes the experimental activity carried out at the Escola Técnica Superior de Enxeñaría at the University of Santiago de Compostela (ETSE-USC) in Spain, during a 4 months research stay under the supervision of Prof. Anuska Mosquera Corral.

The experimental activity was carried out within the framework of the MEDRAR project, funded by Galician Agency for Innovation and co-funded by European Union – European Regional Development Fund.

MEDRAR (MEjora en la DepuRación de Aguas Residuales en pequeños núcleos de población - Improvement in wastewater treatment in small residential clusters) project aimed at the development of a modular wastewater treatment plant applicable to small residential or industrial building clusters. Such modular system was proposed as a sustainable alternative compared to those currently applied in small towns, which often showed lacks in design and efficiency as well as high operational costs. Main objective of the project was then the assessment and validation of each single wastewater treatment unit based on different technologies, in order to map out a plant composed of interlocking process blocks.

Different treatment units would aim at nutrient and organic matter recovery and valorization, as well as at the improved control of micropollutants and pathogenic organisms. Proposed technologies were:

- anaerobic digestion of black water for energy production;
- aerobic biofilm, autotrophic nitrogen removal (ELAN® technology), biofiltration and aerobic granular sludge for nutrient recovery and removal;
- aerobic membrane process for water reuse;
- tertiary treatment, water filtration and purification treatment: ozone, activated carbon, filtration through mussel shells, etc.

Wastewater were considered to originate either from sanitary or combined sewers: for each of them, the modular system would allow to apply a different treatment line in order to match the final destination: reuse, discharge in water body, etc. Integration of treatment units would be managed by a specifically designed control system.

Within this framework, autotrophic nitrogen removal took place in the proposed black water treatment line (Figure 6.1). Black water from separated sanitary sewer would be treated in small anaerobic digester (AD) aimed at the conversion of organic matter into biogas. AD supernatant would be enriched in ammonium, and also contains slowly/not degradable organic micropollutants from pharmaceutical and personal care products (PPCPs) and residual degradable COD. Subsequent single-stage partial nitritation/anammox process at room temperature (ELAN® technology [1]) would be applied in order to remove nitrogen and residual COD, while a tertiary chemical-physical treatment would be used for PPCPs control and water purification.

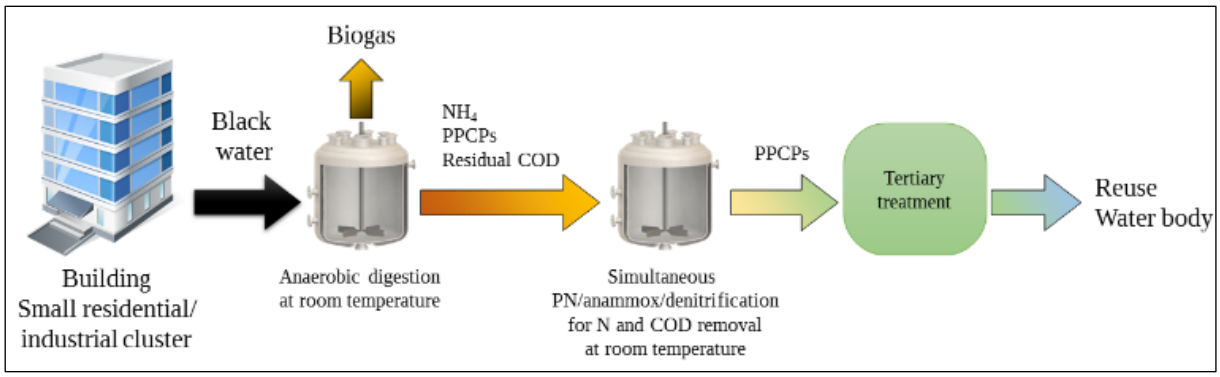

Figure 6.1: Proposed modular plant for black water treatment according to MEDRAR project.

According to the project, such modular treatment line is designed to serve isolated residential clusters or even single buildings (e.g., small reactors can be installed in the basement of big office buildings in industrial areas). Such solution would have to deal with the typical high variability of stream flow rate associated to small towns, which could top at the extreme case of a single office/work building: in this case, wastewater flow rate would be zero during nights, weekends and holydays. In order to face the problem, many solutions could be proposed to regulate the flow rate, such as accumulation tanks; however, the simplest solution would be to run the reactors according to the variable influent flow rate. Thus, the research question resulted in: “regular and prolonged famine periods may affect the process performance and biomass characteristics?”.

The experimental activity carried out at USC during the research stay focused on the assessment of the effects of prolonged intermittent operation on a PN/anammox reactor. Design of the experiment was outlined as follows:

- startup and operation of two 'twins' PN/anammox reactors under the same conditions, until steady-state conditions are achieved;
- application of scheduled stops (nighttime, weekends, holidays) on one of them;
- monitoring of process performance in terms of nitrogen and carbon removal, and biomass activity.

6.2 Materials and methods

6.2.1 Reactors setup

Two glass vessels with a working volume of 4 L were used to carry out the experiment. Reactors were operated as sequencing batch reactors (SBR) with a 3-h cycle configuration (5 min mixed anoxic feeding; 160 min aerated reaction, 10 min settling and 5 min effluent withdrawal). Mechanical mixing (stirring velocity ~40 rpm) was provided by marine impellers operated by overhead stirrers (RW 20 digital, IKA). The influent flow rate was set at 160 mL/min in order to feed a volume of 800 mL per cycle; the resulting volumetric exchange ratio (i.e., the ratio between the influent volume and the total working volume) was 0.2, and the corresponding hydraulic retention time (HRT) was 15 h (0.625 d). Temperature was not controlled, and ranged between 15 and 24 °C; pH was monitored (PHC101 probe, Hach, and HQ40d multimeter, Hach), but not controlled, and ranged between 6.2 and 8.1, depending on the process performance. Dissolved oxygen was monitored (LDO101 probe, Hach, and HQ40d multimeter, Hach), but not controlled.

Feeding and effluent withdrawal were operated by means of two peristaltic pumps; aeration was provided by a compressor (Laboport N 86, KNF), through a submerged glass air stone. Air flow rate was adjusted by means of a gas flow meter (P model, Aalborg).

Process timing was performed via a programmable control system (Simatic S7-200, Siemens).

The reactors were labeled as 'SARD' (Simultaneous AutotRophic Deammonification): one of them was always operated at the same conditions (SARD-C reactor) and served as control reactor, while the other was tested with regular and prolonged operation stops during the night and the weekend (SARD-D reactor).

6.2.2 Inoculum

Reactors were inoculated with mixed nitrifying and anammox granular biomass drawn from the 230 m^3 ELAN plant settled in Guillarei (Pontevedra, Galicia, Spain), and gently provided by FCC Aqualia. Initial total suspended solids (TSS) and volatile suspended solids (VSS) concentrations were 7.5 and 5.8 g/L, respectively (SARD-C) and 4.1 and 3.4 g/L, respectively (SARD-D).

The ELAN® process (Spanish acronym for ELiminación Autotrófica de Nitrógeno, autotrophic nitrogen removal) was developed by FCC Aqualia in Spain. It is based on the use of a SBR with granular biomass to carry out the nitrogen removal by means of the partial nitritation-anammox processes in a single stage. This system is operated in cycles of 3 or 6 h, and the feeding and the aeration are supplied to the reactor during most of cycle duration (90-95%). The ELAN process operates with DO concentrations between 0.8 and 4 mg O_2/L, and mechanical stirring. To maintain the stability of the process, the controlled parameter is the consumed alkalinity to conductivity ratio (alk/cond). The ELAN process was able to treat loads between 0.8 and 1.0 gN/L·d [2,3]. Two full scale plants were started in 2015 in Spain, treating the supernatant from an anaerobic sludge co-digestion (municipal and meat processing wastes) plant, and the anaerobically predigested effluent from a fish canning factory, respectively. Inoculum used in the present experiment was drained from the former plant.

6.2.3 Synthetic influent

Reactors were fed with a synthetic influent simulating the anaerobically digested black water. Synthetic influent provided an ammonium and inorganic carbon (IC) concentration of 300 mgN/L and 240 mgIC/L, respectively. Residual degradable organic matter from anaerobic digestion was estimated equal to 200 mgCOD/L, and provided as sodium acetate (NaAc). Such composition resulted in influent COD/N and molar alkalinity/nitrogen ratios of 0.67 gCOD/gNH_4-N and 0.93 molIC/molNH_4-N, respectively.

Final influent composition was: NH_4Cl, 1.15 g/L; $KHCO_3$, 2.0 g/L; $CH_3COONa{\cdot}3H_2O$, 0.43 g/L; KH_2PO_4, 18 mg/L; K_2HPO_4, 47 mg/L; $MgSO_4$, 49 mg/L; trace elements solution, 0.2 mL/L. Trace elements solution was prepared according to Vishniac and Santer [4]. Final influent pH was 7.9±0.2.

6.2.4 Analytical methods

Ammonium, nitrite and nitrate were measured via spectrophotometric measurements, according to APHA Standard Methods [5]. Influent and effluent organic matter content was expressed as

total organic carbon (TOC) concentration, which was determined using an automatic analyzer (TOC-5000, Shimadzu). Total nitrogen was measured by catalytic thermal decomposition-chemiluminescence method (TNM-L, Shimadzu). TSS and VSS concentrations were determined according to APHA Standard Methods [5]. During the experiment, biomass from both reactors appeared composed partly of granular sludge and partly of suspended flocs. From day 65 onward, biomass was then characterized as it was ('raw' biomass) and with regard to its granular or flocculant fractions. The separation of the two fractions was performed by means of a 250 µm sieve. Smaller granules were further separated by centrifugation (1500 rpm, 5 min).

6.2.5 Mass balance and calculations

Nitrogen mass balance was done taking into account the following five different biological processes and corresponding reactions:

i. ammonia oxidation to nitrite (i.e., nitritation) performed by ammonium oxidizing bacteria (AOB) [6]:

$$NH_4^+ + 1.9852HCO_3^- + 0.07425CO_2 + 1.4035O_2 \rightarrow 0.9852NO_2^- + 1.9852CO_2 + 0.01485C_5H_7O_2N + 2.9406H_2O \quad \text{(Eq. 6.1)}$$

ii. nitrite oxidation to nitrate (i.e., nitratation) performed by nitrite oxidizing bacteria (NOB) [6]:

$$NO_2^- + 0.005NH_4^+ + 0.005HCO_3^- + 0.020H_2CO_3 + 0.471O_2 \rightarrow NO_3^- + 0.005C_5H_7O_2N + 0.008H_2O \quad \text{(Eq. 6.2)}$$

iii. anaerobic ammonium oxidation (anammox) [7]:

$$NH_4^+ + 1.146NO_2^- + 0.071HCO_3^- + 0.057H^+ \rightarrow 0.986N_2 + 0.161NO_3^- + 0.071CH_{1.74}O_{0.31}N_{0.20} + 2.002H_2O \quad \text{(Eq. 6.3)}$$

iv. aerobic heterotrophic carbon removal by ordinary heterotrophic organisms (OHO); acetate was considered as substrate [6]:

$$0.125\ CH_3COO^- + 0.0295NH_4^+ + 0.103O_2 \rightarrow 0.0295C_5H_7O_2N + 0.0955H_2O + 0.0955HCO_3^- + 0.007CO_2 \quad \text{(Eq. 6.4)}$$

v. anoxic heterotrophic carbon removal (denitrification); acetate was considered as substrate [6]:

$$NO_3^- + H^+ + 0.33NH_4^+ + 1.45\ CH_3COO^- \rightarrow 0.5N_2 + 0.33C_5H_7O_2N + 1.60H_2O + 1.12HCO_3^- + 0.12CO_2 \quad \text{(Eq. 6.5)}$$

The following assumptions were also assumed in order to simplify calculations:

1. biomass growth, and subsequent ammonia-nitrogen uptake for cell synthesis, was mainly ascribed to OHO growth;
2. all influent ammonium converted to N_2 gas which was not used for biomass growth was used by PN/anammox process;
3. nitratation (i.e. nitrate production from nitrite by NOB) was negligible compared to nitrate production by anammox and nitrate consumption by denitrifying bacteria;
4. nitratation and denitrification did not occur simultaneously.

Calculations were then carried out as follows.

From rough data (influent and effluent concentrations), ΔTN and $(\Delta TOC)_{TOT}$ were calculated:

$$\Delta TN = (NH_4^+\text{-N} + NO_2^-\text{-N} + NO_3^-\text{-N})_{inf} - (NH_4^+\text{-N} + NO_2^-\text{-N} + NO_3^-\text{-N})_{eff} \quad \text{(Eq. 6.6)}$$

$$(\Delta TOC)_{TOT} = (TOC)_{inf} - (TOC)_{eff} \quad \text{(Eq. 6.7)}$$

An initial estimation of total nitrogen removed for bacteria growth ($N_{biomass}$) was done according to the first initial assumption, i.e. estimating OHO growth. To do that, reactions reported in (Eq. 6.4 and (Eq. 6.5 were considered, resulting in a nitrogen uptake of 0.1376 and 0.1355 mgNH4-N/mgTOC for aerobic and anaerobic reaction, respectively. Thus, an average value of 0.1365 mgNH_4-N/mgTOC was assumed as representative of overall heterotrophic bacteria growth (Eq. 6.8).

$$N_{biomass} = 0.1365 \times (\Delta TOC)_{TOT} \quad \text{(Eq. 6.8)}$$

Anammox contribution to nitrogen mass balance was estimated according to the stoichiometry proposed by Lotti et al. [7] (Eq. 6.3), corresponding to 1.972 and 0.014 gN converted into dinitrogen gas and biomass, respectively, per gram of NH_4-N removed. Hence, (Eq. 6.9, (Eq. 6.10, (Eq. 6.11 and (Eq. 6.12 were used to estimate ammonium and nitrite removal, nitrate production and ammonium uptake for biomass synthesis by anammox bacteria.

$$(\Delta NH_4^+\text{-N})_{AMX} = \frac{\Delta TN}{1.9862} \quad \text{(Eq. 6.9)}$$

$$(\Delta NO_2^-\text{-N})_{AMX} = 1.146 \times (\Delta NH_4^+\text{-N})_{AMX} \quad \text{(Eq. 6.10)}$$

$$(\Delta NO_3^-\text{-N})_{AMX} = 0.161 \times (\Delta NH_4^+\text{-N})_{AMX} \quad \text{(Eq. 6.11)}$$

$$(\Delta NH_4^+\text{-N})_{AMX\_biomass} = 0.0142 \times (\Delta NH_4^+\text{-N})_{AMX} \quad \text{(Eq. 6.12)}$$

When the measured nitrate production (Eq. 6.13) was higher than the estimated nitrate production by anammox bacteria, nitratation was considered to occur during the cycle, and

denitrification was neglected; when the effluent contained less nitrate than expected by anammox activity, denitrification was considered and nitratation was neglected, according to the assumption #3 and #4. Therefore, the calculation was split in two different paths (A and B), according to the sign of the difference between $(\Delta NO_3^-\text{-N})_{TOT}$ and $(\Delta NO_3^-\text{-N})_{AMX}$, as reported in Table 6.1.

Table 6.1: Calculation paths A and B.

$$(\Delta NO_3^-\text{-N})_{TOT} = (NO_3^-\text{-N})_{eff} - (NO_3^-\text{-N})_{inf} \quad \text{(Eq. 6.13)}$$

Path A: $(\Delta NO_3^-\text{-N})_{TOT} - (\Delta NO_3^-\text{-N})_{AMX} > 0$

NOB activity not negligible, no denitrification

$$(\Delta NO_3^-\text{-N})_{NOB} = (\Delta NO_3^-\text{-N})_{TOT} - (\Delta NO_3^-\text{-N})_{AMX} \quad \text{(Eq. 6.14)}$$

$$(\Delta NH_4^+\text{-N})_{NOB\_biomass} = 0.005 \times (\Delta NO_3^-\text{-N})_{NOB} \quad \text{(Eq. 6.15)}$$

$$(\Delta NO_3^-\text{-N})_{Denit} = 0 \quad \text{(Eq. 6.16)}$$

Path B: $(\Delta NO_3^-\text{-N})_{TOT} - (\Delta NO_3^-\text{-N})_{AMX} < 0$

No NOB activity, only denitrification.

$$(\Delta NO_3^-\text{-N})_{NOB} = 0 \quad \text{(Eq. 6.17)}$$

$$(\Delta NO_3^-\text{-N})_{Denit} = (\Delta NO_3^-\text{-N})_{AMX} - (\Delta NO_3^-\text{-N})_{TOT} \quad \text{(Eq. 6.18)}$$

$$(\Delta TOC)_{Denit} = 1.45 \cdot 2 \cdot 12.0107 \times \frac{(\Delta NO_3^-\text{-N})_{Denit}}{14.0067} \quad \text{(Eq. 6.19)}$$

$$(\Delta NH_4^+\text{-N})_{Denit\_biomass} = 0.33 \times (\Delta NO_3^-\text{-N})_{Denit} \quad \text{(Eq. 6.20)}$$

Finally, organic carbon removal and ammonium uptake for biomass synthesis by aerobic OHO were calculated according to (Eq. 6.21) and (Eq. 6.22). Hence, overall N uptake due to biomass growth was calculated as the sum of each contribution. Such value was then assumed as the new initial estimation of total nitrogen removed for bacteria growth ($N_{biomass}$), and calculation was iterated from the top. Iterations stopped when difference from initial and final $N_{biomass}$ values was <0.01 mgN/L.

$$(\Delta TOC)_{OHO\_aer} = (\Delta TOC)_{TOT} - (\Delta TOC)_{Denit} \quad \text{(Eq. 6.21)}$$

$$(\Delta NH_4^+\text{-N})_{OHO\_biomass} = \frac{(\Delta TOC)_{OHO\_aer}}{0.125 \cdot 2 \cdot 12.0107} \cdot 0.0295 \cdot 14.0067 \quad \text{(Eq. 6.22)}$$

6.2.6 Specific activity assessment

Respirometric assays were carried out in order to determine specific aerobic heterotrophic activity (SA_{OHO_Aer}), as well as specific ammonium- and nitrite-oxidation activity (SA_{AOB} and SA_{NOB} activity, respectively). Tests were performed according to Lòpez-Fiuza et al. [8], using a Biological Oxygen Monitor (BOM, YSI model 5300) with oxygen selective electrodes, model YSI 5331, equipped with a computer data acquisition system. The instrument was a discontinuous respirometer equipped with 15 ml vials with working volume of 10 ml.

The biomass employed in each assay was drained from reactor and continuously aerated for at least 30 min before being used, in order to remove completely the possible remaining substrate. Biomass was then resuspended in each vial and diluted with phosphate buffer solution, in order to achieve a concentration of 1-2 gVSS/L. In AOB and NOB tests, $NaHCO_3$ solution was added to the suspension in order to provide a final concentration of 2.5mM (0.21 mg/L). In OHO activity test, a nitrification inhibitor, i.e., 100 µL of allithiourea (ATU), 1 g/L, was added before starting the test. Also, in AOB activity test, a NOB activity inhibitor (i.e., 100 µL of sodium azide NaN_3, 1 g/L) was added before starting the test. Vials were placed in a thermostatically controlled chamber (20°C) with a magnetic stirring system. The initial pH was adjusted to 7 and air was used to obtain the initial level of dissolved oxygen saturation. Subsequently, aeration was stopped and DO was monitored along with time. After ~2-5 min the oxygen uptake rate (OUR, $mgO_2/L \cdot min$) corresponding to endogenous respiration was determined (OUR_{end}); Then, substrate was injected, causing a higher OUR, reflected by a steeper slope (corresponding to OUR_{max}). At the end of the assay, TSS and VSS concentrations of the suspension were determined. Specific activities (SA) were then calculated as follows:

$$SA_{AOB} = \frac{(OUR_{max} - OUR_{end})}{X_{VSS}} \cdot \frac{1}{3.43} \cdot \frac{1440\ (min/d)}{1000\ (mg/g)} \quad \left[\frac{gNH_4^+\text{-}N}{gVSS \cdot d}\right] \qquad \text{(Eq. 6.23)}$$

$$SA_{NOB} = \frac{(OUR_{max} - OUR_{end})}{X_{VSS}} \cdot \frac{1}{1.14} \cdot \frac{1440\ (min/d)}{1000\ (mg/g)} \quad \left[\frac{gNO_2^-\text{-}N}{gVSS \cdot d}\right] \qquad \text{(Eq. 6.24)}$$

$$SA_{OHO\_Aer} = \frac{(OUR_{max} - OUR_{end})}{X_{VSS}} \cdot \frac{1440\ (min/d)}{1000\ (mg/g)} \quad \left[\frac{gCOD}{gVSS \cdot d}\right] \qquad \text{(Eq. 6.25)}$$

Substrate addition consisted of 0.1 mL of ammonium (3.5 gN/L), nitrite (3.5 gN/L) or acetate solution (10 gCOD/L) for AOB, NOB and aerobic OHO assessment, respectively.

Phosphate buffer solution consisted of (concentration in g/L): KH_2PO_4 (3.31), K_2HPO_4 (3.97), $MgSO_4 \cdot 7H_2O$ (1.84), $MgCl_2 \cdot 10H_2O$ (1.52), NaCl (0.8) and a nutrient solution (5 mL/L) as described by Lòpez-Fiuza [8].

Each assay was performed in triplicate.

As to specific anammox activity (SAA) and specific denitrification activity (SA_{Denit}) assessment, manometric batch tests were carried out according to the methodology described by Dapena-Mora et al. [9]. The assays were performed in vials with a total volume of 38 mL and a volume of liquid of 25 mL, each closed with a gas-tight coated septum. The vials were inoculated with biomass drained from the reactors, previously washed and resuspended in phosphate buffer (KH_2PO_4, 0.14 g/L and K_2HPO_4, 0.75 g/L; initial pH value was 7.8). Final biomass concentration was 1.5-3.0 gVSS/L. The headspace and liquid phase were sparged with argon to remove dissolved oxygen. The vials were placed in a thermostatic shaker (HWY-200-D, Lan technics; shaking speed, 150 rpm) until stable conditions were reached. Assays were performed at a fixed temperature of 20°C (average working temperature of reactors); specific anammox activity (SAA) was also replicated at 30°C (reference temperature for anammox activity). Then the substrates were added (total volume added, 1 mL) and pressure was equalized to the atmospheric one. The substrates consisted of ammonium+nitrite (both 70 mgN/L) and acetate+nitrate (200 mgCOD/L and 25 mgN/L, respectively) for anammox and denitrification test, respectively.

The production of N_2 gas was tracked along with time by measuring the overpressure in the headspace at fixed time. Overpressure was measured via a handheld pressure transducer (Model PSI-5, Centrepoint electronics). At the end of the test, gas composition of each vial was analyzed via gas chromatography (Hewlett Packard 5890A with TCD) in order to confirm the production of N_2; also, TSS and VSS concentrations were determined.

Overpressure time courses typically showed an initial lag phase, followed by a linear increase of gas production along with time, and a final stationary phase. When no more overpressure increase was observed within 30-60 min, stationary phase was reached and assays were stopped. Maximum slope α (mmHg/min) was determined through linear regression of overpressure linear increase phase. Subsequently, nitrogen production rate and specific activity were calculated according to (Eq. 6.26 and (Eq. 6.27, respectively.

$$\frac{dN_2}{dt} = \alpha \cdot \frac{V_G}{RT} \quad \left[\frac{\text{mol } N_2}{\text{min}}\right] \qquad \text{(Eq. 6.26)}$$

$$SAA\ ,\ SA_{Denit} = \frac{dN_2/dt}{VSS \cdot V_L} \cdot 14.0067 \cdot 2 \cdot 1440 \quad \left[\frac{gN_2\text{-}N}{gVSS\ \cdot\ d}\right] \qquad \text{(Eq. 6.27)}$$

Where: V_G and V_L, headspace gas and liquid phase volumes in each vial, respectively (L); R, gas constant (62.3637 mmHg·L/mol·K); T, temperature (K).

Each assay was performed in triplicate.

6.3 Results

The experimental activity had a total duration of 90 days. On day 0, both reactors were started up. The key operational parameter that needed to be adjusted in order to reach the optimal process performance was aeration flow rate. Indeed, high flow rate led to excess in oxygen availability, resulting in high DO level (>2 ppm) during most of the cycle duration, which inhibited anammox activity and promoted the nitrite conversion to nitrate; conversely, low flow rate resulted in limiting DO level, and consequently in poor ammonium and organic carbon removal efficiency. An optimal flow rate was found between 1.5-2.0 NL/min.

After 28 days of operation, stable performance was considered to be achieved for both reactors. Hence, regular stops were applied on reactor D. Regular stops consisted of reactor not operated (no feeding, no stirring, no aeration and no withdrawal) for 4 of the normal 8 daily cycles, from Monday to Friday, and not operated at all on Saturdays and Sundays, as depicted on Figure 6.2. Thus, each reactor D operation stop ranged between 12 and 60 hours.

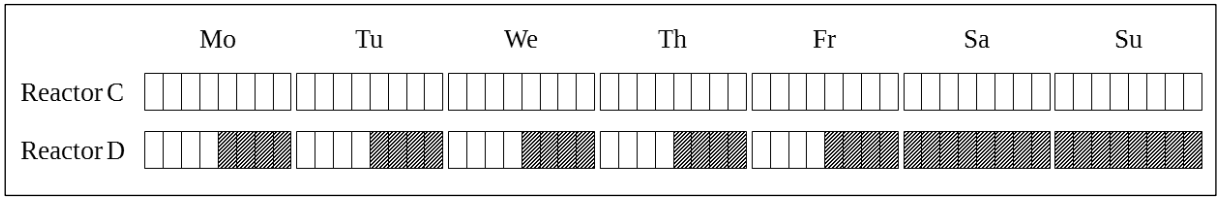

Figure 6.2: Continuous and discontinuous operation of reactors C and D, respectively. White rectangles, normal operation cycle; grey rectangles, not operated cycle.

6.3.1 Nitrogen and organic carbon removal and mass balance

The discontinuous operation on reactor D automatically resulted in a sharp decreasing (-64%) in applied nitrogen loading rate (NLR) and organic loading rate (OLR): expected values changed from 0.48 to 0.17 gN/L·d, and from 0.32 to 0.11 gCOD/L·d, respectively.

Time course of the main process performance indicators are depicted in Figure 6.3, Figure 6.4 and Figure 6.5. During the whole experimentation, the most influential factor on process performance was the air flow rate. Troubles in adjusting the flow rate caused the fluctuating performance observed in the startup phase, up to day 20, and on days 58-68. However, stable performance was achieved and maintained in both reactors.

Generally, process performance in reactor D was not apparently affected by the regular stops applied. Total nitrogen removal averaged out at 84% and 88% in reactors C and D, respectively,

although a significant fluctuation was observed, mostly ascribed to failure in air flow control. Effluent nitrite and nitrate concentrations averaged out at 2.7 $mgNO_2$-N/L and 6.4 $mgNO_3$-N/L, and 4.0 $mgNO_2$-N/L and 7.4 $mgNO_3$-N/L for reactor C and D, respectively. Remarkably, observed acetate removal efficiency was mostly lower than nitrogen removal (Figure 6.5). Average values of the main process performance indicators (TN and TOC removal efficiency, NRR/NLR ratio) are reported in Table 6.2: statistical analysis supported the hypothesis that average values of the two reactors were not significantly different ($p>0.3$).

Mass balance calculations allowed to ascribe most of the observed nitrogen removal to the partial nitritation/anammox metabolism (92±8% and 93±8% for reactor C and D, respectively), while denitrification and assimilation accounted for 11% and 9% in reactor C, respectively, and 9% and 9% in reactor D, respectively. However, the error in quantification of different metabolisms averaged out at 11% for both reactors.

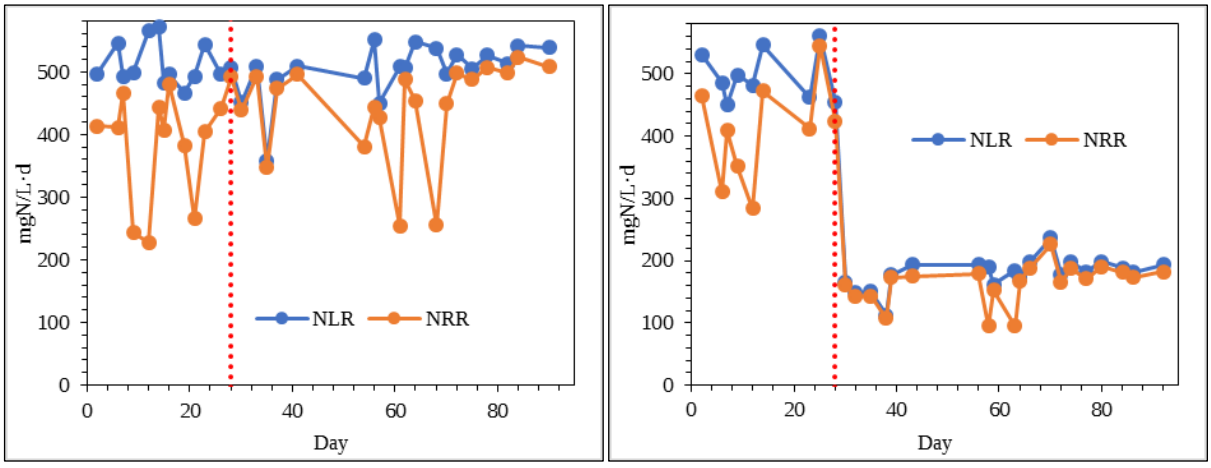

Figure 6.3: Nitrogen loading rate and nitrogen removal rate time profiles in SARD-C and D reactors (left and right, respectively). Red dashed line corresponded to day 28.

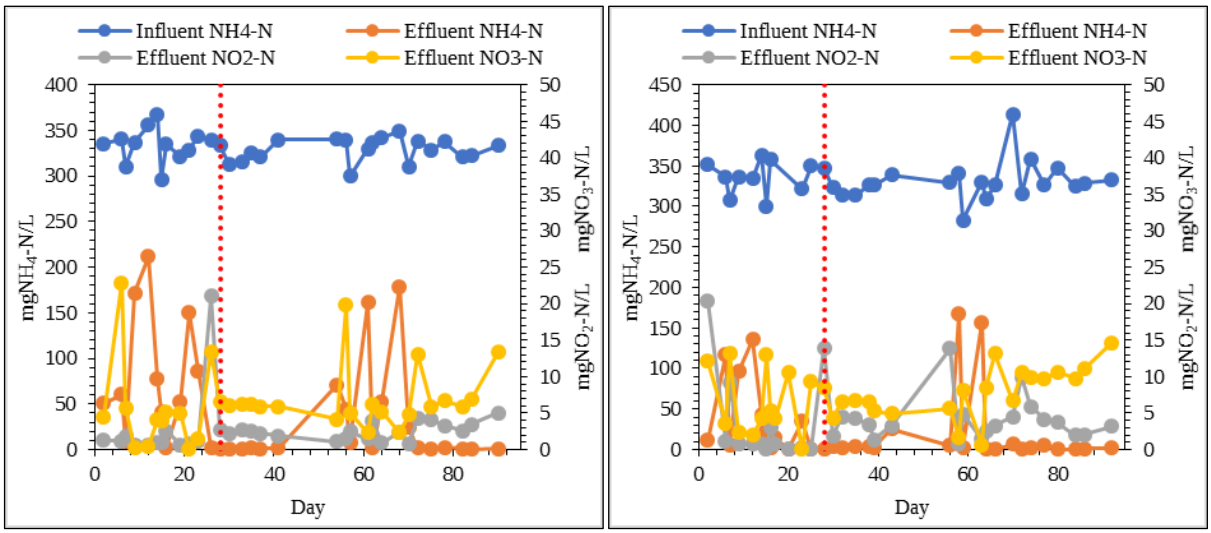

Figure 6.4: Time profiles of influent and effluent nitrogen forms in SARD-C and D reactors (left and right, respectively). Red dashed line corresponded to day 28.

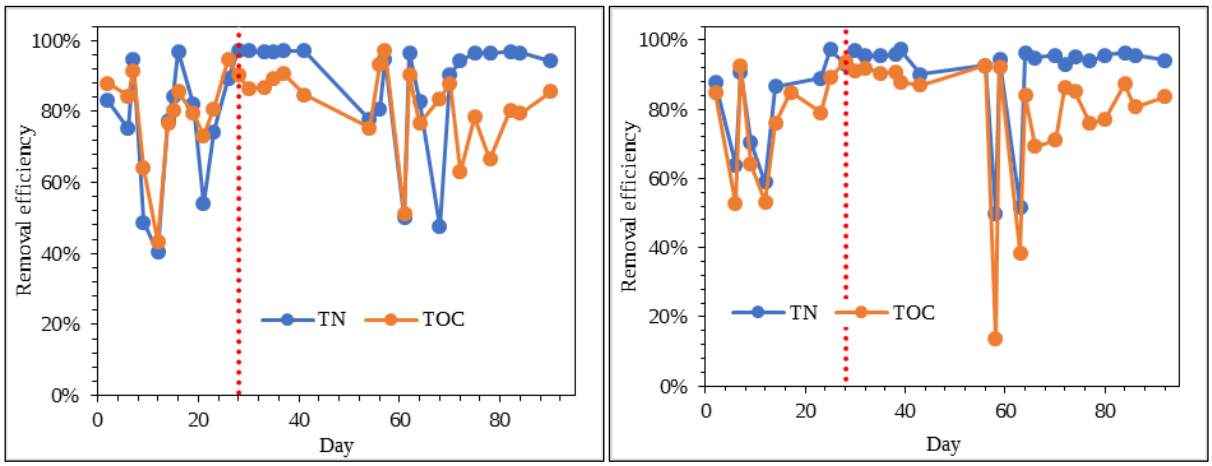

Figure 6.5: Time profiles of total nitrogen and total organic carbon removal efficiencies in SARD-C and D reactors (left and right, respectively). Red dashed line corresponded to day 28.

Table 6.2: Average values of the main process performance indicators.

	Reactor C	Reactor D
TN removal efficiency	84±17%	88±17%
TOC removal efficiency	81±12%	78±12%
NRR/NLR ratio	84±15%	88±13%

In order to better highlight the evolution of the different analytes during a single working cycle, specific characterization was performed on both reactors when stable performance was observed. A typical resulting time profile of the different analytes is showed in Figure 6.6.

Pulse feeding (20% of total reactor volume in 5 minutes) resulted in an initial increase of ammonium and TOC and a corresponding decrease in nitrite and nitrate concentration, only partially caused by dilution. Indeed, decrease in nitrate appeared faster than nitrite decrease, suggesting an active consumption, i.e. denitrification. Subsequently, nitrate started to increase quite linearly, while quite stable nitrite levels were observed; at the same time, ammonium progressively decreased. Aeration started after 5 min; however dissolved oxygen remained below 0.5 ppm during most of the cycle. Increase in DO concentration was detected after 120-150 min, corresponding to a residual ammonium concentration <20 mgN/L; simultaneously, nitrite started to slowly accumulate. Remarkably, TOC showed an unexpected profile, with a quite stable concentration during most of the cycle, followed by a decreasing trend occurring in parallel to oxygen raise.

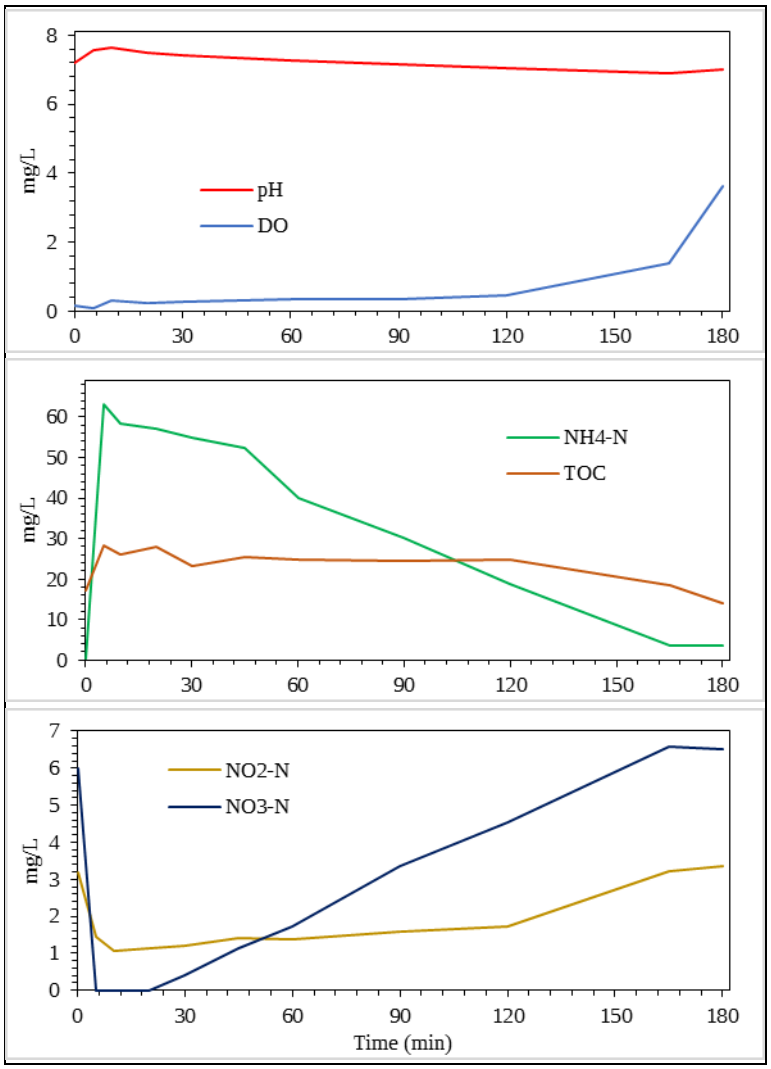

Figure 6.6: Time profiles of different parameters during a single cycle operation.

6.3.2 Solids

Reactor and effluent solid content during the experimental activity are depicted in Figure 6.7. During the startup phase (days 0-28) an increase in biomass concentration was observed in both reactors: inoculum originated from a full scale plant treating real wastewater, thus a positive effect of the synthetic feeding and controlled environment was expected. In both reactors, suspended biomass (already present in the inoculum) increased rapidly: as a consequence, progressive depletion in settling capacity was observed and measured through sludge volume index assessment (Table 6.3).

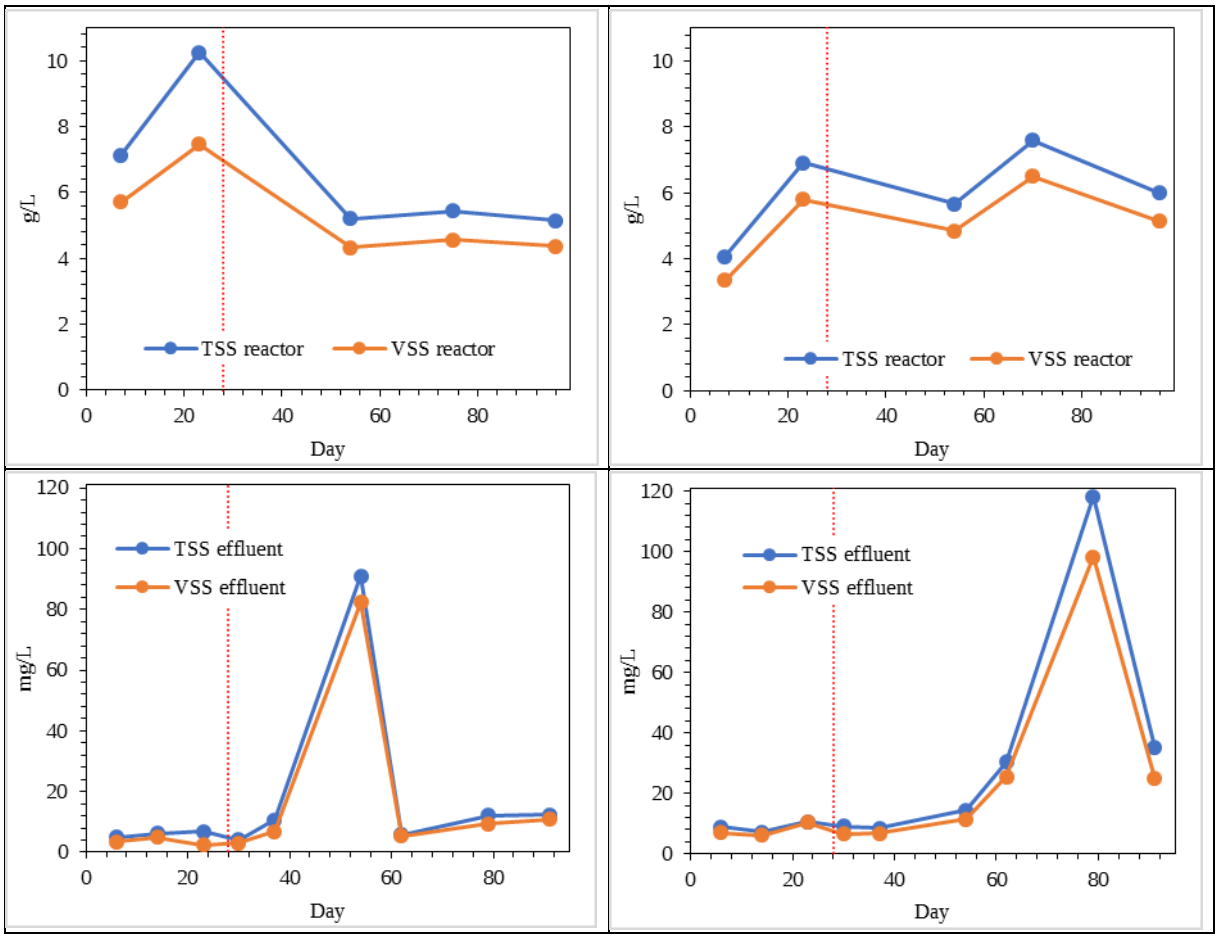

Figure 6.7: Time courses of reactor and effluent solids content in SARD-C and D reactors (left column and right column, respectively). Red dashed line corresponded to day 28.

Remarkably, increase in poorly settling flocculant biomass content avoided the correct sedimentation of granules too; however, supernatant appeared well clarified. Eventually, such increasing trend led to detect relatively high solids content in the effluent from reactor C (~90 mgTSS/L on day 53); consequently, sludge started to be washed out from the reactor. Subsequently, reactor biomass concentration decreased (from 7.5 gVSS/L on day 23 to 4.3 gVSS/L on day 54) as well as effluent solid concentration. However, sludge continued to show poor settling capacity, resulting in a slow, constant increase in effluent solid concentration, while biomass in reactor C appeared to have stabilized at 4.4±0.1 gVSS/L.

As to reactor D, discontinuous operation apparently slowed down the development of suspended biomass. Interestingly, effluent solids concentration showed the same behavior observed in reactor C, but it was apparently shifted forward in time. Such behavior may be ascribed to the reduced organic and nitrogen loading rate, which limited biomass growth; however, progressive depletion of settling capacity and deterioration of effluent quality was only slowed down, but not avoided.

Table 6.3: Sludge settling capacity during the experimental activity.

	Reactor C (mL/gTSS)			Reactor D (mL/gTSS)		
Day	SVI5	SVI8	SVI30	SVI5	SVI8	SVI30
2	64	na	na	na	na	na
23	94	94	73	139	136	83
55	192	192	180	175	170	136
90	192	192	183	167	167	151

Starting from day 65, biomass characterization was also performed on both the isolated suspended and granular fractions. Noticeably, correct separation was difficult to obtain, thus such measures were likely affected by significant errors. However, as reported in Table 6.4, in the last part of the experiment, reactor C showed an increase in granular fraction, while flocculant fraction appeared stable.

Table 6.4: Observed sludge fractions during final experimental phase.

Day	Biomass fraction	Reactor C		Reactor D	
		TSS	VSS	TSS	VSS
75	Mixed (g/L)	5.4 ± 0.2	4.6 ± 0.1	7.6 ± 1.1	6.5 ± 1.1
90		5.2 ± 0.1	4.4 ± 0.1	6.0 ± 0.2	5.1 ± 0.2
75	Granular (%)	43 ± 2 %	45 ± 2 %	33 ± 5 %	36 ± 6 %
90		66 ± 7%	64 ± 6%	55 ± 4%	55 ± 4%
75	Flocculant (%)	43 ± 2 %	49 ± 2 %	63 ± 6 %	63 ± 7 %
90		41 ± 3%	52 ± 3%	40 ± 2%	44 ± 3%

In reactor D a significant decrease in flocculant fraction was observed, together with a decrease in total biomass concentration and an increase in granular fraction. Such difference may be ascribed to reduced organic and nitrogen loading rate, as well as to prolonged anaerobic starvation, which limited the development of fast-growing suspended bacteria.

6.3.3 Specific activity

Specific anammox activity did not appear to be influenced by the different operating conditions in reactors C and D (Figure 6.8). SAA measured at 30 °C averaged out at 0.432 and 0.459 gN/gVSS·d in reactors C and D, respectively, although a decreasing trend was observed in the last part of the experimental activity. SAA at 20°C, differently, showed a decreasing trend in the first phase of the experiment, then stable values stabilized were observed. Average values were 0.201 and 0.215 gN/gVSS·d for reactors C and D, respectively.

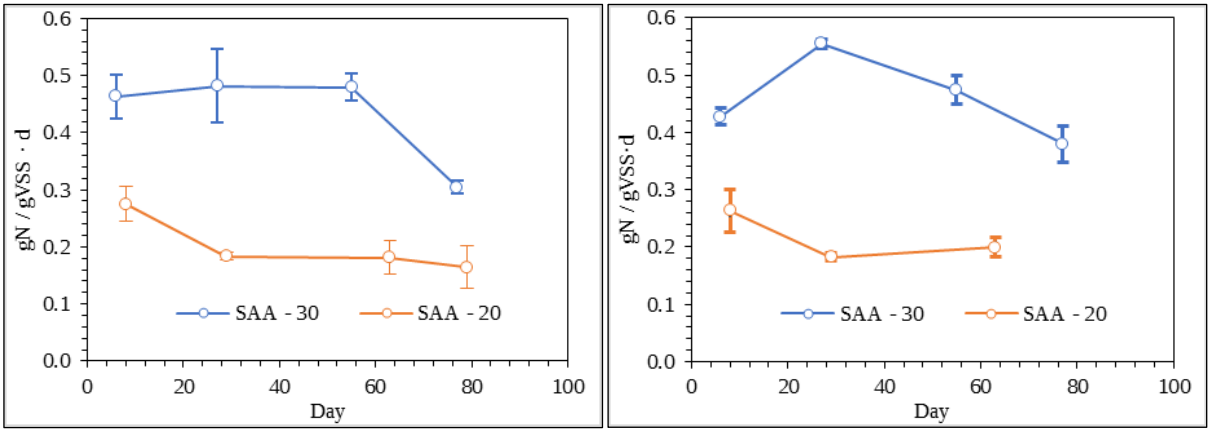

Figure 6.8: Specific anammox activity, measured at 30°C and 20°C in SARD-C and D reactors (left and right, respectively).

On days 80-85, SAA at 30°C, as well as other specific activity assays, was also measured on isolated granular and flocculant biomass fractions of both reactors; results are showed in Figure 6.10. As expected, anammox bacteria were mostly located on granular aggregates; however, relevant activity was detected in suspended biomass, indicating the presence of highly active small anammox granules, or even suspended anammox bacteria.

AOB specific activity showed fluctuating values (Figure 6.9), which were mainly ascribed to errors in data collections and interpolations, due to little measured changes in OUR before and after the substrate addition. Measured ammonium oxidation activities were surprisingly low compared to overall ammonia oxidation efficiency, i.e. ranging between 12 and 48 mgNH_4-N/gVSS·d whereas a conventional nitrifying activated sludge resulted in 36-120 mgNH_4-N/gVSS·d [10]. Less surprisingly, AOB activity in reactor D progressively decreased compared to reactor C, as expected due to prolonged exposure to anoxic conditions. Moreover, AOB were expected to be located on the surface of the granules; differently, assays carried out withon isolated fractions showed that AOB activity was almost totally due to flocculant biomass in both reactors (Figure 6.10). Throughout the whole experimental period, no nitrite oxidation activity was observed.

As to heterotrophic bacteria, both aerobic and anoxic, constantly increasing trends were observed in reactor C (Figure 6.9). A different result was found in reactor D: aerobic OHO activity showed a clear decrease after day 28, i.e. after regular stops started to be operated, while anaerobic OHO activity, on the contrary, showed the same increasing behavior as reactor C. Predictably, heterotrophic bacteria were present in suspended biomass more than granular biomass (Figure 6.10).

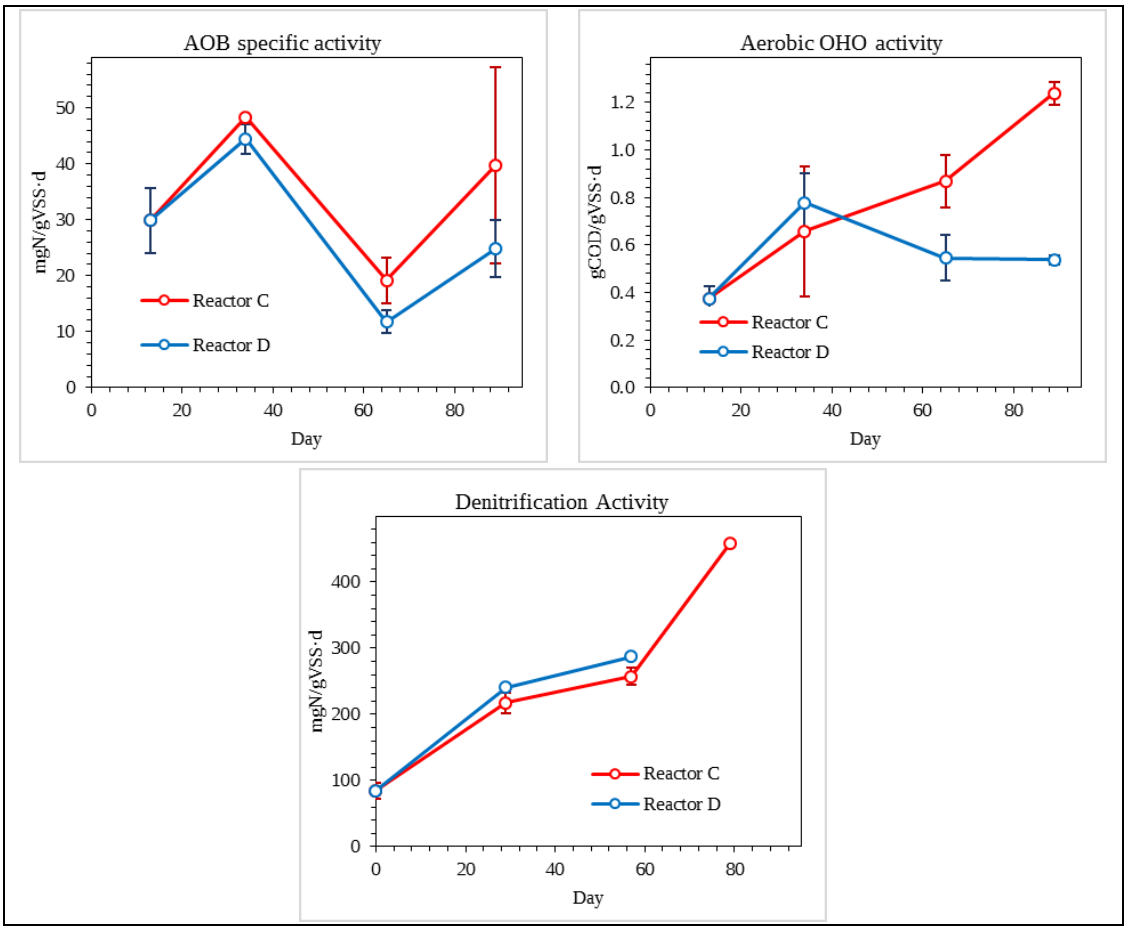

Figure 6.9: Results of AOB, aerobic and anaerobic OHO activity test. NOB activity was never detected.

6.4 Discussion

In recent years, many researches focused on the assessment of effects of starvation and reactivation on anammox process performance, as well as on microbiological or biochemical characterization of bacterial community [11–13]. Previous studies indeed pointed out at the sensitivity of anammox organisms to the environmental changes [12]. Carvajal-Arroyo et al. [11] were among the first to investigate the response of anammox bacteria to starvation, reporting increased sensitivity of anammox organisms to nitrite toxicity. More recent studies focused on the assessment of the best strategies to recover a satisfactory activity after prolonged starvation times: for instance, Zhang et al. [14] tested the effect of periodic (every 10 days) substrate addition in a 30-days-long starvation period. Results showed that 'intermittent' starvation led to a higher endogenous decay coefficient compared to persistent starvation, as well as to an exacerbated decrease in specific anammox activity, whereas no significant difference was observed in the heme *c* contents.

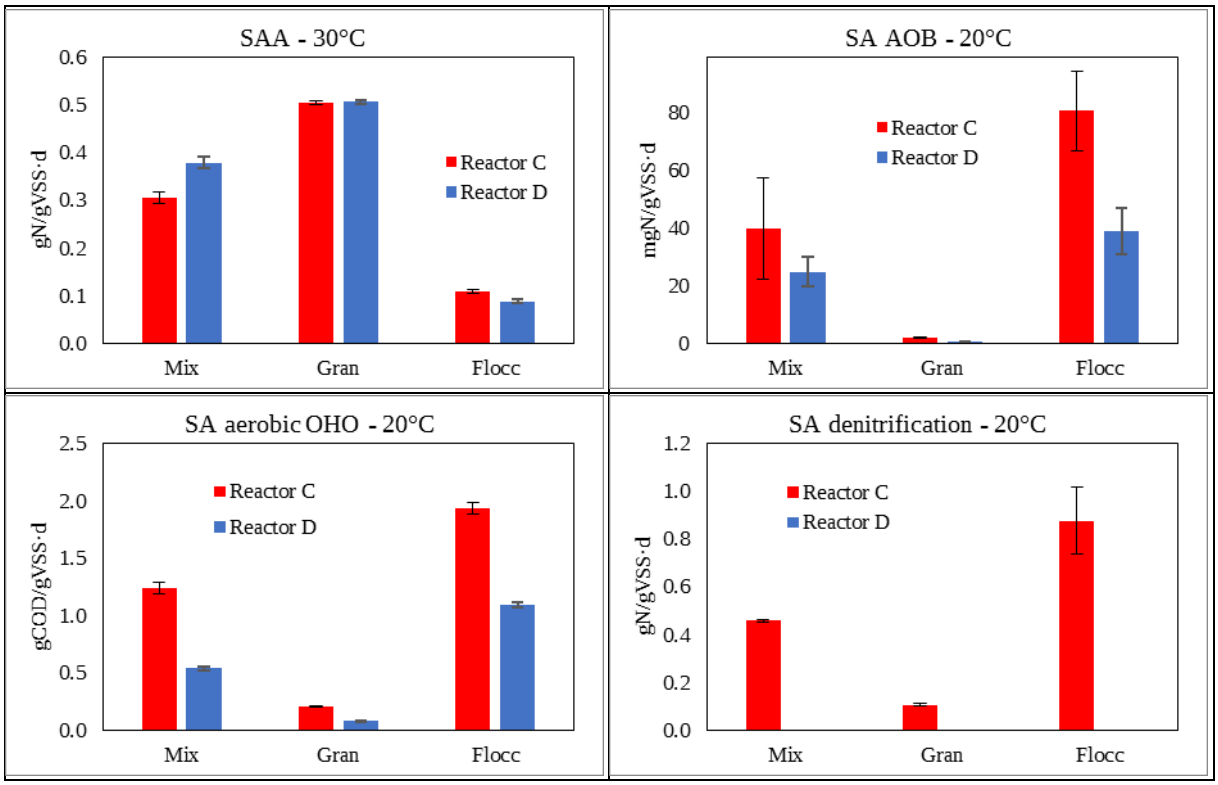

Figure 6.10: Results of the different specific activity assays performed on isolated granular and flocculant biomass fractions.

A similar result was reported by Ma et al. [15] in two short-term anammox starvation experiments (4.5 and 1.67 days, under anaerobic and anoxic conditions, respectively), which resulted in no significant decay of the anammox cells, but in significant SAA decreasing after both anaerobic and anoxic starvation, thus confirming that activity decrease was more important than cell decay in anammox bacteria subjected to short-term starvation. Also, successive activity batch tests indicated almost completely SAA recovery. However, in the anoxic starvation experiment (1.67 days) SAA only increased by 33.98 ± 3.32%, while in the anaerobic starvation experiment (4.5 days) a 100% recovery was observed. Such results indicated that anaerobic (more than anoxic) starvation could provide an effective storage strategy for anammox sludge.

In the present experiment, regular anoxic starvation occurred, lasting from 0.5 to 2.5 days, for 72 days. Corresponding SAA values, both at 20°C and 30°C, did not show a significantly different behavior compared to the non-starved biomass (Figure 6.8); yet, biomass used in SAA assays was drained from reactor D just before the cycle reactivation. Such result is in contrast with those reported by Ma et al. [15], suggesting that anammox biomass could quickly acclimate to such operating conditions. Remarkably, in the cited study Zhang et al. [14] reported that acetate addition significantly accelerated short term recovery of enriched anammox

suspended-growth cultures after starvation, but reduced anammox activity over the longr term in suspended- and attached-growth cultures. Whether the presence of acetate in the influent medium might have contributed to the anammox resilience to starvation observed in the present study, however, could only be hypothesized.

A recent investigation on repeated short-time starvation and reactivation cycles was performed by Ye et al. [12], and the same authors reclaimed theirs to be the first description of the repeated short-term starvation of the anammox sludge. Proposed starvation conditions were: reactor sat idle with no substrate addition; temperature controlled at 27°C; starvation time, 1-5 days; operation time after starvation, 5-48 days. Results indicated that the repeated starvation could increase the recovery rate, providing a pathway to enhance the resilience of the starved anammox sludge. Moreover, by studying the performance and apparent activities of anammox bacteria in the short-term starvation (1–5 days) of the recovery culture, the inhibition due to the starvation was aggravated by prolonging the starvation time, while the activity and tolerance of the anammox sludge was enhanced when the same starvation was repeated. Such results are in good agreement with the findings of the present experiment, suggesting that anammox process can be successfully operated under regularly repeated short-time starvation and reactivation operation.

In the present experiment, operating conditions applied on reactor D mostly affected AOB and aerobic heterotrophic activity, likely due to the exposure to prolonged oxygen starvation, more than substrate starvation. AOB activity in reactor D progressively decreased compared to reactor C (approximatively -38%). Torà et al. [16] tested different ammonium starvation strategies on an highly enriched AOB biomass originating from a full nitritation reactor, concluding that fully anaerobic starvation condition was the best alternative to maintain AOB activity, compared to anoxic and aerobic conditions. However, prolonged anaerobic starvation was also recently proposed as successful strategy for the rapid achievement of AOB selection over NOB in nitritation reactors [17]. Such results could not allow to confirm whether long-term operation under frequent anoxic starvation/aerated operation cycles significantly cause deterioration of nitritation process. However, ammonia oxidizing bacteria are strictly aerobic, slow-growing organisms which could likely be inhibited by periodic oxygen starvation, while OHO are able to rapidly switch between aerobic and anoxic conditions [6].

6.5 Conclusions

In the present experiment, two lab-scale SBR performing simultaneous PN/anammox and organic carbon removal were inoculated with ELAN biomass and successfully operated for 28 days at NLR and OLR of 0.48 gN/L·d and 0.32 gCOD/L·d, respectively, using a synthetic influent. Subsequently, intermittent operation comporting repeated, regular substrate and oxygen starvation and reactivation was applied for 62 days on one of the two reactors, in order to simulate the discontinuous operation of the process implemented at full scale within the framework of MEDRAR project. The other reactor was instead operated under continuous operation and served as control. No remarkable difference in overall process performance was observed between the two reactors. Statistical inference did not highlight a significant difference on average values of TN and TOC removal efficiencies, as well as of NRR/NLR ratio. Small differences were observed in biomass settling capacity and effluent quality, ascribed to reduced organic loading rate on reactor D, leading to a slower heterotrophic suspended biomass growth. No significant differences were measured in specific anammox activity as well as denitrification activity, while repeated oxygen starvation might have caused a decrease in AOB and aerobic OHO activity in reactor D.

No other study has previously investigated the effects of repeated short-term starvation-reactivation periods on a complex nitrifying/anammox/heterotrophic biomass as that used in the present study. Results of the present study demonstrated the feasibility of long-term operation of such process under the proposed conditions. However, further investigations are needed, in particular regarding the feasibility of long-term operation of such process treating the real wastewater, the assessment of process resilience when longer starvation periods are applied, and the emissions of greenhouse gas such as nitrous oxide.

6.6 References

[1] J. Vázquez-Padín, I. Fernádez, M. Figueroa, A. Mosquera-Corral, J.L. Campos, R. Méndez, Applications of Anammox based processes to treat anaerobic digester supernatant at room temperature, Bioresour. Technol. 100 (2009) 2988–2994. doi:10.1016/j.biortech.2009.01.028.

[2] N. Morales, A. Val Del Río, J.R. Vázquez-Padín, R. Gutiérrez, R. Fernández-González, P. Icaran, F. Rogalla, J.L. Campos, R. Méndez, A. Mosquera-Corral, Influence of dissolved oxygen concentration on the start-up of the anammox-based process: ELAN®,

Water Sci. Technol. 72 (2015) 520–527. doi:10.2166/wst.2015.233.

[3] J.R. Vázquez-Padín, N. Morales, R. Gutiérrez, R. Fernández, F. Rogalla, J.P. Barrio, J.L. Campos, A. Mosquera-Corral, R. Méndez, Implications of full-scale implementation of an anammox-based process as post-treatment of a municipal anaerobic sludge digester operated with co-digestion, Water Sci. Technol. 69 (2014) 1151–1158. doi:10.2166/wst.2013.795.

[4] W. Vishniac, M. Santer, the Thiobacilli, 12, Bacteriol. Rev. 21 (1957) 195–213. http://www.ncbi.nlm.nih.gov/pmc/articles/PMC180898/.

[5] APHA, Standard Methods for the Examination of Water and Wastewater, 21 st, American Public Health Association, 2005. https://books.google.it/books?id=buTn1rmfSI4C.

[6] F.L. Burton, G. Tchobanoglous, R. Tsuchihashi, H.D. Stensel, I. Metcalf & Eddy, Wastewater Engineering: Treatment and Resource Recovery, McGraw-Hill Education, 2013. https://books.google.it/books?id=6KVKMAEACAAJ.

[7] T. Lotti, R. Kleerebezem, C. Lubello, M.C.M. van Loosdrecht, Physiological and kinetic characterization of a suspended cell anammox culture, Water Res. 60 (2014) 1–14. doi:10.1016/j.watres.2014.04.017.

[8] J. López-Fiuza, B. Buys, A. Mosquera-Corral, F. Omil, R. Méndez, Toxic effects exerted on methanogenic, nitrifying and denitrifying bacteria by chemicals used in a milk analysis laboratory, Enzyme Microb. Technol. 31 (2002) 976–985. doi:10.1016/S0141-0229(02)00210-7.

[9] A. Dapena-Mora, I. Fernández, J.L. Campos, A. Mosquera-Corral, R. Méndez, M.S.M. Jetten, Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production, Enzyme Microb. Technol. 40 (2007) 859–865. doi:10.1016/j.enzmictec.2006.06.018.

[10] F. Elena, R. Alberto, pH-Stat Titration to Assess Nitrification Inhibition, J. Environ. Eng. 127 (2001) 698–704. doi:10.1061/(ASCE)0733-9372(2001)127:8(698).

[11] J.M. Carvajal-Arroyo, D. Puyol, G. Li, A. Swartwout, R. Sierra-Álvarez, J.A. Field, Starved anammox cells are less resistant to NO2- inhibition, Water Res. 65 (2014). doi:10.1016/j.watres.2014.07.023.

[12] L. Ye, D. Li, J. Zhang, H. Zeng, Resuscitation of starved anaerobic ammonium oxidation

sludge system: Impacts of repeated short-term starvation, Bioresour. Technol. 263 (2018) 458–466. doi:10.1016/j.biortech.2018.04.126.

[13] X. Ma, Y. Wang, Anammox bacteria exhibit capacity to withstand long-term starvation stress: A proteomic-based investigation of survival mechanisms, Chemosphere. 211 (2018) 952–961. doi:10.1016/j.chemosphere.2018.07.185.

[14] Z.Z. Zhang, X. Buayi, Y.F. Cheng, Y.H. Zhou, H.Z. Wang, R.C. Jin, Anammox endogenous metabolism during long-term starvation: Impacts of intermittent and persistent modes and phosphates, Sep. Purif. Technol. 151 (2015) 309–317. doi:10.1016/j.seppur.2015.07.066.

[15] X. Ma, Y. Wang, S. Zhou, Y. Yan, X. Lin, M. Wu, Endogenous metabolism of anaerobic ammonium oxidizing bacteria in response to short-term anaerobic and anoxic starvation stress, Chem. Eng. J. 313 (2017) 1233–1241. doi:10.1016/j.cej.2016.11.019.

[16] J.A. Torà, J. Lafuente, J.A. Baeza, J. Carrera, Long-term starvation and subsequent reactivation of a high-rate partial nitrification activated sludge pilot plant, Bioresour. Technol. 102 (2011) 9870–9875. doi:10.1016/j.biortech.2011.08.008.

[17] W. Liu, Q. Yang, B. Ma, J. Li, L. Ma, S. Wang, Y. Peng, Rapid Achievement of Nitritation Using Aerobic Starvation, Environ. Sci. Technol. 51 (2017) 4001–4008. doi:10.1021/acs.est.6b04598.

Conclusions

The research described in the present work was focused on the application of the two-stage partial nitritation (PN)/anammox process for the treatment of the anaerobic supernatant (AD) of a two-stage anaerobic digestion of organic fraction of municipal solid waste (OFMSW) and food waste (FW).

The feasibility of the proposed treatment was first preliminarily evaluated by starting and operating a PN continuous flow stirred tank reactor (CSTR) and a granular anammox sequencing batch reactor (SBR) using a synthetic influent, in order to determine the best operating conditions. The PN reactor was successfully operated under the following conditions: nitrogen loading rate (NLR), 1.5 gN/L·d; hydraulic retention time (HRT), 1 d, dissolved oxygen concentration (DO), ≥1.5 ppm. Acute toxicity effect of the target wastewater, i.e. the supernatant originating from the two-stage AD of FW, on the nitrifying biomass was assessed by means of batch tests: results showed an acute inhibiting effect of such wastewater. Differently, when in-situ prolonged exposure tests were carried out on PN biomass, no toxic effects were observed. Such result was mainly ascribed to the dilution rate in the chemostat, which avoided biomass inhibition, suggesting the combination of AD-FW wastewater high alkalinity and dilution rate in the chemostat as a possible key factor to avoid inhibition, and the feasibility to start up the process using the target wastewater as the only influent.

In the PN unit, nitrous oxide gaseous emissions were assessed under different operating conditions. Measured N_2O emissions in the off-gas were lower than most of the values reported in previous studies. Unlike many of the results previously reported in literature, where increase in ammonia oxidation rate or decrease in DO level appeared to trigger nitrous oxide production, reactor configuration adopted in this study (i.e., non-aerated settling and discharge phases were avoided) coupled with a continuous aeration strategy led to minimization of anoxic conditions. As a consequence, this contributed to the reduction of N_2O emissions even at low, as long as not process-limiting, dissolved oxygen concentrations.

Granular anammox SBR was able to withstand the same NLRs applied to the PN unit, and the increase of influent NO_2/NH_4 molar ratio led to an increase in nitrogen removal efficiency.

In a second phase, the target wastewater, i.e. the supernatant originating from the two-stage AD of FW, was fed into the PN/anammox process. Such process was applied for the first time to the treatment of the liquid fraction produced by two-stage anaerobic digestion of municipal solid waste Main issue faced during the experiment concerned the adjustment of the operational

parameters in order to produce a suitable effluent, especially in terms of nitrite to ammonium molar ratio. Different solutions were proposed and tested.

Nitritation was first achieved within 42 days in a semi-continuous batch reactor seeded with conventional activated sludge and fed with the target wastewater only. Startup strategy involved intermittent aeration and pH control to avoid NH_3 production and subsequent stripping, and occasional $NaHCO_3$ addition to regulate alkalinity; semi-continuous batch mode allowed prolonged biomass/wastewater contact and solid retention, promoting biomass acclimation and ammonia-oxidizing bacteria (AOB) growth; nitrite-oxidizers inhibition was mainly ascribed to both free ammonia and free nitrous acid inhibition.

The semi-continuous batch reactor was then converted into a CSTR system. Stable PN was achieved treating the target wastewater only, under the following operating conditions: T=35°C, DO=3 ppm, HRT≥0.6 d and NLR≤2.5 gN/L·d. Because of high influent alkalinity/ammonium ratio (1.3 mol/mol), PN effluent mostly resulted in a NO_2/NH_4 molar ratio higher than optimum range requested by subsequent anammox unit (1.6-2.1). Decreasing DO and increasing NLR were tested as control parameters for the adjustment of the effluent nitrite/ammonium molar ratio. While DO regulation led to process failure, NLR regulation initially seemed to be effective; however, biomass was not able to withstand NLR≥2.5 gN/L·d.

Anammox SBR unit was operated in a fed batch mode at an average NLR of 1.5 gN/L·d. Synthetic influent was progressively replaced by PN effluent according to an exponential law, in order to promote a gradual acclimation of the biomass. 100% pre-treated target wastewater was eventually fed, and chemical addition was used to correct the influent NO_2/NH_4 molar ratio. Stable good process performance was observed throughout phase 1 and 2. Subsequently, influent NO_2/NH_4 molar ratio was corrected by mixing pre-treated and untreated target wastewater. Such solution was tested for the first time using effluent from AD of OFMSW. A decrease in anammox performance was observed at first, but process recovery was achieved within 20 days. Average NRE ranged between 87 and 90%. When target wastewater was fed, specific anammox activity (SAA) averaged out at 0.44 gN_2-N/gVSS·d.

Digital color characterization of anammox granular biomass and subsequent representation through CIE Lab color space was applied for the first time on a system fed with real wastewater. Although results were not statistically well correlated to other performance indicator or to anammox bacteria abundance, some issues were pointed out about the need for measurement protocols considering possible interference by mixed liquor/influent wastewater color and

suspended solids content. In this sense, a protocol introducing a blank assessment was proposed. Moreover, chroma C* was identified as a more representative color parameter.

In conclusion, the application of the two-stage PN/anammox process to the treatment of ammonium-rich liquid by products of two-step AD of OFMSW, aimed at the production of H_2 and CH_4, as part of the development of a novel integrated system for the management and valorization of solid waste, was assessed. Such result may potentially allow to increase the sustainability of the entire OFMSW treatment chain.

Further studies are needed in this direction, in particular concerning the development of such technologies at a greater scale, and the assessment of the environmental footprint of the entire treatment process.