DIPLOMARBEIT

Effect of scale and configuration on partial nitritation and anammox process

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von

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durch

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Wien, am Dezember 2022

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Affidavit

I hereby declare that I have authored this master thesis independently and that I have not used any assistance other than that which is permitted. The work contained herein is my own except where explicitly stated otherwise. All ideas taken in wording or in basic content from unpublished sources or from published literature are duly identified and cited, and the precise references included.

I further declare that this master thesis has not been submitted, in whole or in part, in the same or a similar form, to any other educational institution as part of the requirements for an academic degree.

I hereby confirm that I am familiar with the standards of Scientific Integrity and with the guidelines of Good Scientific Practice, and that this work fully complies with these standards and guidelines.

Vienna, 12.2022 Agime BEQAJ *(manu propria)*

Preface

This study was carried out at the Institute of Sanitary Engineering and Water Pollution Control (BOKU), within the framework of the DEKO Project led by DI Dr. Norbert Weissenbacher and supervised by Univ. Prof. Dipl.-Ing. Dr.nat. techn. Thomas Ertl. The main scope of the project relied in the implementation of small and medium municipal treatment plants treating reject wastewater by deammonification.

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Abstract

The continuous disposition of people to concentrate in urban areas has risen challenges regarding the discharged wastewater and its treatment. Partial nitritation and anammox is a cost-effective method for treating municipal wastewater rich in ammonia. In the frame of this study, the influence of scale and configuration on the partial nitritation and anammox was assessed, while alkalinity was reduced. Two semi-scale reactors (375 L) and a 3 L lab reactor were set up to carry out the experiment. Ammonium and other parameters were kept constant while the alkalinity was reduced gradually. Furthermore, a mesh was integrated into one of the semi-scale reactors, to create two compartments. A particle measurement was undertaken to see which reactor had larger anammox granules. Particle measurement showed that the one-compartment system had 40% of size 50-150µm and 43% were of size 10-50µm, whereas the two-compartment system had 34% particles in the range of 50-150µm and 27% of 10-50µm. The integrated mesh did assist in retaining larger anammox granules in the two-compartment system. N-compound evaluation showed that the control strategy to regulate the microorganism consortia in the bioreactor worked well. The average nitrogen removal efficiency after supplemental alkalinity reached up to 76% for the one-compartment system and 69% for the two-compartment system. However, an accumulation of nitrates was present in both semi-scale reactors and the small-scale reactor. In one-compartment system (SBR) and two-compartment system (continuous) the average nitrogen removal efficiency during the alkalinity limitation experiment was 0.0125 and 0.0180 kg N removal per m^3 aeration. Also, the small-scale reactor was comparable in this regard with 0.0024 kg N removal per m^3 aeration. The 3 L reactor showed the least sensibility with respect to the alkalinity limitation, while the one-compartment bioreactor was the most affected by a faster reduction of removal rate. Further optimization of the ammonium oxidizing bacteria could possibly improve the performance of partial nitritation and anammox process, especially in the two-compartment system (continuous) with the potential of lower nitrous oxide emissions.

Kurzfassung

Die ständige Neigung der Menschen, sich in städtischen Gebieten zu konzentrieren hat zu neuen Herausforderungen in Bezug auf das eingeleitete Abwasser und seine Behandlung geführt. Die Kombination der partiellen Nitritation und des Anammox Prozesses ist eine kosteneffiziente Methode, um kommunale Abwässer reich an Ammonium zu behandeln. Im Rahmen dieser Arbeit wurde der Einfluss von Maßstab und Konfiguration auf die partielle Nitritation und Anammox während einer Alkalinitätsreduzierung untersucht. Zur Durchführung der Untersuchung wurden zwei halbtechnische Reaktoren und ein 3 L Labor Reaktor zum Einsatz gebracht. Ammonium und andere Parameter wurden konstant gehalten, während dessen wurde die Alkalinität schrittweise reduziert. In einen der halbtechnischen Reaktoren wurde durch ein Sieb ein Reaktor mit zwei Kammern geschaffen. Eine Partikelmessung wurde durchgeführt, um zu sehen, in welchem Reaktor mehr Anammox Granulen mit größerem Durchmesser enthalten. Die Partikelmessung hat gezeigt, dass das Einkammersystem prozentual 40% Partikel in der Größe 50-150 µm und 43% in der Größe 10-50 µm beinhaltet, wohingegen im Zweikammersystem 34% Partikel in der Größe von 50-150 µm und 27% in der Größe von 10-50 µm wuchsen. Das kontinuierlich betriebene Zweikammersystem mit dem eingesetzten Sieb konnte größere Anammox Granulen zurückhalten. Die Auswertung der Stickstoffverbindungen zeigte, dass die Strategie für die Regulierung der im Reaktor vorhandenen Mikroorganismen gut funktionierte. Die durchschnittliche Stickstoffentfernung nach künstlicher Alkalinitätszufuhr im Einkammersystem erreichte 73 % und im Zweikammersystem 69 %. Dennoch war sowohl in den halbtechnischen Reaktoren als auch im kleintechnischen Reaktor eine Ansammlung von Nitrat vorhanden. Die durchschnittliche Stickstoffentfernung für einen m^3 Belüftungsvolumen, während der Alkalinitätsuntersuchung war 0.0125 kg N im Einkammersystem (SBR) und 0.0180 kg im Zweikammersystem (kontinuierlich). Der 3 L Reaktor hatte mit 0.0024 kg vergleichbare Ergebnisse. Der 3 L Reaktor zeigte die geringste Sensibilität bezüglich Alkalinitätslimitierung, während das Einkammersystem am meisten davon betroffen war, indem die Abbauleistung schneller zurückging. Eine weitere Optimierung von Ammonium oxidierenden Bakterien könnte die Leistung der partiellen Nitritation und des Anammox Prozesses verbessern, besonders in dem kontinuierlichen Zweikammersystem mit dem Potential geringerer Lachgasemissionen.

1. Introduction

Due to the tendency to concentrate in urban areas, the urban human population is expected to increase to 68% by 2050 (UN DESA, 2018). The modern life inevitably makes people gather in large urban areas. The demands of production and human consumption alter land use and cover biodiversity and hydro systems locally to regionally. Moreover, urban waste discharge affects local to global biogeochemical cycles and climate (Grimm et al., 2008). Therefore, the amount of wastewater discharged is also increasingly causing nutrient pollution which enfolds concentrations of nitrogen and phosphorous higher than the ecosystem can actually manage. The consequence is eutrophication and an overall reduced water quality. Different approaches can be taken into consideration in order to deal with this issue. The wastewater can be managed through biological, physical, and chemical processes.

Wastewater treatment plants (WWTPs) are one of the most common measures of modern environmental biotechnology to treat municipal wastewater. Wastewater treatment removes a large fraction of nutrients before the resulting effluent is discharged into receiving water bodies (Gücker et al., 2006).

Conventional wastewater nitrogen removal systems require a lot of energy for nitrification, and often an external organic carbon source for denitrification (Beata Szatkowska & Bjarne Paulsrud, 2014). During the nitrification, the ammonia is converted to nitrites (NO_2^-) and then to nitrates (NO_3^-) by the nitrifying bacteria under anaerobic conditions. Thereafter nitrates are converted to gaseous nitrogen (N_2) by the denitrifying bacteria (Bertino, 2010).

The biological way of treating wastewater is considered a favorable alternative in regard to the protection of the environment and the costs of treatment. Still, biological wastewater treatment is a costly endeavor, whereby biological nitrogen removal significantly contributes to it.

Anaerobic ammonium oxidation (anammox) bacteria offer a way to remove N more efficiently in comparison to conventional nitrogen removal (Figure 1). They have been used to treat wastewaters rich in ammonia for more than two decades because of their unique metabolic ability to combine ammonium and nitrite to form nitrogen gas (Kuenen, 2008).

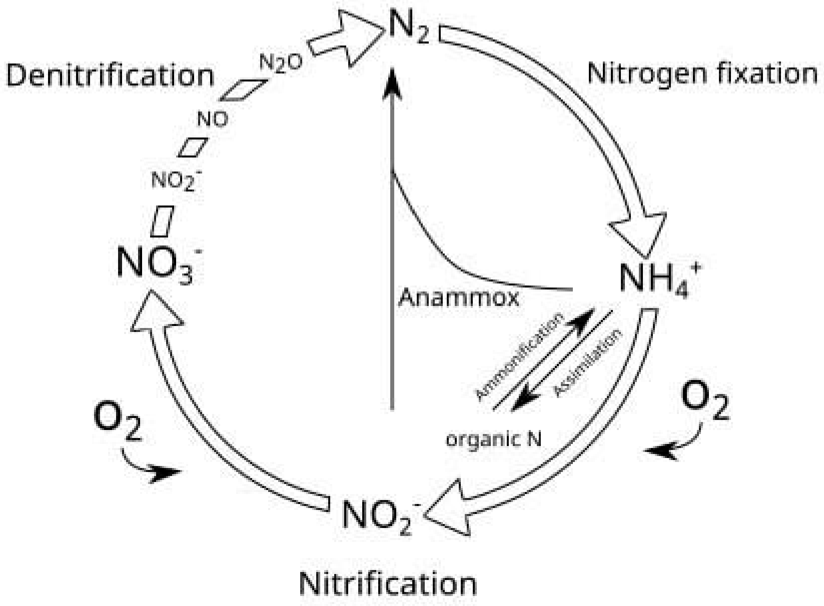


Figure 1. Nitrogen cycle adapted from Dang and Chen (2017).

With the increased demand for clean water, the wastewater treatment quickens the natural process of water purification. The goal is to promote sustainable wastewater treatment and subsequently mitigate aquatic pollution. Usually, there are two basic steps in the treatment of wastewater: the primary and the secondary. Once the wastewater is collected in sewers or other facilities, it is then transported to treatment plants. During the primary treatment, the water flows through a screen, where large objects like sticks or alike present in raw wastewaters are removed. Next, there is a grit chamber where solids and smaller particles settle down and are removed from wastewater. Afterwards, the water goes into a sedimentation tank where organic and inorganic matter sediment at the bottom of the tank. This is later removed and can serve as a land fertilizer after further preparatory steps. The generated effluent then undergoes secondary treatment, which uses biological processes to clean the wastewater (Figure 2).

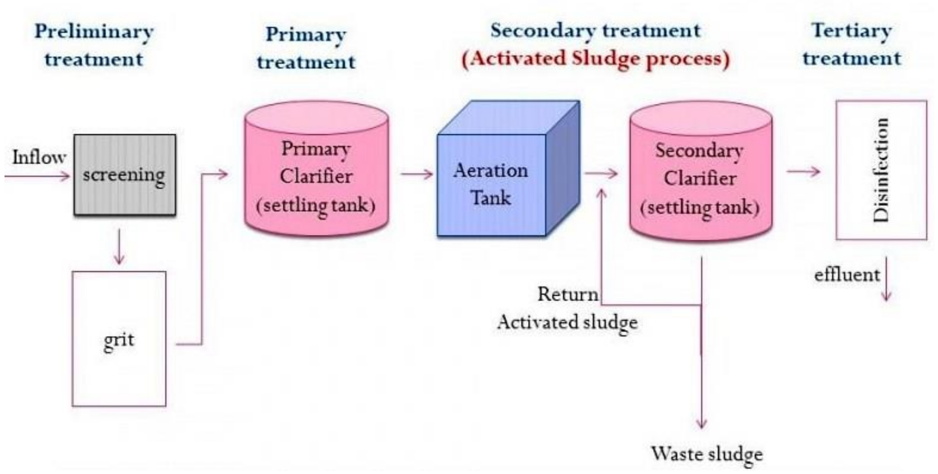


Figure 2. Illustration of the wastewater treatment process (*Water treatment and greywater recycling – INTEWA Wiki*, o. J.)

During the last two decades, various configurations have been developed for the treatment of wastewaters rich in ammonia, such as municipal reject wastewater, which comes from sludge dewatering. The sludge has originated from a previous secondary wastewater treatment. Basically, the wastewater enters the primary process where the solid waste sediments, then it goes to further secondary processing in the wastewater treatment plant. During the secondary wastewater treatment, most of the organic matter is removed by employing bacteria during the activated sludge process. After this process, reject wastewater emerges after the digested sludge is dewatered.

The utilization or application of this technology requires stoichiometrically far less oxygen in comparison with other technologies and does not need an additional external carbon source. External is called the carbon source that is not found in the influent wastewater rather than added as an external supplement to make the denitrification process more efficient. The application of external carbon sources for denitrification becomes necessary for wastewater treatment plants that have to meet very stringent effluent nitrogen limits (Cherchi et al., 2009).

Deammonification is a two-step process: the aerobic conversion of ammonium to nitrate (autotrophic nitrification) followed by the subsequent anaerobic oxidation of residual ammonium by nitrite to nitrogen gas. Both steps are catalyzed by different consortia of organisms, i.e., a population of aerobic autotrophic ammonia oxidizers (AOBs) and a consortium of anaerobic autotrophic ammonia oxidizers (anammox) (Wett et al., 2014).

The process is carried out in the two-compartment system for each step or, as an alternative approach, both process steps are operated in a single-sludge system (Sliekers et al., 2002)

A sufficient amount of alkalinity in the influent wastewater plays a fundamental role in the nitrogen removal efficiency during partial nitritation. Therefore, the assessment of the alkalinity concentration in the influent is of central importance in maintaining an optimal nitrogen removal rate because the production of the appropriate nitrite/ammonium mixture depends on the ammonium/alkalinity ratio in the influent. For the oxidation of ammonium to nitrite, two proton equivalents are produced per mol of ammonium converted (Fux et al.,2002). Thus, it is interesting to evaluate how varying alkalinity concentrations present in influent can affect the performance of different scaled systems. During scaling-down, a crucial task is trying to keep all the variables constant and simulate at the laboratory scale the conditions which micro-organisms meet at the production scale (N.M.G. Oosterhuis, 1984). Hence it is important to investigate the variation and the effect different parameters have on the biology behavior and finally its performance.

Another focusing point when designing and operating a WWTP is the release of greenhouse gas emissions (GHG). Direct GHG emissions include emissions of methane (CH_4) and nitrous oxide (N_2O) that can be biologically produced during wastewater and sewage sludge treatment (Parravicini & Karl, 2016). Nitrous oxide emissions from all of its various environmental sources are currently the single most important ozone-depleting substance emission and are expected to remain the largest throughout the 21st century (Wang et al., 2014). The global warming potential of N_2O is 265–298 times that of CO_2 for a 100-year timescale. The N_2O emitted today remains in the atmosphere for more than 100 years, on average (US EPA, o. J.) The gas N_2O is produced in biological nutrient removal systems during autotrophic nitrification and heterotrophic denitrification. Although the nitrification step involves both ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB), it is widely accepted that NOB does not contribute to N_2O production (Law et al., 2012).

The deammonification process remains a robust method for the implementation of WWTPs. The application of this technology requires stoichiometrically far less oxygen demand, 63% less aeration (Qiu et al., 2021), in comparison with other technologies and does not need an additional external carbon source. External refers to the carbon source that is not found in the influent wastewater rather than added as an external supplement to make the denitrification process more efficient. The use of biological conversion methods and the application of compact operation technology does allow us to save operation and investment costs (Seyfried et al., 2001).

2. Objectives

The aim of the study was to investigate the impact of configuration and scale of a bioreactor for wastewater treatment on partial nitritation and anammox process and ultimately the nitrogen removal efficiency.

To corroborate the scale dependency, two semi-scale bioreactors and a 3 L lab scale bioreactor were set up. The amount of alkalinity was reduced stepwise and proportionally in all three systems, while ammonium load and other parameters were kept constant. Furthermore, in order to gain an understanding if minor changes in configuration can lead to different results, a mesh was added to one of the semi-scale bioreactors to create two compartments. The goal of mesh application was to help retain larger anammox granules and subsequently assist in the improvement of nitrogen removal efficiency.

The thesis starts with an introductory part, where a general concept of the wastewater treatment issue is laid out. Followed up by the main objectives of this thesis and the approach to implement them. Next, the basic fundamentals of biological nitrogen removal from wastewater are presented. The necessary materials and the applied methods during the experimental work are written in Chapter 4. The data obtained are shown in Chapter 5, along with the discussion. At the end of the thesis is a summary and the deduced conclusions.

3. Fundamentals

3.1 Anammox

Since their discovery, anaerobic anammox bacteria have continued to raise attention on the mechanisms they operate and their application in the efficient removal of nitrogen in WWTP. This is not surprising considering the 90% reduction of operational costs that the implementation of the anammox process would achieve (van Dongen et al., 2001). Although numerous studies have been conducted regarding the pathways they undertake, the whole process is not fully understood.

Anaerobic ammonium oxidation occurs widely in marine ecosystems, but its occurrence, distribution and contribution in freshwater ecosystems and especially in extreme environments, remains largely unknown (Zhu et al., 2015). They were first discovered in a WWTP in the Netherlands in denitrifying fluidized bed reactor treating effluent from a methanogenic reactor (Mulder et al., 1995).

Anammox bacteria do not conform to the typical characteristics of bacteria but instead share features with all three domains of life: Bacteria, Archaea, and Eukarya (Niftrik & Jetten, 2012a). They do have unique biological properties: the occurrence of hydrazine as a free intermediate of catabolism, the biosynthesis of ladderane lipids and the role of cytoplasm differentiation (Strous et al., 2006). Being chemolithoautotrophs of the phylum *Planctomycetes*, they have three independent cell compartments bounded by bilayer membranes (listed from outside to inside): the paryphoplasm, riboplasm and anammoxosome (Figure 3) (van Teeseling et al., 2013).

The paryphoplasm is a region between the cytoplasmatic membrane and the intracytoplasmatic membrane (Fuerst, 2013) in which the cell division ring in anammox bacteria is situated (Van Niftrik et al., 2009)

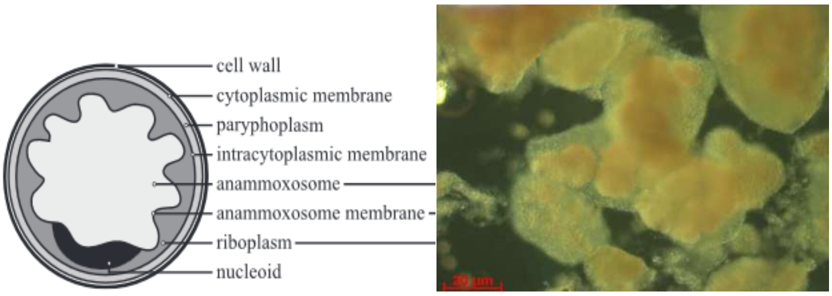


Figure 3. Schematic drawing of anammox structure (left) (Niftrik et al., 2008); own darkfield micrograph of anammox granule (right).

The innermost ribosome-free compartment is the anammoxosome, which is the locus of the anammox catabolism (Van Niftrik et al., 2009). It occupies most of the cell volume and is bound by the anammoxosome membrane (Niftrik & Jetten, 2012b). The membrane is constituted of unique lipids called ladderanes, these lipids are comprised of three to five linearly concatenated cyclobutane moieties with cis ring junctions, which occur as fatty acids, fatty alcohols, alkyl glycerol monoethers, dialkyl glycerol diethers and mixed glycerol ether/esters (Sinninghe Damsté et al., 2005). Ladderane lipids occur in the membrane of the anammoxosomes. Since these lipids are unique to anammox bacteria they can be used as biomarkers for anammox cells and the anammox reaction. It is assumed that such a membrane is essential to maintain concentration gradients during the exceptionally slow anammox metabolism and to protect the remainder of the cell from the toxic anammox intermediates (Sinninghe Damsté et al., 2002). The highly toxic hydrazine (N_2H_4) and nitric oxide (NO) are the two intermediates of this process (Niftrik & Jetten, 2012b).

The first step involves the reduction of nitrite to nitric oxide by nitrate reductase. Ammonium is then combined with nitric oxide by hydrazine hydrolase to form hydrazine (Figure 4) (Niftrik et al., 2008).

Currently, five genera have been reported: *Candidatus* Brocadia, *Candidatus* Kuenenia, *Candidatus* Scalindua, *Candidatus* Anammoxoglobus, and *Candidatus* Jettenia (X.-R. Li et al., 2009). The indicated bacteria derive their energy for growth from the conversion of ammonium and nitrite into dinitrogen gas in the complete absence of oxygen (Table 1) (M. S. M. Jetten et al., 2009)

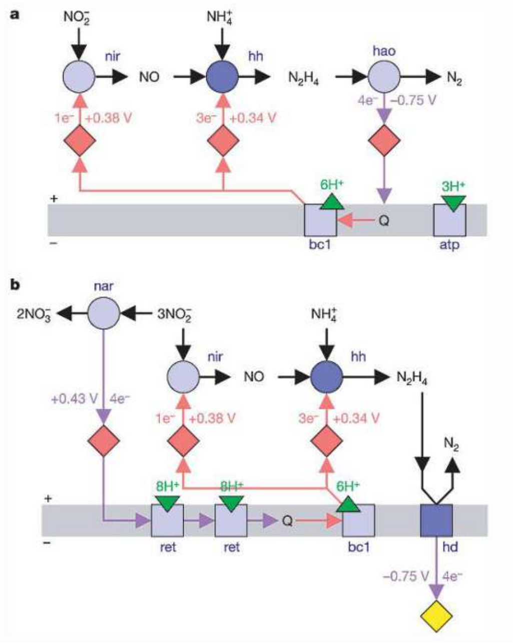


Figure 4. a. Anammox central catabolism with nitric oxide as an intermediate, electron transport and energy conservation; b, combination of central catabolism with nitrate reductase to generate high potential electrons for the acetyl-CoA pathway. Three-letter abbreviations below enzymes refer to the genes and operons. The dark blue 'hh' and 'hd' are gene clusters. Red diamonds, cytochromes; yellow diamond, ferredoxin; red arrows, reductions; purple arrows, oxidations (Strous et al., 2006).

Their main properties are:

- Obligate anaerobe
- Coccoid cells < 1µm
- Doubling time of 11-20 days

Previously it was thought that anammox bacteria can occur from 20 to 43°C (Strous et al., 1998), but more recent studies evidenced their presence also at 60°C and 85°C at deep-sea hydrothermal vents (Byrne N et al., 2009).

Table 1. Physiological characteristics of some anammox bacteria species: *Ca*. B. anammoxidans, *Ca*. Jettenia caeni, *Ca*. Brocadia sinica, *Ca* .Scalindua.

Physiological characteristics	*Ca*.Brocadia. anammoxidans	*Ca*.Jettenia caeni	*Ca*.Brocadia sinica	*Ca*.Scalindua
Growth temperature °C	20-43 °C	20-42.5°C	25–45 °C	10 to 30°C

Physiological characteristics	*Ca*.Brocadia. anammoxidans	*Ca*.Jettenia caeni	*Ca*.Brocadia sinica	*Ca*.Scalindua
Growth pH	6.4-8.3	6.5-8.5	6.5–8.8	6.0 to 8.5
Growth rate (h^{-1})	0.0027 h^{-1}	0.0020 h^{-1}	0.0041	0.0020
Doubling time (days)	10.7	n. d	7	14.4
Biomass yield (mmol-C [mmol-NH_4^+]$^{-1}$)	0.066	0.056	0.062	0.030
Tolerance:				
NO_2^- (mM)	7	11	< 16	7.5
NH_4^+ (mM)	n.d	> 20	n. d	> 16
Sulfide (µM)	110	540	n. d	
Salinity (mM)	n. d	68 mM	< 513 mM	1.5-4.0
References	Schmidt et al. 2002, Sinninghe Damsté et al. 2002, Jetten et al. 2005	Ali et al. 2015	Oshiki et al. 2011	Awata, Oshiki, and Kindaich 2013, Awata et al. 2015

There are many factors known for inhibiting the anammox process. These represent the main challenge in optimizing the process.

Many inhibitory substances are present in wastewater such as substrates (nitrites and ammonia), organic matter (nontoxic organic matter and toxic organic matter), salts, heavy metals, sulfides and phosphates. The effect of inhibitors can be diminished by optimal management of parameters like pH, temperature, dissolved oxygen and loading regulation (Jin et al., 2012)

The impact of inhibitors varies among different operating configurations, experimental methods and physical structure of sludge (biofilm or granular sludge, flocculent sludge).

The inhibitory effect of the substrate has been mentioned by different authors (Table 2 and Table 3) in the run of various scale and operational established anammox facilities.

Although nitrite has a key functional role in the anammox process as an electron acceptor their presence at high levels causes inhibition of anammox activity. However, to date, there is no consensual explanation as to the mechanism of nitrite inhibition nor how the inhibition is impacted by variations in the physiological status of anammox cells (Arroyo & Maria, 2013). Many authors have reported reversible and irreversible effects of nitrite (Fux & Siegrist, 2004; Kimura et al., 2010; Strous et al., 1999).

Table 2. Effect of nitrite inhibition on the anammox activity; * repeated exposure.

Reference	Concentration NO_2-N		Effect
	mM	mg L^{-1}	
Strous, Jetten, and Kuenen 1999	7.14	100	Complete inhibition
Lotti, van der Star, et al. 2012	28.57	400	IC_{50}
	42.85	600	60% activity decrease
Kimura et al. 2010	>19.57	>274	Activity decrease
	>53.57	>750	10% activity decrease
Bettazzi et al. 2010	4.286	Spiked 60 mg	Activity decrease
	2.143	>30 mg*	Loss of activity
Egli et al. 2001	13.21	185	Loss of activity
Dapena-Mora et al. 2007	>25	>350	IC_{50}

Reference	Concentration NO_2-N		Effect
	mM	mg L^{-1}	
López, Puig, and Ganigue 2008	7.27	101.8	Activity decrease
Raudkivi et al. 2016	~6.07	~85 (MBBR)	IC $_{50}$
	7	98 (SBR)	IC $_{50}$
	17.14	240 (UASB)	IC $_{50}$
Arroyo and Maria 2013	27.42	384	IC $_{50}$
	35.71	500	Complete inhibition
Isaka, Sumino, and Tsuneda 2007	20	280	Inhibition

Ammonium is the second substrate of the anammox process, but it has been suggested as a substrate-inhibitor because of the action of its unionized form, free ammonia (Puyol et al., 2014)

Ammonium can be transported into the cell by ion pumps in the cytoplasmic membrane. Ammonia then diffuses out through the cell membrane. A futile cycle is created that results in cytoplasmic acidification and extracellular alkalinization (Martinelle et al., 1996).

As seen in Dapena-Mora et al. (2007), 770 mg L^{-1} NH_4-N caused a semi-inhibitory effect IC$_{50}$ and that free ammonia is the actual inhibitor rather than NH_4^+ itself. In relation to this, a very important parameter is the pH. The concentration of free ammonia as a proportion of the total ammonia concentration is pH-dependent. As described by Jaroszynski, Cicek, and Oleszkiewicz (2012) at pH range 7-8, the decrease in anammox activity was independent of pH and related only to the concentration of free ammonia (FA), and when FA exceeded 2 mg N L^{-1} it manifested inhibition at nitrogen removal rate.

Table 3. Effect of the concentrations of FA and NH_4^+ on the anammox activity

Reference	Inhibitor	Concentration NH_3-N or NH_4-N (mg L^{-1})	Effect
Jaroszynski, Cicek, and Oleszkiewicz 2012	NH_3	2	Activity decrease
Jung et al. 2007	NH_3	1.7	Inhibition
Fernández et al. 2012	NH_3	38 100	IC_{50} 80% activity decrease
Dapena-Mora et al. 2007	NH_4	770	IC_{50}
Waki et al. 2007	NH_3	90	Inhibition

The application of a control pH strategy is fundamental in order to manage the ratio NH_4^+/NH_3 and to keep the FA concentration levels low enough to hinder restriction on the anammox process. An optimal operation of anammox systems can be maintained at 20-25 mg L^{-1} (Fernández et al., 2012), however other studies report various FA concentrations (Table 3).

Wastewater composition is determined qualitatively and quantitatively by its source, private or industrial discharge. Therefore, different types of wastewater arise having a divergent content (Table 4).

Table 4. Different types of wastewater (Henze et al., 2008).

Wastewater from society	Wastewater generated internally in treatment plants
Domestic/municipal wastewater	Thickener supernatant

Wastewater from institutions	Digester supernatant
Industrial wastewater	Reject water from sludge dewatering
Infiltration into sewers	Drainage water from sludge drying beds
Stormwater	Filter wash water
Leachate	Equipment cleaning water
Septic tank wastewater	

Current constituents of the common wastewater can also manifest inhibitory effects on the anammox process. Typical constituents in municipal wastewater with inhibitory capacity are sulphide, phosphate, dissolved oxygen, and salts (Table 5).

Table 5. Present constituents in municipal wastewater (Henze et al., 2001).

WW constituents	Description	Effect
Microorganisms	Pathogenic bacteria, viruses and worm eggs	Risk when bathing and eating shellfish
Biodegradable organic Materials	Oxygen depletion in rivers, lakes, and fjords	Fish death, odours
Other organic materials	Detergents, pesticides, fat, oil and grease, coloring, solvents, phenols, cyanide	Toxic effect, aesthetic inconveniences, bioaccumulation in the food chain
Nutrients	Nitrogen, phosphorus, ammonium	Eutrophication, oxygen depletion, toxic effect
Metals	Hg, Pb, Cd, Cr, Cu, Ni	Toxic effect, bioaccumulation

WW constituents	Description	Effect
Other inorganic materials	Acids, for example, hydrogen sulphide, bases	Corrosion, toxic effect
Thermal effects	Hot water	Changing living conditions for flora and fauna
Odour (and taste)	Hydrogen sulfide	Aesthetic inconvenience, toxic effect

Since the anammox process is carried out under anaerobic conditions the presence of oxygen induces an inhibitory effect. Bacteria oxidizing ammonia to nitrite requires oxygen, whereas bacteria converting ammonia and nitrite to dinitrogen gas are anaerobic and their activity is only reversibly inhibited by oxygen (Szatkowska et al., 2004). According to Carvajal-Arroyo et al. (2013), the dissolved O_2 showed moderate inhibition (IC_{50}=2.3-3.8 mg L^{-1}). A loss of 30–50% of the total nitrogen in the ocean is attributed to the anammox process. This happens in ocean oxygen minimum zones (OMZ), where the results showed that O_2 is a major controlling factor for anammox activity in OMZ waters. The zone where the N-loss can occur is primarily controlled by the O_2 sensitivity the of anammox itself. An estimation of the upper limit of anammox is 20 µmol L^{-1} (Kalvelage et al., 2011).

One of the common constituents of the wastewater is sulphide, which is present in three forms: hydrogen sulfide gas (H_2S), non-volatile ionic species hydrogen sulfide (HS^-) and sulfide (S^{2-}). The ratio of each of the three species is pH-dependent. At pH 6, 90% of the sulfide is present as H_2S. The higher the H_2S concentration, the greater the tendency for it to volatilize. Conversely, at pH 10, 100% of the sulfide is present as S^{2-}. The rate of sulfide production depends on the concentrations of sulfate ions, organic matter, and dissolved oxygen, as well as on other factors such as pH, temperature, retention time, stream velocity, and surface area (www.oxy.com, 2017). Sulfides are known to affect the bacterial activity. The performance of the anammox system was halved at a sulfide-S level of 32 mg L^{-1} within 13 days (Jin et al., 2013). Similar results were reported also by Carvajal-Arroyo et al. (2013) where at pH=7.5 and pH=7.3 hydrogen sulfide was the most severe inhibitor, with 50% inhibitory concentrations

(IC_{50}) as low as 0.03 mM undissociated H_2S, whereas dissolved O_2 showed moderate inhibition ($IC_{50} = 2.3–3.8$ mg L^{-1}).

Table 6.The inhibition concentration of various salts and the effect on anammox activity.

Reference	Inhibitor	Operation mode	Inoculation sludge (dominant species)	Concentration	Effect
van de Graaf et al. 1996	KH_2PO_4	Batch	Denitrifying FBR sludge	5 or 50 mM	Loss of activity
				1 mM	No effect
Dapena-Mora et al. 2007	Phosphate	Batch	*Ca*. Kuenenia stuttgartiensis	21mM	IC_{50}
Egli et al. 2001	Phosphate	Batch	Nitrifying RBC biofilm	20 mM	No inhibition
van de Graaf et al. 1996	Sulfide	Batch	Denitrifying FBR sludge	1 or 5 mM	Increase of anammox activity
Dapena-Mora et al. 2007	Sulfide	Batch	Anammox bacteria	1 or 5 mM	Increase

Besides ammonium, high salinity is a concerning parameter of WWTP as this can impede WW treatment by inhibiting bacterial growth (Table 6).

Shock loads from 5 to 60 g L^{-1} NaCl were applied to the anammox bacteria for a period of 12 h. The increase in shock loads was linked to a decrease in the sludge retention time (SRT) of the reactor between 2.9 and 22.5 d and a biomass decrease of 0.8-37.4%. When the 10 g L^{-1}

NaCl load was exceeded, the reactor was prone to lose too much biomass. With further shock loads, the anammox bacteria started to show adaptation (Ma et al., 2012). The effect of salinity on the anammox process in different system growths showed that the attached growth system (biofilms) outperformed the suspended growth system and that the freshwater bacteria sustained a salt shock load up to 4 kg NaCl/m^3 (Yi et al., 2011).

The effect of NaCl on anammox bacteria was tested by adding every two weeks 5 g and 2.5 g NaCl to the ammonium removal process via partial nitration/anammox. Anammox bacteria grew well with the 2.5 g strategy, whereas with the 5 g strategy a complete growth inhibition occurred at 15 g L^{-1} salinity. Furthermore, it proved that conductivity is not applicable for monitoring the process when WW is with increased salinity (Malovanyy et al., 2015).

The presence of heavy metals in wastewater represents another source of inhibition (Table 7), mostly due to industrial water discharge. Heavy metals are not biodegradable, can accumulate in organisms and exhibit a toxic effect. This is further exacerbated when heavy metals build persistent complexes with residues of antibiotics, leaked from livestock farms where they are commonly used (Khurana et al., 2021).

Table 7. List of some reported heavy metals as inhibitors of anammox activity.

Reference	Inhibitor	Concentration (mg L^{-1})	Effect
Daverey et al. 2014	Zn	6.9	IC_{50}
Zhang et al. 2016	Cu (II); Zn (II)	16.3; 20.0	20.1% inhibition
Li et al. 2015	Cu; Zn; Cd; Ni;	4.2; 7.6; 11.2; 48.6	IC_{50}
Kimura and Isaka 2014	Ni ; Cu ; Co ; Zn ; Mo	5; 5; 5; 10; 0.2	>10% decreased activity
Lotti, Cordola, et al. 2012	Cu; Zn;	1.9; 3.9	IC_{50} (24h exposure)

In relation to the anammox activity and nitrogen removal rate, the antibiotics investigated by Lotti, Cordola, et al. (2012) after 24h exposure the half maximal inhibitory concentration (IC_{50}) for sulfathiazole results to be 650 and for oxytetracycline 1.100 mg L^{-1} respectively. Furthermore, even under prolonged exposure (14 days), the anabolism and catabolism reactions were active indicating that anammox bacteria can endure extreme conditions.

Another inhibitor of the anammox activity is the toxic organic matter (OM). Basically, in wastewaters, there is always organic matter such as detergents, pesticides, solvents, phenols, fats, oils and grease (Henze et al., 2001), that influence the growth and survival of present microorganisms. The adverse effect of OM on the anammox bacteria can be explained by two different mechanisms (Jin et al., 2012):

- the outcompetion of two different biological populations

  The heterotrophic bacteria compete with the autotrophic anammox bacteria. Since the growth rate of heterotrophic bacteria is higher than that of anammox, the last is subsided reducing the nitrogen removal efficiency.
- the so-called *metabolic pathway conversion inhibition*

  The anammox bacteria show substrate diversity. Instead of using ammonium, the organic matter is consumed via different metabolic pathways.

As the coexistence of OM and nitrogen outlines a hurdle in the application of the anammox process, the strategy investigated by Tomar, Gupta, and Mishra (2015) with the usage of anaerobic granular sludge simultaneously facilitated the nitrogen removal and also the OM.

3.2 Nitrogen removal in WWTP

Biological processes are preferred and held for the most (cost-)effective processes in the field of wastewater treatment (Tchobanoglous et al., 1991). A current limitation on the widespread application of autotrophic N-removal processes is the difficulty associated with growing large quantities of anammox biomass, due to the very low growth rate (0.003 h^{-1}) and biomass yield (0.07 Cmol/mol substrate) of anammox bacteria (Strous et al., 1998). Wastewater properties, space requirements, feasibility, available budget and also environmental issues are taken into account when a WWTP is established.

3.2.1 Physical-chemical methods

Different methods for nitrogen removal from wastewaters have been used for a long time and some are still in use. The selection and application of one is mainly achieved by taking into consideration the operational costs, complexity, cost of chemicals and wastewater characteristics. In comparison with biological methods, physical-chemical methods have the advantage of not being sensitive to the compounds present in wastewater and requiring less space. However, their disadvantages are not trivial. The cost of chemicals, high level of maintenance and energy requirements are just a few to name.

The current methods for the treatment of WW according to the United States Environmental Protection Agency (https://nepis.epa.gov/, 1973) are:

1. Ammonia stripping

In water exists an equilibrium between ammonium and ammonia strongly dependent on the pH and the temperature.

$$NH_4^+ + OH^- \rightleftharpoons NH_3 + H_2O$$

When the pH of the water drops below 7 ammonia will be present in ionic form (NH_4^+). A pH above 12 will cause ammonia to dissolve to gas (NH_3.), so in the pH range of 7-12 both forms can be found −ammonium ions and dissolved gas. The ratio of these two will change with temperature and pH. This technique is suitable for ammonia removal in the range of 10-100mg L^{-1} given that pH and air temperature remain stable (www3.epa.gov, 2000).

2. Selective ion exchange

Selective ion exchange for the removal of ammonia is a method based on the affinity of an ion exchanger which has a high selectivity for ammonium ions over other cations present in wastewater. The natural zeolite clinophlolite is used, however, filtration is required prior to ion exchange to prevent the fouling of the zeolite. The efficiency of ammonium removal rises to 90-97 %. Other N-compounds like nitrate, nitrite and organic nitrogen are not affected by this process (Sedlak, 1991).

3. Breakpoint chlorination

Addition of chlorine to the wastewater containing ammonia nitrogen results in the reaction to form nitrogen gas as a product. Firstly, the ammonia reacts with the hypochlorous acid to form chloramines, then with the further addition of chlorine to the breakpoint, the nitrogen gas is formed.

At diluted NH_3-N concentrations below 1mg L^{-1} chlorine reacts with ammonia and results in various chloramines:

$$Cl_2 + H_2O \rightarrow HOCl + HCl$$

$$NH_4^+ + HOCl \rightarrow NH_2Cl + H_2O + H^+$$

$$NH_2Cl + HOCl \rightarrow NHCl_2 + H_2O$$

$$NHCl_2 + HOCl \rightarrow NCl_3 + H_2O$$

The chlorine dosage reaches the breakpoint around a 10:1 ratio of Cl: NH_3-N. Monochloramine is oxidized under slightly alkaline conditions to nitrogen gas.

$$2NH_2Cl + HOCl \rightarrow N_2 + 3HCl + H_2O$$ (Pressley et al., 1972)

This WW treatment method comes with advantages and disadvantages. It is highly efficient, but on the other hand it has high operation costs and it creates a high acidity during the process; each mole ammonium oxidized generates four mole acids, which brings extra work, since the acidity has to be neutralized by alkaline substances such as CaO (Qi, 2018).

3.2.2 Conventional Nitrification/Denitrification

For quite a while the nitrogen removal technologies engagement has been in optimizing the conventional nitrification and denitrification pathways of ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) (Nifong et al., 2013). During the first step of nitrification, the free ammonia and saline ammonia are oxidized by *Nitrosomonas spp*. bacteria (AOB) into nitrite and then secondly the nitrite is converted to nitrate by *Nitrobacter spp*. bacteria (NOB).

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + 2\,H^+$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^-$$

Additionally, during the nitrification according to the reaction below 7.4 g of alkalinity as $CaCO_3$ is consumed.

$$NH_4^+ + 2HCO_3^- + 2O_2 \rightarrow NO_3^- + 2CO_2 + 3H_2O$$ (Nifong et al., 2013)

As mentioned by Henze et al. (2001) during nitrification the alkalinity is consumed, which may cause a decrease in the pH-value in case the consumption exceeds the alkalinity present in wastewater. However, this can be compensated by combination with denitrification (Table 8).

Table 8. Oxygen consumption and alkalinity consumption by nitrification and denitrification, adapted by Henze et al. (2001). * Value in practice.

Process	Oxygen consumption			Alkalinity consumption	
	$\frac{mol\ O_2}{mol\ N}$	$\frac{g\ O_2}{g\ N}$	$\frac{g\ O_2{}^*}{g\ N}$	$\frac{eqv.alk}{mol\ N}$	$\frac{eqv.alk^*}{mol\ N}$
Nitrification	2.0	4.57	4.3	2.0	1.9
Denitrification	-1.25	-2.86	-2.4	-1.0	-0.8
Nitrification+Denitrification	0.75	1.71	1.9	1.0	1.1

Afterwards the process of reduction of nitrate to nitrite and subsequently to gaseous nitrogen is carried out by heterotrophic organisms (Fux & Siegrist, 2004). The denitrifying bacteria are facultative organisms that preferably use dissolved oxygen over nitrate as a source for metabolism and oxidation of organic matter since the energy generated per unit weight of organic matter metabolizes is higher (Bertino, 2010). Requirements for the process to occur are: a) nitrogen in the form of nitrates; b) an organic carbon source, and c) an anaerobic environment (Ishizuka et al., 1995). In this process the transfer of electrons is done by the organic matter as an electron donor and the nitrate or nitrite as electron acceptor, the electron donor may be already present in the wastewater as OM or added, as the most common external carbon source used is the methanol (Liu et al., 2007).

A typical wastewater denitrification process having the methanol as a sole carbon source is shown below (Metcalf & Eddy, 2003).

Wastewater: $C_{10}H_{19}O_3N + 10NO_3^- \rightarrow 5N_2 + 10CO_2 + H_2O + NH_3 + 10OH^-$

Methanol: $5CH_3OH + 6NO_3^- \rightarrow 3N_2 + 7CO_2 + 7H_2O + 6OH^-$

Despite the fact that other substrates are competent of inducing the denitrification like succinic acid, ethanol, acetic acid (Kesserű et al., 2002), glucose, sucrose, glycerol, and mannitol and also plant residues (straw and alfalfa) (Catanzaro & Beauchamp, 1985), methanol is mostly used.

As an end product in most of the cases is the gaseous nitrogen N_2, but during this process is also generated the nitrous oxide (Liu et al., 2007), known for its harmful greenhouse effect.

3.2.3 Innovative technologies for biological nitrogen removal

Although the classical nitrogen removal processes have been in application for a while, new innovative technologies are taking the attention with respect to energy saving, infrastructure, and operational assistance. These processes include the single reactor system for high ammonia removal over nitrite (SHARON) process, which involves the part conversion of ammonium to nitrite; anammox process, which involves anaerobic ammonium oxidation; and the completely autographic nitrogen removal over nitrite (CANON) process, which involves nitrogen removal within one reactor under oxygen-limited conditions (Annachhatre & Khin, 2004).

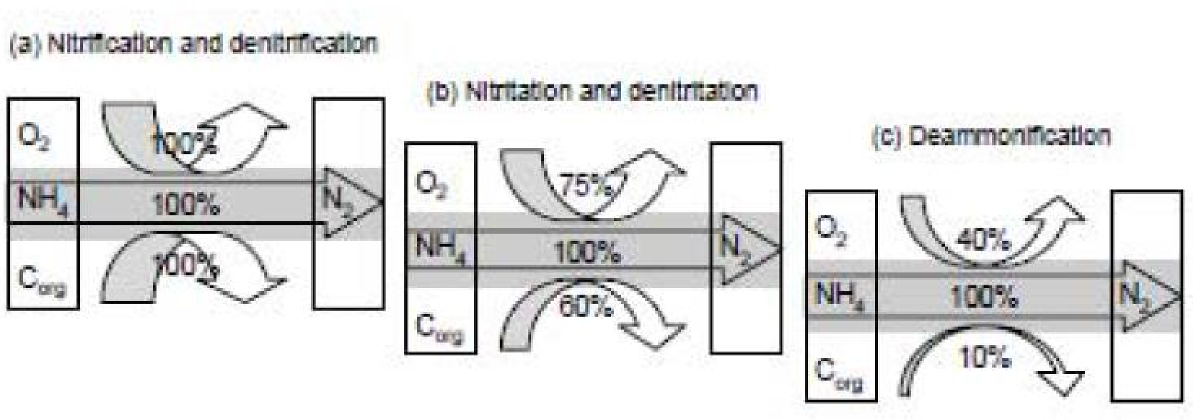


Figure 5. Nitrogen removal process comparison regarding O_2 and C demand (Wett, 2007)

3.2.3.1 Nitritation-Denitritation (SHARON®)

Single reactor system for High-rate Ammonium Removal Over Nitrite, this system sludge digester effluent can be treated separately, NH_4-N in the sludge digester effluent is oxidized in the Sharon reactor to NO_2-N for only 50%. The mixture of nitrite and ammonia is ideally suited as an influent for the anammox process where ammonium and nitrite are anaerobically converted to N_2-gas and water (van Dongen et al., 2001). The SHARON process operates at a high temperature (30–40 °C) and pH (7–8). The process is performed without sludge retention. This enables the prevention of nitrite oxidation, leading to lower operational costs (Hellinga et al., 1998b). The performance is usually carried out in separate reactor compartments with continuous flow, where during the process, ammonium is oxidized under aerobic conditions to nitrite (Nitritation). The produced nitrite is in turn reduced and heterotrophically denitrified to nitrogen gas under anoxic conditions by using an external carbon source (Denitritation) (Bertino, 2010).

- Nitrification:

$$NH_4^+ + 1.5O_2 \rightarrow NO_2^- + H_2O + H^+$$

- Denitrification:

$$NO_3^- + 4g\ COD + H^+ \rightarrow 0.5N_2 + 1.5\ sludge$$

- Summary:

$NH_4^+ + 1.5O_2 + 2.4\ COD \rightarrow 0.5N_2 + H_2O + H^+ + 0.9$ sludge (Henze et al., 2001)

To obtain successful nitritation/denitritation, a differential between ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) rates should be obtained to be able to wash out NOB based on SRT control (Regmi et al., 2014). A key process factor is the temperature (above 25°C) at which the maximum specific growth rate of the desired ammonium oxidizers is higher than that of the "undesired" nitrite oxidizers. At the operational temperature of 35°C, the maximum specific growth rate of nitrite oxidizers is approximately only half of the one for the ammonium oxidizers (0.5 and 1 day^{-1}, respectively) (Hunik, 1993). As stated by Van Hulle et al. (2007) on the kinetics of SHARON process indicating the existence of a pH interval between 6.5 and 8 and a temperature interval from 35 to 45 °C where the biomass activity is maximal.

The reduced need for oxygen means that the process needs 25% less aeration, also the reduction of nitrite to nitrogen gas requires 40% less carbon source (Henze et al., 2001).

Overall processing costs are less than 50% of other techniques (Hellinga et al., 1999). Nitrogen removal efficiency via the nitrite route under low DO concentrations showed good results (Aslan & Dahab, 2011), likewise in the study by van Kempen et al. (2011) the conversion rates of 90% were possible and the process was shown to be stable.

3.3 Study-specific Introduction

3.3.1 Partial nitritation/Anammox

An alternative to the traditional systems is represented by the innovative biological process named anammox that anaerobically converts ammonium into N_2. In comparison to the conventional processes, this new process requires a lower amount of oxygen, has no need for organic carbon supply and is highly efficient with any concentration of ammonium (Figure 5) (Ding et al., 2015). Due to the partial nitritation where ammonium is converted to nitrite but not to nitrate reduces the amount of oxygen needed. The conditions should favor the growth of the anammox organisms considering their low growth rate and possible inhibition effects. Therefore DO and nitrite concentrations should be kept as low as possible and biomass washout should be limited (S. W. H. Van Hulle et al., 2010) The oxidation of ammonium to nitrite shortens the pathway of the process as described by Strous, Jetten, and Kuenen (1999).

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

3.3.1.1 Partial nitritation/Anammox in separate reactors (two-reactor system)

This process has also been called the combined SHARON®-Anammox ® process. The process is performed in two reactors (Figure 6). In the first aerobic reactor, about 50 % of ammonium is partially nitrified to nitrite (Bertino, 2010), then the process is overtaken by anammox bacteria in an anaerobic reactor where as an end product is generated the nitrogen gas N_2.

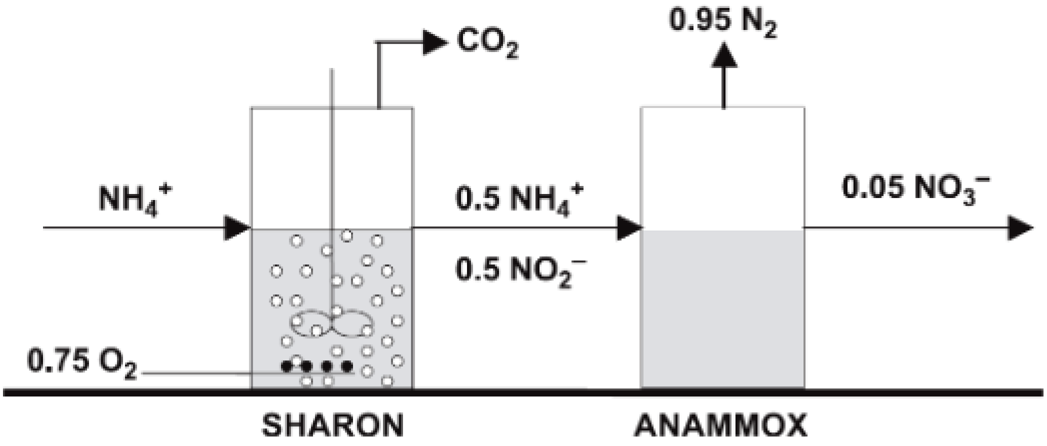


Figure 6. Schematic overview of the combined process SHARON®-Anammox (Khin & Annachhatre, 2004).

Compared to conventional nitrification/denitrification, the combined system saves 50% on required oxygen, 100% on the external carbon source and reduces CO_2 emission by more than 100% (the combined process actually consumes CO_2) (Van Loosdrecht & Jetten, 1998).

Moreover, the effluent of the SHARON process was ideally suited as an influent for the anammox reactor (van Dongen et al., 2001), the influent for the anammox process must be composed of NH_4-N and NO_2-N in a ratio 1:1 (Galí et al., 2007). The nitrite-oxidizing activity should be suppressed and ammonium should only be oxidized for about 50% to nitrite, therefore, several important parameters should be controlled like the free ammonia (FA, NH_3) and free nitrous acid (FNA, HNO_2) concentration, the temperature, pH and DO concentration (S. W. H. Van Hulle et al., 2010).

3.3.1.2 Partial nitritation/Anammox in one single reactor (one-reactor system)

To this process, several names are attributed like CANON (Completely Autotrophic Nitrogen removal Over Nitrite), DEMON® based on pH strategies and aeration control, SNAP (Single-stage Nitrogen removal using the Anammox and Partial Nitritation), and aerobic/anoxic deammonification. Therefore a generalization in one term of the partial nitritation/anammox in one single reactor system would be deammonification (Bertino, 2010). According to Płaza, Trela, and Gut (2003), low oxygen concentrations below 0.5 mg O_2 L^{-1} in combination with pH-values around 8.2-8.3 and temperature of 30°C were required to successfully operate the deammonification process. Partial nitritation requires a stoichiometric oxygen demand of only 40% compared to complete nitrification (Wett et al., 2007). Furthermore, the addition of an external carbon source is not needed due to the autotrophic denitrification to N_2 (Sliekers et al., 2002) (Figure 7). Since it is operated in only one reactor the infrastructure demand is lower, yet requires maintenance from skilled technical workers.

As stated by Nielsen M et al. (2005) it is critical to have online information about the chemical conditions within the reactor. Considering the continuous threat of reactor failure due to oxygen overloading and subsequent nitrite poisoning of the anammox biomass probably necessitates information about NO^{-}_2 status, likewise, information about increasing NO^{-}_3 concentration would indicate the growth of NO^{-}_2 oxidizing bacteria. Hence, according to Wett (2007) three parameters that need to have a sufficiently accurate adjustment, to keep appropriate boundaries and avoid the impact of ammonia inhibition, nitrite toxicity, and inorganic carbon limitation, are:

- Time control defines operation cycles of 8 hours each, involving a fill/react phase, a settling period and a decant period.
- pH is affected by the two successive processes, the partial nitritation reaction depresses the pH and the anaerobic ammonia oxidation reaction elevates the pH, so according to the pH signal, the aeration intervals are regulated.
- DO control, the set-point of dissolved oxygen (DO control) control is specified at a low range, close to 0.3 mg L^{-1} in order to prevent rapid nitrite accumulation and to maintain continuous repression of the second oxidation step of nitrite to nitrate.

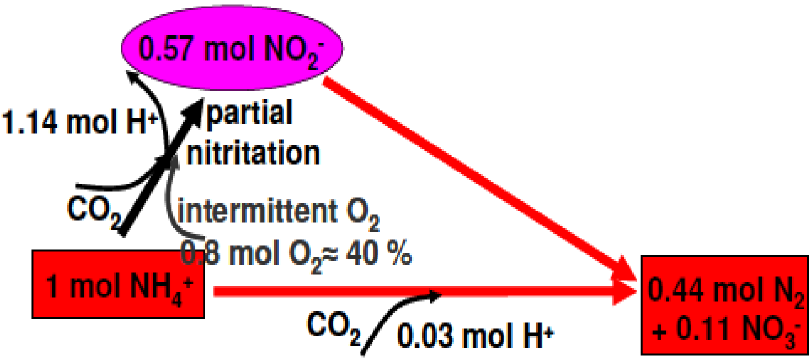


Figure 7. Partial nitritation and anaerobic ammonia oxidation as the two steps of the deammonification process (Wett et al., 2007).

Many full-scale installations based on PN/A technology have been implemented around the world. A summary done by Lackner et al. (2014) revealed that more than 50% of all PN/A installations are sequencing batch reactors, 88% of which are being operated as single-stage systems, and 75% are used for sidestream treatment of municipal wastewater. Nevertheless, the decision to implement a one or two-stage configuration system is based on various factors depending on the goal and results (Table 9 and Figure 8).

Table 9. A comparison of the main operational advantages of single and two-stage configurations of fully autotrophic nitrogen removal, adapted from Ángeles, Luis, and Anuska (2016).

Single Stage Configuration	Two-Stage Configuration
Simplification of reactor control and operation, lower investment costs	Each stage operation can be optimized individually
Continuous depletion of nitrite, avoiding toxic concentrations for anammox	More effective nitritation suppression in the first stage
Lower N_2O emissions compared to two-stage (Vlaeminck et al., 2012)	Influent COD oxidation is performed in the first stage, avoiding anammox to be overgrown by heterotrophs
	Lower risk of dissolved oxygen inhibition over anammox

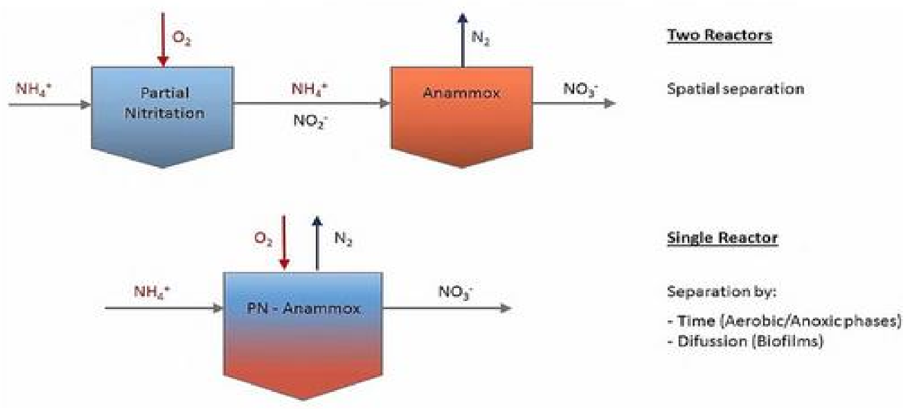


Figure 8. Schematic overview of one and two-stage configurations of PN/A (Ángeles et al., 2016)

3.3.2 Alkalinity in the wastewater treatment

During the process of nitrification, 7.14 g of alkalinity is consumed per gram of N oxidized (B. Li & Irvin, 2007).

The production of the appropriate nitrite/ammonium mixture depends on the alkalinity/ammonium ratio in the influent. For the oxidation of ammonium to nitrite, two proton equivalents are produced per mol of ammonium converted. In the second reaction, the nitrite produced together with the remaining ammonium as an electron donor is converted to nitrogen gas (Fux et al., 2002).

According to Bagchi, Biswas, and Nandy (2010a) the control of alkalinity and (DO) concentrations in influent to maintain an alkalinity to ammonia ratio of less than 8 and DO loading of less than 0.06 mg O/(mg N day), respectively, was required for inhibiting nitratation and enhancing partial nitritation and anammox. Also, by regulating the bicarbonate alkalinity the process of NOB elimination can be improved.

Hence a sufficient amount of alkalinity is considered to be a prerequisite for an optimal partial nitration/anammox process.

4. Materials and Methods

4.1 Anammox Inoculum Source

The biomass used for inoculation was collected at WWTP located in Strass im Zillertal, Tyrol, Austria at the effluent sieve of the DEMON® process. For the inoculation, anammox granules produced from sludge liquor treatment are seeded to the mainstream, retained and enriched by a hydro-cyclone classifier selecting for the high-density sludge fraction from the waste stream (Wett, 2007). Prior to inoculation, the biomass was preserved for a week at room temperature. In the two-compartment bioreactor the anammox biomass was firstly introduced in the anoxic zone, however it diffused into the aerobic zone as well. The biomass had a VSS of 4.2 g L^{-1} and ISS 2.8 g L^{-1}.

4.2 Pilot plant facility

The wastewater treatment facility was composed of two semi-scale bioreactors: a one-compartment system reactor (1-CS) and a two-compartment system reactor (2-CS), two effluent tanks and a switch box linked with a PC for controlling and regulation. To evaluate the PN/A process within the bioreactor, several methods were brought to use.

The reactors were supplied with reject water from the WWTP Stockerau (Austria) (Table 10). Additional NH_4HCO_3 was put in reject WW to reach the ammonium levels for the research purposes. Initially, the NH_4-N concentration was increased to ~800 mg L^{-1} and later up to ~1200 mg L^{-1}.

Table 10. The characteristics of reject WW when it was at first received.

Origin	NH_4-N [mg L^{-1}]	COD filtered [mg L^{-1}]	Alkalinity [mmol L^{-1}]	pH
WWTP Stockerau	80-140	90-100	10-15	7.6-7.8

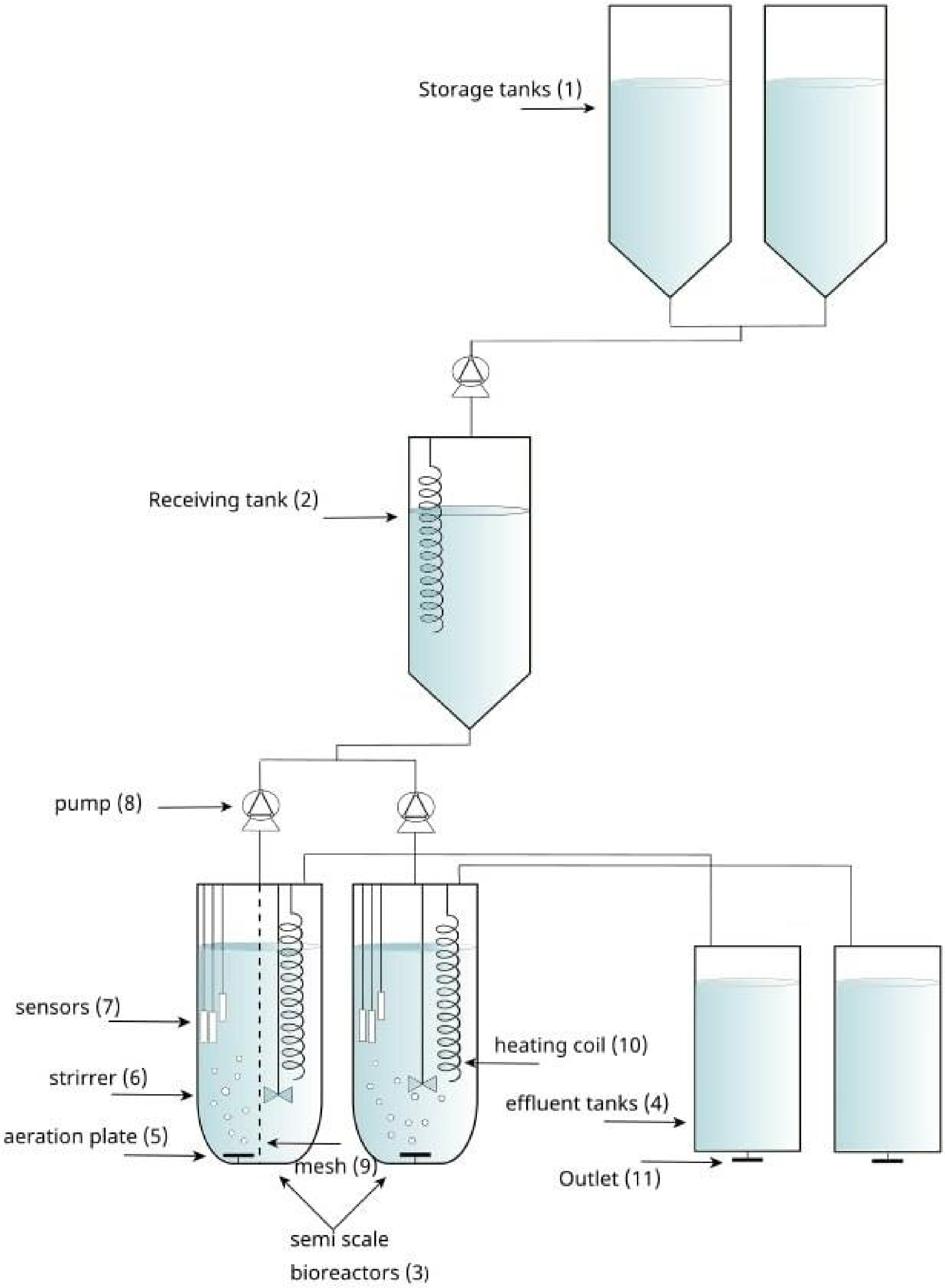


Figure 9. A schematic representation of the pilot plant facility: (1) storage tanks (5 m³); (2) receiving tank (1.3 m³); (3) semi-scale bioreactors; (4) effluent tanks (1m³); (5) aeration plate; (6) stirrer; (7) pH sensor, redox and oxygen sensor; (8) peristaltic pump; (9) mesh-1 mm; (10) stainless steel heating coil and (11) outlet.

4.2.1 Constitution of the semi-scale bioreactors

The two semi-scale bioreactors that carried the PN/A process were set up at the technical hall at the Institute of Sanitary Engineering and Water Pollution Control (BOKU). They were constructed of PE-plastic tanks and operated with a working volume of 375 L and dimensions 500 x 500 x 2000 mm. Each reactor had a separate bottom outlet and for temperature regulation heating coils made of stainless steel were installed in which hot water 40°C was provided from a heating pump.

Each reactor was equipped with:

- pH sensors (SensoLyte SE. WTW. DE). (Orbisint CPS11. Endress Hauser. CH) and measuring transducers (pH 298. WTW. DE). (Liquidsys M. Endress Hauser. CH)
- Optical oxygen sensors (Oxymax W COS61. Endress Hauser. CH) and measuring transducers (Liquidsys M. Endress Hauser. CH) for dissolved oxygen control.
- Redox sensors (SensoLyte PL. WTW. DE) and measuring transducers (pH 293. WTW. DE) for Oxidation-Reduction Potential (ORP)–control.
- Conductivity sensors (Condumax. Endress Hauser. CH)
- Volume flow controller (Aera Mass Flow Contoller. Advanced Energy. USA) for controlling and regulating the ventilation rate.
- Diaphragm gas meters (brand) for monitoring the ventilation rate and regulating the volume flow controller.

There were two reactor configurations implemented: one-compartment system and two-compartment system (Figure 10).

The two-compartment system reactor was divided into two reaction zones, an aerobic and anoxic zone, separated by a mesh with 1mm pores. With the intention to that the nitrification process takes mainly place in the aerobic zone (left) while the anammox process takes place in the anoxic zone (right) (Figure 9).

The aeration was performed through an aerator plate (AQUACONSULT, AT) placed in the middle of the reactor, whereas in the two-compartment system the aeration was carried only on the nitrification side.

In order to maintain a homogenous condition of the biomass, reactors were equipped with propeller stirrer fixed on top of the reactor. The reactors were each fed from a receiving tank through peristaltic pumps (Watson Marlow, USA), which regulated the inlet load into the reactors.

Both reactors had separate tanks where the effluent wastewater was initially discharged. After sampling, the effluent wastewater was released into the drain.

Figure 10. Overview of the two semi-scale bioreactors and the control system: A two-compartment and B one-compartment(left) and the control center with PC (right).

4.2.2 Constitution of the lab scale bioreactor 3L

For further alkalinity investigation a SBR bioreactor was set up with a working volume of 3 L (Figure 11). The reactor was placed in a water bath made of plastic (350 x 250 x 250 mm). The regulation and the constant maintenance of temperature at 35 ° were done by using an immersion circulator (Haake C1. DE). The influent wastewater taken from a storage tank was brought to the bioreactor with the help of a dosing pump (Minipuls 2. Gilson. UK). The effluent wastewater was also carried out through a dosing pump initially into a 240 ml sample container and then discharged into a reservoir. The aeration was conducted using an aquarium pump (ProSilent a 200. JBL. CH) and a stirrer.

The bioreactor was equipped with pH, redox and oxygen sensors. Each of the sensors was connected to a multifunction meter (Multi 3410. WTW. DE). The data was monitored and recorded on-line.

Figure 11. The 3 L bioreactor immersed in a heated water bath (right) and the control PC (left).

The whole process was fully automated from a PC with the same program that was used for the 375 L bioreactors.

4.3 Regulation and control strategy

Several parameters were measured online through the sensors placed in the bioreactor. The measurements were recorded in real-time on the attached PC.

To have the process run under optional conditions, upper and lower limits (set points) were set for pH and DO. The temperature was set at 35°C. It is known that temperatures above 30°C favor selectively the wash-out of NOB (Hellinga et al., 1998a).

The set point for DO ranged from 0-0.3 and 0.1-0.5 mg L^{-1}. Normally the DO set point was kept at 0.3 mg L^{-1}, but in case of high NOB and instability was set below this value. This was due to the avoidance of prolonged aeration periods. The pH limits were set in the ranges 7.01-7.015 and 7.13-7.125. During the aeration, the pH value drops, while without aeration it rises. The forced aeration was run according to the schema in Figure 12. The cycle time (SBR) consisted of a reaction period (210 min), a settling phase (15 min) and a decant phase (15 min), in total 240 minutes cycle period.

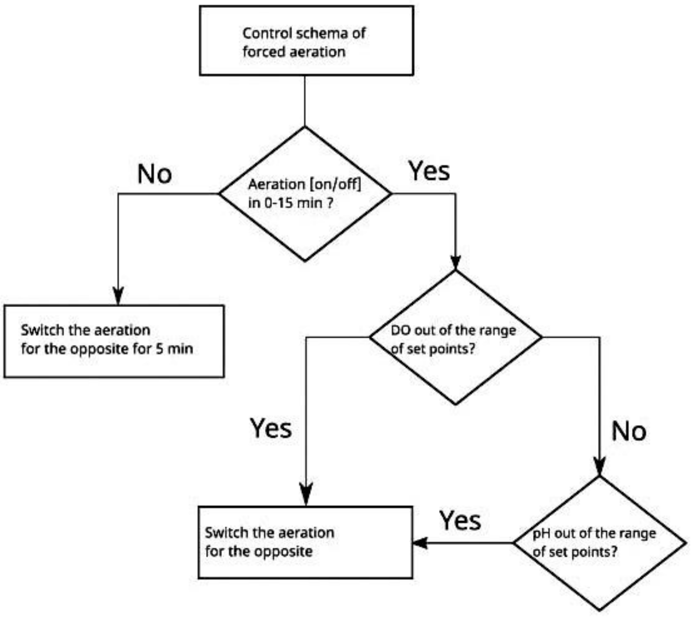


Figure 12. The algorithm of forced aeration should automatically stabilize the pH pattern: If aeration has not been on within the last 15 minutes, then switch aeration on. If aeration has not been off within the last 15 minutes, then switch aeration off. If aeration has been switched on in the last 15 minutes, then check if the DO is within the range of set points; if no then switch aeration[on/off], if yes then check if the pH is within the range of set points, if yes then switch aeration for the opposite[on/off].

The control program was accessible through desktop where parameters could be optimized. Critical values of N-compounds in influent and effluent wastewater require regular check-ups. If NO_2-N value in the influent increase above 15 mg L^{-1}, aeration intensity needs to be reduced. A value of NO_2-N over 20 mg L^{-1} in the effluent wastewater indicates a possible inhibition of anammox bacteria. Measures required are DO checkup and settling time reduction. If NO_3-N (effluent) increases over 20%, NH_4-N (influent) signal a high amount of NOB, respectively a failure to suppress them. Measures required are: temperature check, pH set points adjustment, DO checkup and settling time reduction. If NH_4-N concentration in effluent surpasses 100 mg L^{-1} NH_4-N, first the loading rate must be reduced. If pH drops under 7, $NaHCO_3$ should be added.

4.4 Standard methods for sludge characterization

Methods chosen to characterize sludge were based on their feasibility to be applied to a semi-scale wastewater treatment plant. Straightforward practicability and time efficiency were the main criteria.

4.4.1 Suspended solids and volatile suspended solids

For the SS and VSS determination samples were collected from the influent (direct from the reactor) and effluent. A sample of 100 mL was retrieved and thoroughly mixed. The sample was partitioned into 50 mL for duplicate determination and muffled for ~2 hours at 105°C. Next, it was placed in an exicator to reach room temperature and weighted to determine SS. Afterwards the sample was put in a muffle furnace for 2 h at 550 °C to determine the amount of VSS.

The measurements were conducted according to DIN 38409 (1987) and DIN EN 12879 (2001) standards. Sludge age was obtained by determination of the total solids under aeration in the reactor divided by the total solids in the effluent.

4.5 N-compounds determination via spectrophotometric measurement

Samples were collected every second day from the influent and effluent to determine the amount of NH_4-N, NO_3-N and NO_2-N, from which the nitrogen removal rate was derived. The samples were analyzed in a Hach Lange photometer (Lange Lasa 50) using standard cuvette tests (Hach, USA) (Figure 13). A sample of 0.2 mL of the appropriate N-compound was added to the corresponding cuvette. The cuvette was closed and briefly shaken. After 15 minutes the cuvette was analyzed. Necessary dilutions were undertaken so that the spectrophotometrically generated value is within the range of detection.

Figure 13. Standard cuvette tests used to detect spectrophotometrically the amount of ammonium (green cuvette), nitrates (yellow-orange cuvette) and nitrites (red cuvette).

4.6 Evaluation of anammox biomass performance

4.6.1 SAA determination

In the framework of this research, the SAA determination was based on two key methods: manometric measuring system as introduced by Dapena-Mora et al. (2007) and Yang and Jin (2012) and the utilization of stoichiometry of anammox reaction to derive its activity.

The method established by Dapena-Mora et al. (2007) and Yang and Jin (2012) was based on the batch test by Buys et al. (2000) used to determine the denitrifying activity. This method was adapted for anammox biomass by Dapena-Mora et al. (2007).

Experimental procedure:

After 100 ml synthetic wastewater was added to the hermetically sealed 250 ml vials, the vials were flushed with 99.99% nitrogen gas to keep out the O_2 and to obtain anoxic conditions. Next, they were placed in the dark in a shaking incubator (35°C, 150 rpm). Just before the incubation additional 20 ml synthetic wastewater with 420 mg L^{-1} of NH_4-N and 420 mg L^{-1} of NO_2-N was injected through the rubber septum into the bottles to reach the concentration of 70 mg L^{-1} N. Samples of the liquid phase were collected at 30 minutes intervals for 4 hours and diluted with H_2SO_4. A UV-Vis spectrophotometer (Tecan Reader: Perkin Elmer Multimode Plate Reader EnSpire 2300) was used to measure NH_4-N, NO_3-N and NO_2-N. N-compounds were derived according to the stoichiometric reaction. All assays were conducted in duplicates.

4.7 Particle Size Determination

Particle size gives additional information about the quality and the performance of the biological process.

According to Vlaeminck et al. (2010) large granular microbial aggregates improve the performance of the PN/A. The granule size may have an impact on the optimal range of oxygen concentration, meaning that the range of oxygen concentration in established PN/A systems becomes broader for larger particles (E. I. P. Volcke et al., 2010). Samples were collected direct from the bioreactor (aerobic and anaerobic compartment) and the effluent. The measurement was conducted with a Particle Track device (Mettler Toledo: Particle Track G400) with Focused Beam Reflectance Measurement technology, which enables measurement of particle size and count in real time (Figure 14). The probe-based instrument was immersed directly into the sample container. Settings were set to 300 rpm and a measurement was obtained every 5 seconds. After each analyzed sample the sensor tube was rinsed with acetone.

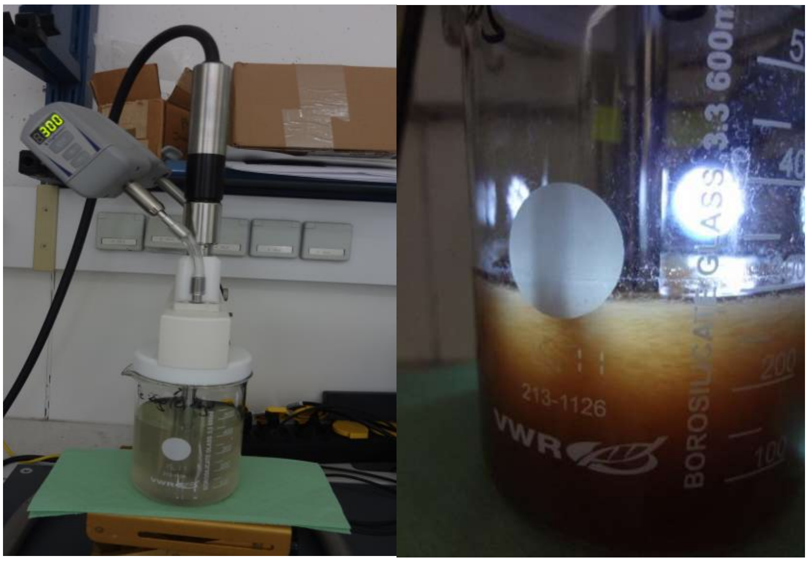


Figure 14. The setup of the Particle Track device (left) and probe-based instrument immersed in the sample collected directly from the bioreactor (right): granules can be seen with the naked eye.

4.8 Alkalinity limitation experimental set up

The two semi-scale bioreactors and the 3 L lab scale bioreactor (already described in subsections 4.2.1 and 4.2.2) were used to conduct the alkalinity limitation experiment. The working volume for the semi-scale bioreactors was 375 L. The operating characteristics and parameters were set to be constant for all three bioreactors. The alkalinity was reduced gradually over the course of the experiment. Samples were collected daily to determine the N-compounds and the amount of alkalinity in the system. The monitoring of several parameters (pH, DO, ORP) was done online utilizing the same control program.

4.9 Data analysis

Data visualization and descriptive analysis (data pivoting and summary) were conducted in Microsoft Excel 2016. Statistical tests were done in SPSS (Version 28.0.0.0) and chosen in accordance with the parameter distribution after the Shapiro-Wilk test. Image processing was done using the software Picasa 3 (Version 3.9.141). Technical drawing was done in Inkscape (Version 1.1). Values are reported as mean ± standard deviation, unless otherwise specified.

5. Results and Discussion

5.1 Assessment of the influent and effluent wastewater parameters

5.1.1 Nitrogen compound parameters

The nitrogen feed concentration was initially increased up to 800 mg L^{-1}. Due to further artificial addition of the NH_4HCO_3 in influent wastewater, the nitrogen load was ultimately brought up to ~1200 mg L^{-1} to assure a steady level.

Although the nitrogen removal efficiency numerically in June was better, the NH_4-N load was still low, therefore the stability and the performance of the semi-scale pilot plants were questionable at this point. Based on the data from N-compounds in influent and effluent wastewater (Table 11, Figure 15 and Figure 16), the implemented strategy to control the microorganism abundance in bioreactors (DO, pH, temperature, aeration), respectively to promote AOB and suppress NOB worked well. Since the sampling for N-compounds was not conducted directly from the bioreactor, nitrite concentrations may be underestimated (Table 2). Moreover, there is no information on the prevailing NH_4-N/NO_2-N ratio in the reactor. A low concentration of concentration of NO_2-N and NH4-N in the effluent is a good indicator for a successful PN/A process.

The average concentration of nitrites during the operating time in the effluent of 1-CS and 2-CS was 4.0±7.9 mg L^{-1} and 6.1±23.3 mg L^{-1}, respectively. A progressive increase in concentrations of NO_3-N in effluent wastewater was observed in both systems. However, in 2-CS the accumulation of nitrates was slightly higher with a maximum of 387±99.5 mg L^{-1} and an average of 199.1± 99.5 mg L^{-1}, while in 1-CS NO_3-N reached the maximum at 342±84.8 mg L^{-1} and the average of 172.7±84.8 mg L^{-1}. As stated by Lackner et al. (2014) the main reason for the accumulation of nitrite-oxidizing bacteria and therefore nitrate, is a too high oxygen supply to the reactor which is not necessarily detected as an increase in DO concentration. The upper limit for dissolved oxygen concentration was set at 0.3 mg L^{-1}.

Table 11. Average N-compounds values [mg L^{-1}] (NH_4-N, NO_2-N and NO_3-N) in influent and effluent WW during the operating time in 1-CS and 2-CS and their respective N-removal efficiency [%] on monthly basis. 1-CS remained during the whole time in SBR mode, whereas the 2-CS was shifted to continuous mode from November; values highlighted in grey color were retrieved while the system was in continuous mode.

Month	NH_4-N influent [mg L^{-1}]		NH_4-N effluent [mg L^{-1}]		NO_2-N influent [mg L^{-1}]		NO_2-N effluent [mg L^{-1}]		NO_3-N influent [mg L^{-1}]		NO_3-N effluent [mg L^{-1}]		N-removal efficiency [%]	
	1-CS	2-CS	1-CS	2-CS	1-CS	2-CS	1-CS	2-CS	1-CS	2-CS	1-CS	2-CS	1-CS	2-CS
June	395.9	395.9	50.1	54.7	29.1	29.1	2.5	2.4	57.0	57.0	45.2	63.8	81.4	77.0
	±181.7	±181.7	±41.9	±47.7	±12.3	±12.3	±4.5	±1.9	±14.7	±14.7	±30.9	±27.7	±5.2	±8.7
July	822.4	822.4	83.7	69.0	17.5	17.5	2.4	4.1	38.0	38.0	132.6	126.1	74.8	77.2
	±240.4	±240.4	±62.0	±46.0	±18.2	±18.2	±1.1	±2.2	±21.3	±21.3	±31.6	±33.2	±5.9	±6.6
August	1005.1	1005.1	151.1	110.4	33.6	33.6	8.1	2.8	38.0	38.0	140.9	144.5	72.1	75.9
	±69.0	±69.0	±31.1	±25.1	±9.7	±9.7	±20.8	±1.3	±11.0	±11.0	±54.5	±23.3	±6.8	±3.1
September	1070.0	1070.0	115.1	98.5	42.5	42.5	5.9	3.0	32.3	32.3	192.6	189.5	72.4	74.3
	±165.2	±165.2	±82.3	±73.7	±15.3	±15.5	±2.7	±0.9	±19.8	±19.8	±43.0	±40.2	±4.9	±4.7
October	1120.8	1120.8	85.4	79.9	7.3	7.3	6.0	1.9	36.1	36.1	260.6	297.5	70.4	69.3
	±107.6	±107.6	±51.3	±26.5	±8.0	±8.0	±3.6	±0.7	±30.6	±30.6	±38.2	±34.6	±9.7	±8.3
November	1243.1	1243.1	50.6	82.5	3.2	3.0	2.7	3.5	3.1	2.4	241.9	320.7	75.7	69.0
	±146.1	±146.1	±45.8	±34.7	±1.0	±0.7	±1.6	±1.3	±1.5	±0.5	±42.2	±28.7	±3.9	±3.8
December	1348.4	1348.4	119.4	149.3	0.6	0.7	1.3	27.7	1.3	1.3	257.8	296.1	72.5	64.9
	±63.8	±63.8	±85.1	±68.9	±0.4	±0.6	±0.8	±62.4	±0.2	±0.2	±18.5	±30.5	±5.1	±6.6

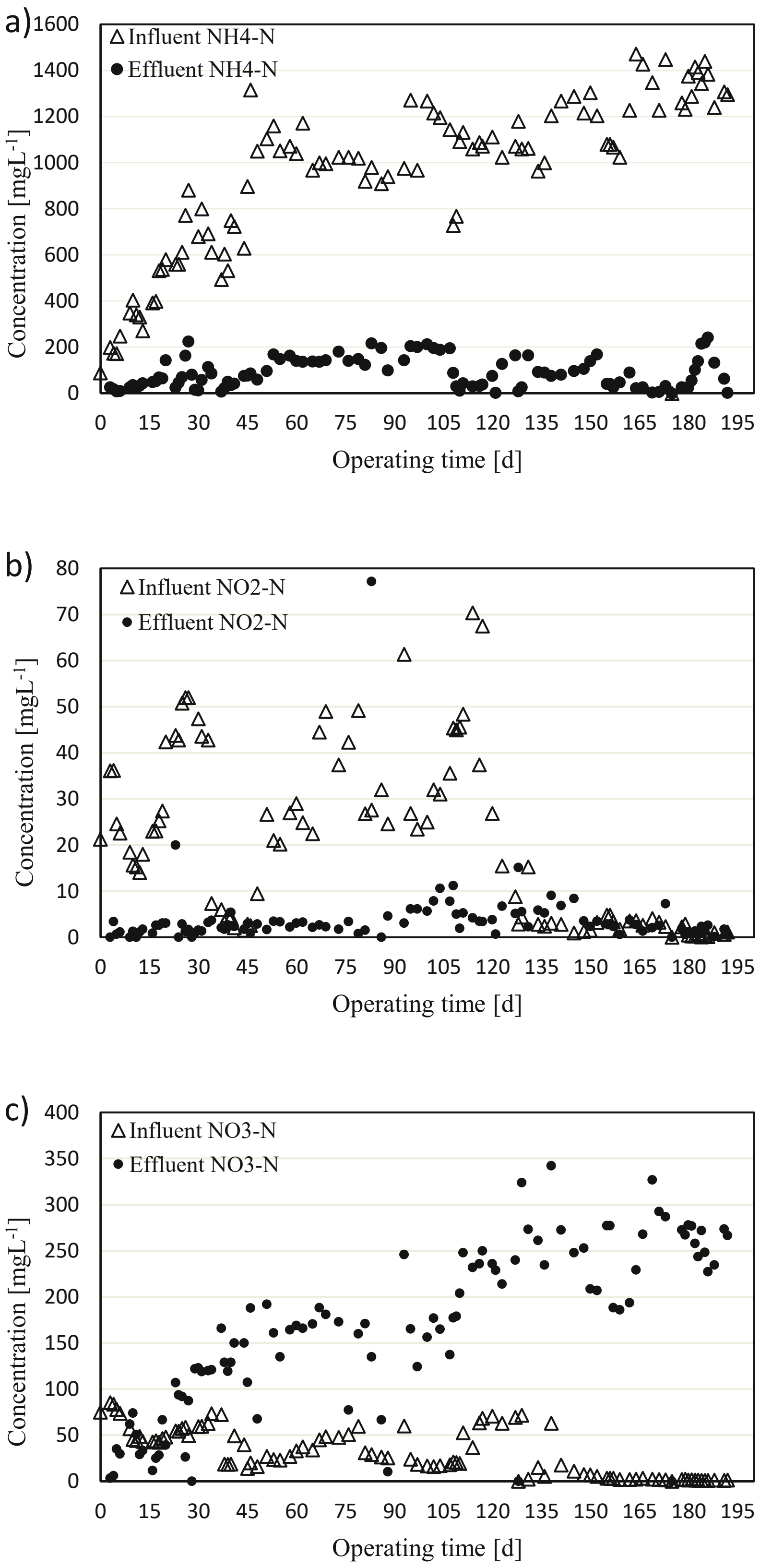


Figure 15.A visual presentation of the concentrations of influent and effluent N-compounds (NH$_4$-N, NO$_3$-N and NO$_2$-N) in 1-CS.

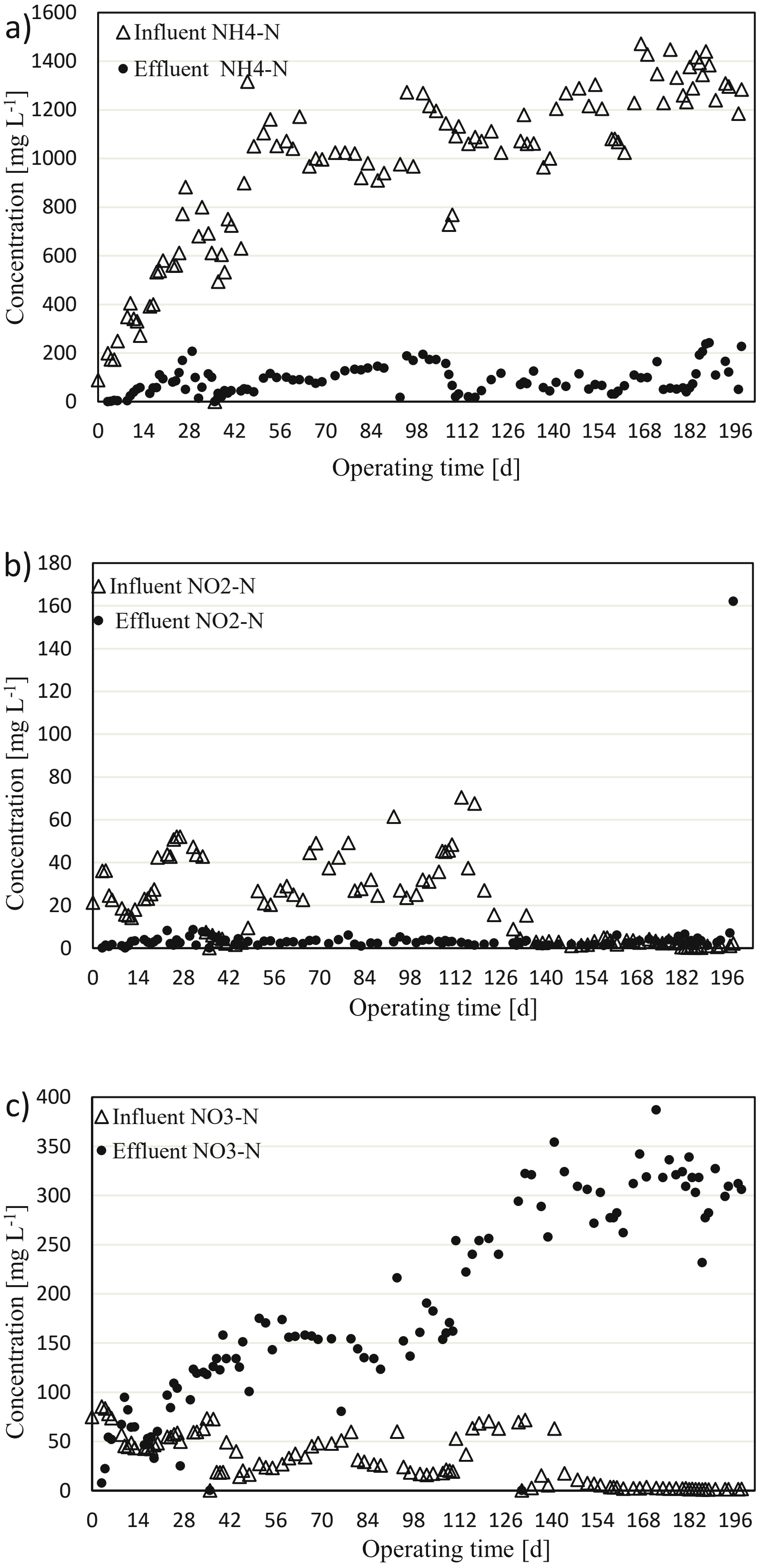


Figure 16. A visual presentation of the concentrations of influent and effluent N-compounds (NH_4-N, NO_3-N and NO_2-N) in 2-CS.

The two pilot plants did not have the exact same configuration. To the 2-CS a mesh was added between the nitritation and anammox side. The null hypothesis assumed that there is no significant difference between the two semi-scale reactors in the matter of N removal efficiency.

The variable "N-removal efficiency" in each group was normally distributed (Shapiro-Wilk's test, 1-CS: p=0.164, n=7; 2-CS: p=0.244, n =7). The skewness and kurtosis for 1-CS was 1.479 (SE=0.794) and 2.474 (SE= 1.587) respectively, while for 2-CS was -0.597 (SE=0.794) and -1.262 (SE=1.587).

There was no significant difference between the monthly means of N-removal efficiency of each bioreactor (1-CS: 74.2±3.6 vs 2-CS: 72.5±4.7, t= 0.736, p=0.476, two-sided). The effect size showed a moderate magnitude of the effect (Cohen d= 0.393). Equal variances were assumed upon results of Levene's test (F= 1.736, p= 0.212).

The 1-CS and 2-CS showed comparable behavior regarding N removal on monthly basis. The mesh addition did not prompt any significant change on the performance of the system. NO_3-N increase in the effluent followed the same pattern too.

5.1.2 Total Organic Carbon (TOC)

The negative effect of organic carbon on PN/A is mentioned by various authors. According to Lackner, Terada, and Smets (2008) the presence of organic carbon can cause disruption on the PN/A process efficiency by suppressing anammox bacteria activity or promoting the growth of heterotroph bacteria (HB) which can use the COD for denitrification and compete with anammox bacteria for nitrite and space. (Fu et al., 2021) revealed that when the influent TOC concentration was up to 200 mg L^{-1}, the activity of anammox bacteria was seriously inhibited, as an increase of organic matter favors the denitrification pathway. As a consequence heterotrophic denitrifiers outcompete the slow-growing anammox bacteria (Val del Rio et al., 2018). However, there is no consensus about the impact of organic carbon on the balance between anammox and denitrification (De Cocker, 2018).

In the present study, the average amount of TOC in the influent wastewater was 194.80 ± 81.54 mg L^{-1}.

Since the TOC was not determined on regular basis, there is a sparsity in the available data from this study. Therefore, it was not possible to assess the effect of TOC on the PN/A process.

Nevertheless, during the operation there was no indication that TOC impaired the PN/A process (Figure 17).

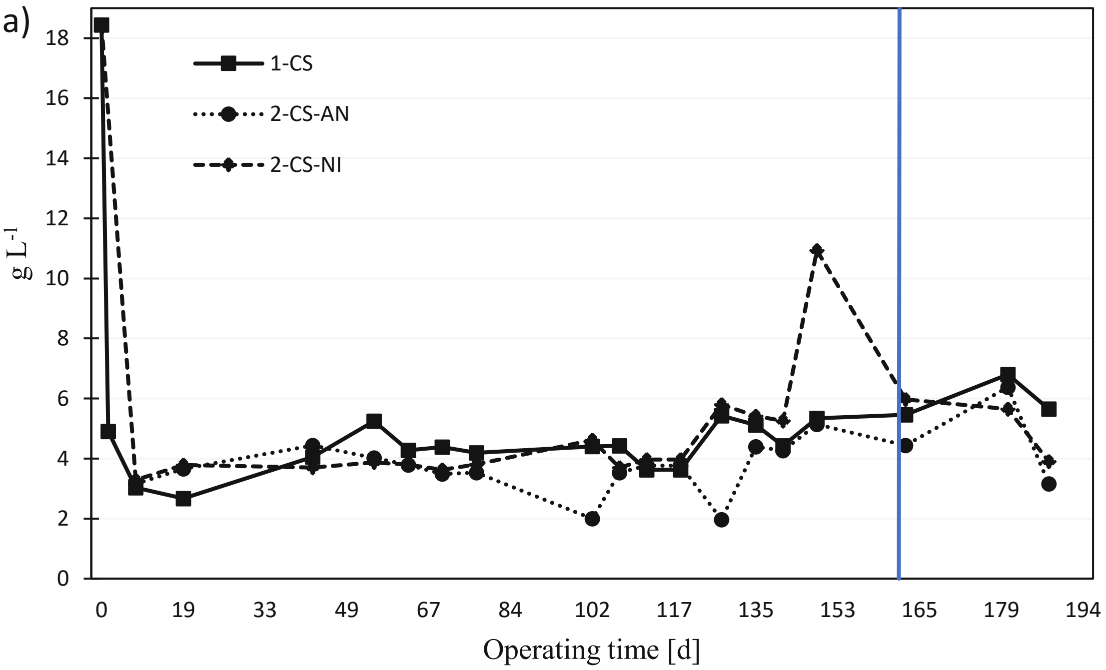
Figure 17. Influent total organic carbon (TOC; mg L^{-1}) vs. nitrogen removal rate (NRR; %).

5.1.3 Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS)

Wastewater may contain large quantities of suspended solids. TSS analysis is a standard in the wastewater industry. Concentration of TSS and VSS was determined via gravimetric analysis described in more detail in sector 4.4.1. An appropriate biomass retention is highly important due to the slow growth rate of the anammox bacteria (Fernández et al., 2008). Applying the mesh in the two-compartment bioreactor should assist in the biomass retention.

In both semi-scale bioreactors, the value of VSS was in the range between 74-89% of TSS. The concentration of suspended solids decreased quickly over the first operation days. Despite a peak of TSS in the two-compartment bioreactor-nitration side (2-CS-NI), there was no significant accumulation of TSS observed in each of the semi-scale bioreactors during the operating time. The peak at t=149[d] in the 2-CS-NI and the sudden increase of TSS may be due to a sampling error and should be interpreted with caution. This doubt is supported by the fact that there is no such increase in biomass in the two-compartment bioreactor-anammox side (2-CS-AN). At day 161 the two-compartment bioreactor was switched from SBR mode to continuous mode (the vertical blue line) (Figure 18). Nevertheless, there was no significant loss of biomass observed in 2-CS-AN as expected.

Due to a technical problem, some biomass from the 2-CS landed in the effluent, most of it was recovered. This could also explain differences observed in the particle measurement, described in more detail in subsection 5.3. Furthermore, it could have impacted the sludge age, as the sludge age during the month of November in 2-CS continuous and 1-CS SBR was 26 and 42 days respectively. However, TSS values generally showed high variability.



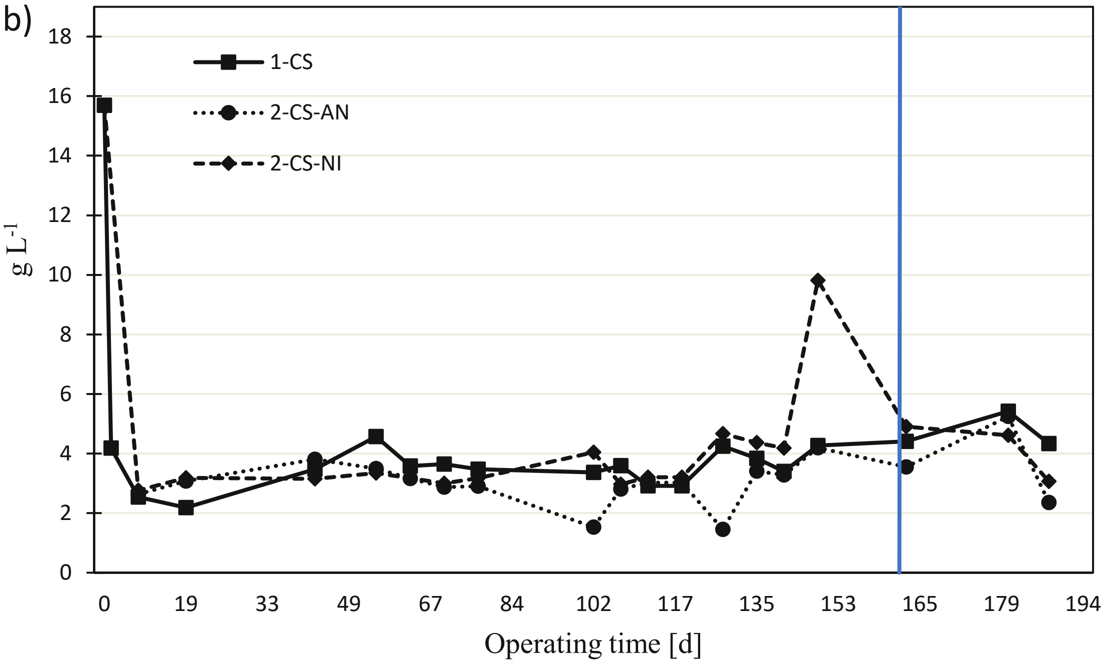


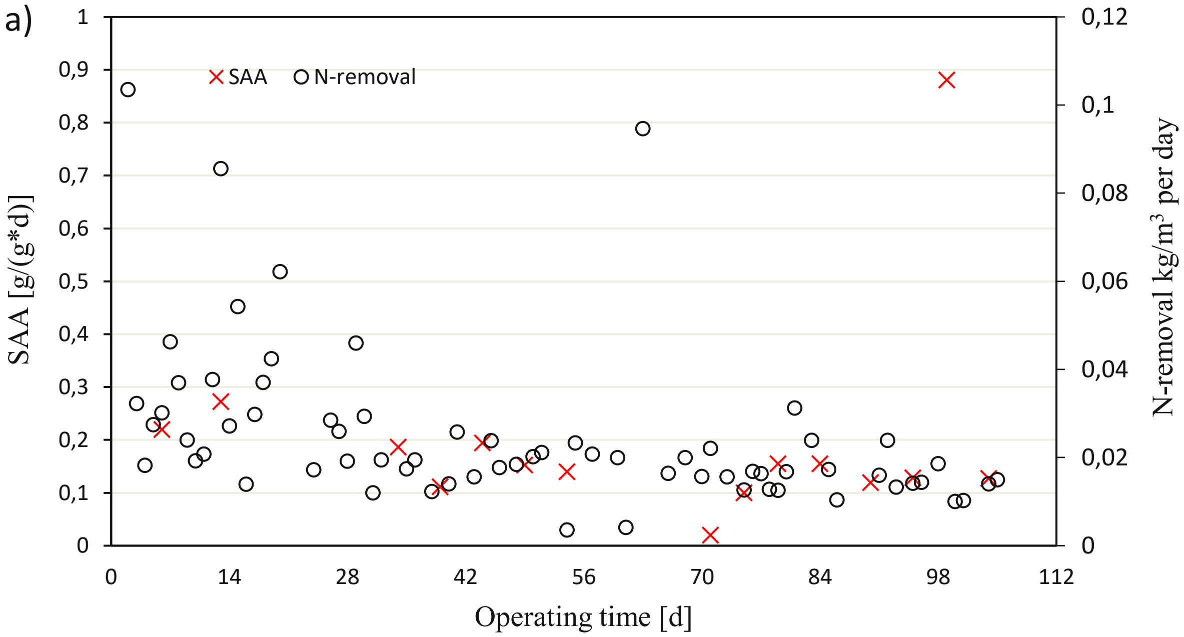
Figure 18. Total (TSS) and volatile (VSS) suspended solids concentrations of samples collected in the bioreactor, one-compartment system (1-CS; ■-solid line), two-compartment system-anammox (2-CS-AN; ●-dotted line), two-compartment system-nitrification (2-CS-NI; ◆-dashed line). a) TSS [g L^{-1}] and b) VSS [g L^{-1}]. The blue vertical line denotes when only the 2-CS was switched to continuous mode, the 1-CS remained in SBR mode.

5.2 Specific Anammox Activity (SAA)

For the determination of SAA samples were collected directly from the bioreactors. There are many factors known to influence the anammox activity (See Table 6 and Table 6). During a study run by Malovanyy, Plaza, and Trela (2009) it was shown that that activity of anammox bacteria increases proportionally with temperature in the range 15-37.5 °C. The maximum specific anammox activity (0.0286±0.0003g N_2–N g VSS^{-1} d^{-1}) was observed at temperature of 43°C and at pH 7.5 (Daverey et al., 2015). However, maintaining higher temperatures is associated with higher energy requirements. In the semi-scale pilot plants, the temperature was set at 35°C and the pH was in range of 6.94 to 7.57. During the period that SAA was measured, the average value in 1-CS was 0,197± 0,197 [g N_2–N g VSS^{-1} d^{-1}] and the average N removed in kg/m^3 aerated/d was 0,025±0,018. In 2-CS the SAA values in anammox side and Nitritation side were 0,199±0,179 and 0,205±0,145 respectively, and the corresponding average N removal 0,026±0,013. The SAA measurement as a monitoring tool for anammox biomass fitness proved to be redundant due to high time-consuming (~6h) and was not bringing new information that was already gained by other methods. As seen in Figure 19, the nitrogen removal efficiency corresponded to the anammox activity. Since the SAA is measuring concretely the performance of anammox bacteria, this indicated that the limiting factor were AOBs. These should be further promoted.

It is reported that supplemental addition of biotin concentration in range of 0.1–1.5 mg L^{-1} promoted the anammox activity, whereas at a biotin level of 1.0 mg L^{-1}, the total nitrogen removal rate increased up to 112% (Q. Li et al., 2021). Furthermore, an improved heme production and an increase in anammox biomass were detected.

Nevertheless, an interesting experiment would be to investigate how the system(s) react with biotin addition during the alkalinity limitation, explained in more detail in subsection 5.4.2. Would the enhancement of anammox activity and biomass result in lessening the susceptibility of the two semi-scale bioreactors? If yes, how strong would this effect be? But more importantly, would the possibly positive results justify the costs for biotin addition at a larger scale?



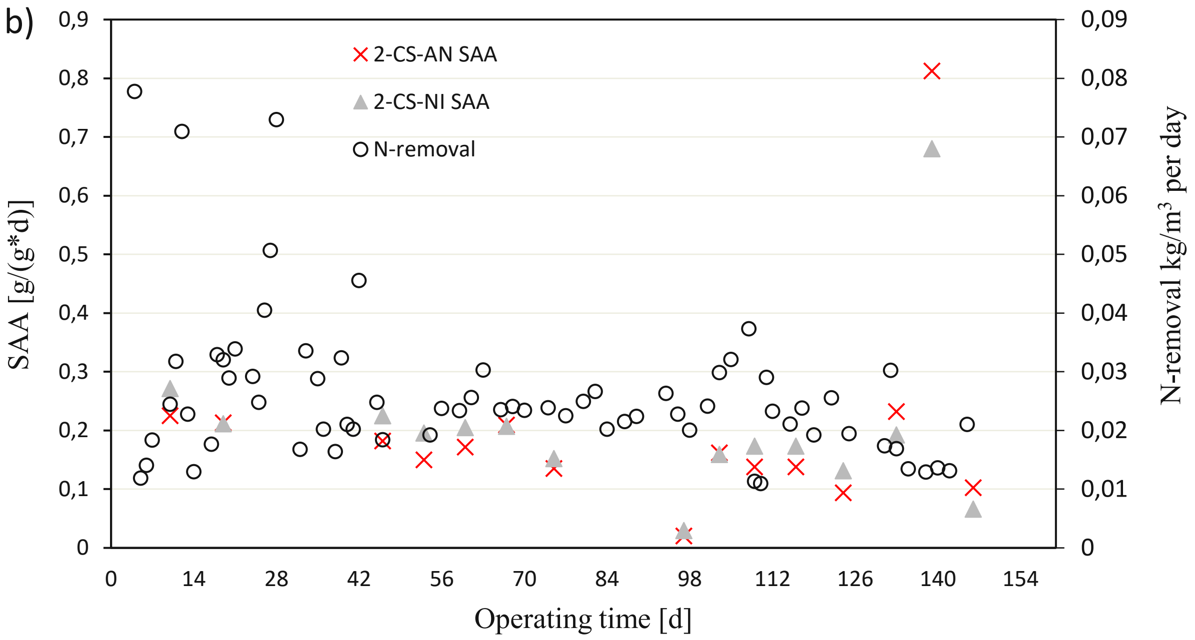


Figure 19. Observation of the nitrogen removal, in kg per m^3 aeration per day, measured spectrophotometrically, in comparison with the specific anammox activity (SAA) [g N2–N /g VSS d]: a) 1-CS and b) 2-CS.

5.3 Particle Size Evaluation

Particle size distribution of anammox bacteria plays an important role in the nitrogen removal efficiency (E. i. p. Volcke et al., 2012), therefore obtaining an insight of the particle size present in bioreactor can potentially facilitate the process of monitoring, based on visual characteristics of anammox bacteria.

By naked eye it was noticeable that the floccular sludge and loose granules remained suspended at the upper part, whereas the dense red anammox granules settled at the bottom of the bioreactor. Wang et al. (2020) reported that anammox granules were composed out of numerous micro-granules, that were capable to aggregate into larger granules, given they are not exposed to external disturbances.

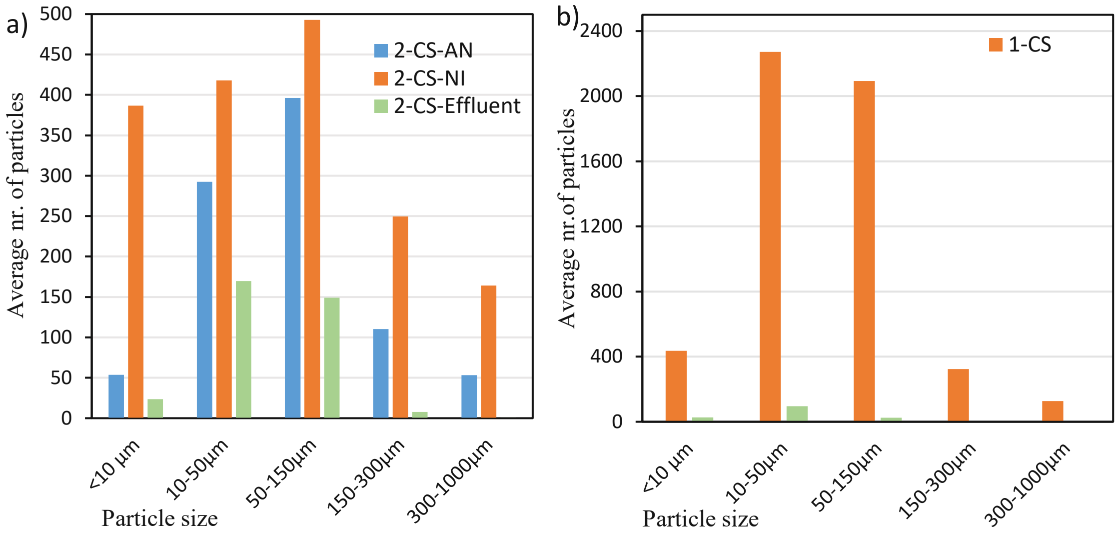


Figure 20. Average number of counted particles and their size[µm] in the two semi-scale pilot plants: a) the two-compartment bioreactor;2-CS-AN (anaerobic compartment), 2-CS-NI (aerobic compartment) and 2-CS-Effluent. b) the one-compartment bioreactor; 1-CS and 1-CS-Effluent.

Attempting to take the most homogenous sample may have caused hydraulic stress to the anammox granulation. As a result, the bigger granules may have disintegrated into smaller ones. Another aspect to be considered in the contribution concerning disintegration is the inactivity during storage time.

There is no obvious consensus about the optimal size of granules or particles, as different working conditions and specifications have led to a variation in results regarding this matter.

Results obtained from Chen et al. (2021) showed that particle size was positively correlated with the settling velocity, furthermore the N removal efficiency and SAA increased with particle size. The particle size was measured only once, and this fact does not allow an inference about any increase or change in the SAA values during the PN/A process.

Chen et al. (2020) revealed that larger granules harbor higher microbial diversity and support more diverse functions than smaller granules.

According to the data obtained from measurements, particles with a size of 50-150µm had the highest abundance (33.96 %), seconded by the particles of size 10-50µm (27.15%) in the two-compartment system. In the one-compartment system the highest abundance was kept by particles with a size of 10-50µm (43.27%) and 50-150µm (39.85%).

The mesh installed in the 2-CS did create a separation of particles (Figure 20). Interestingly larger particles were higher in number in the aerobic compartment, not as expected in the anaerobic compartment without direct aeration. The 2-CS had more particles present in the effluent, especially in the range of 10 to 150µm, indicating a selection of larger particles.

The 1-CS retained in general a higher number of particles in the reactor, according to the measurement, than the 2-CS. The sampling has taken place roughly a month after the 2-CS was shifted to continuous mode.

5.4 The influence of alkalinity in wastewater treatment

5.4.1 NH_4-N/alkalinity ratio impact on nitrogen removal efficiency

Taken that usually there is enough alkalinity present in reject water, mostly due to bicarbonate, carbonate and hydroxide ions, the establishment of an effective PN/A process would be rather feasible. Even though the demand for alkalinity in wastewater was met stoichiometrically, the performance of the system was yet not stable. The system viability increased when an additional amount of $NaHCO_3$ was added to the influent wastewater. The system performance gained better stability under these conditions.

The supplemental feed of alkalinity supported the maintenance of a relatively fair nitrogen removal efficiency of 73%±6.5 in 1-CS and 69%±6.8 in 2-CS respectively. An actual increase in the nitrogen removal efficiency after addition of alkalinity was reported by (Klein et al., 2012).

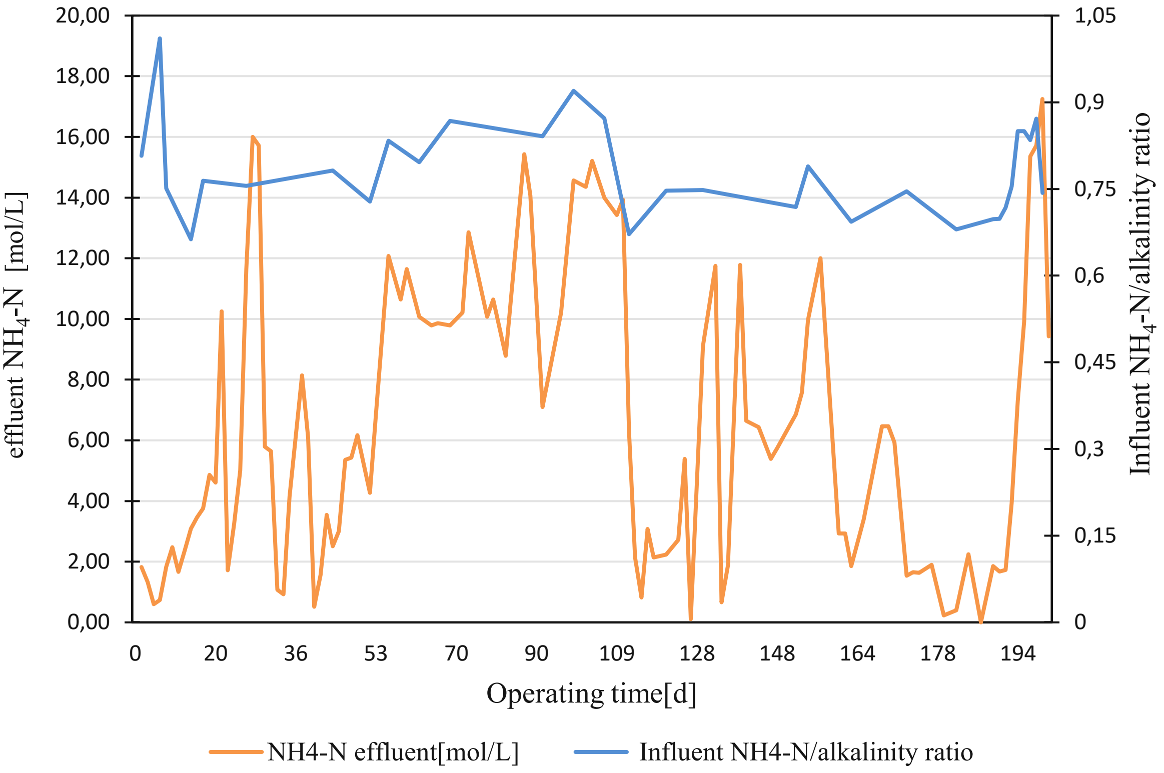


Figure 21. The ratio of influent NH_4-N [mol L^{-1}] and alkalinity [mM L^{-1}] versus the effluent NH_4-N [mol L^{-1}] in 2-CS.

The 2-CS at t=163 days was shifted to continuous mode, while the 1-CS remained all the time in SBR mode. As it is known, the oxidation of ammonia to dinitrogen through partial nitritation and anaerobic ammonium oxidation in a single-stage bioreactor is based on suppressing the nitritation process. Therefore, the activity of NOB has to be inhibited without affecting the activities of AOB and anammox bacteria. Moreover, by assisting and regulating bicarbonate alkalinity, the process of elimination of NOB can be further fine-tuned (Bagchi et al., 2010b).

The ratio NH_4-N/alkalinity decreased in both operating pilot plants. The average ratio of NH_4-N/alkalinity during the entire operating time in the 1-CS was 0.78±0.08, whereas in the 2-CS 0.73±0.08 (Figure 21). However, in both pilot plants the average ratio of NH_4-N/alkalinity before addition of $NaHCO_3$ was 0.81±0.09 and after addition of $NaHCO_3$ was 0.75±0.06.

There was an increase of VSS [g L^{-1}] after the addition of $NaHCO_3$ in the 1-CS by 13%, while in the 2-CS-AN and 2-CS-NI by 11% and 31% respectively (Table 12). The increase of biomass on the nitrification side is also depicted in subsection 5.3 during the particle count and size determination.

Table 12. Volatile Suspended Solids (VSS) [g L^{-1}] in both semi-scale pilot plants before and after the addition of $NaHCO_3$.

Reactor configuration	VSS [g L^{-1}] before addition of $NaHCO_3$	VSS [g L^{-1}] after addition of $NaHCO_3$
1-CS	3.46±0.69	3.97±0.80
2-CS-AN	2.92±0.64	3.28±1.07
2-CS-NI	3.20±0.36	4.67±2.05

According to these data, it can be estimated that a value around 0.75 of the ratio NH_4^+-N/alkalinity offered the system a stable nitrogen removal efficiency. In matters of the overall stability of the pilot plant, it is important to consider the technical problems that occurred during the pilot plant operation starting from power failure to the effluent pipe blockage due to biomass residue.

Table 13. Operating characteristics of the two semi-scale pilot plants, 1-CS in SBR mode and 2-CS in continuous mode during the month of November.

Parameter	Reactor	Average	Median	Minimum	Maximum
Volumetric loading rate [N/m^3/d]	1-CS SBR	0.54	0.57	0.17	0.92
	2-CS continuous	0.55	0.54	0.41	0.76
Aeration [L/h]	1-CS SBR	459	475	98	631
	2-CS continuous	374	361	208	501
Hydraulic loading [L/h]	1-CS SBR	6.7	7.0	1.9	10.0
	2-CS continuous	7.1	7.0	4.0	8.9
Nitrogen removal efficiency [%]	1-CS SBR	76	76	69	83
	2-CS continuous	69	70	59	73

Possible causes that lead to problems when implementing a PN/A process with reject wastewater could possibly be traced back to the dewatering process, more precisely to the sludge conditioning. The low presence of alkalinity or the loss of alkalinity in wastewater treatment plants could also be due to stripping. The pH fluctuations may lead to increased CO_2 stripping, for example due to the addition of iron chloride during sludge treatment.

5.4.2 The impact of alkalinity limitation

The investigation was conducted with the both semi-pilot scale reactors and the 3 L lab-scale reactor. The 3 L lab-scale reactor was inoculated with sample from the bigger system and the same control strategy and set points where used. A short summary of the parameters of the two semi-scale pilot plants before the investigation took place can be found in Table 13. The systems were analyzed regarding their robustness to the alkalinity limitation. The alkalinity in the system was reduced stepwise.

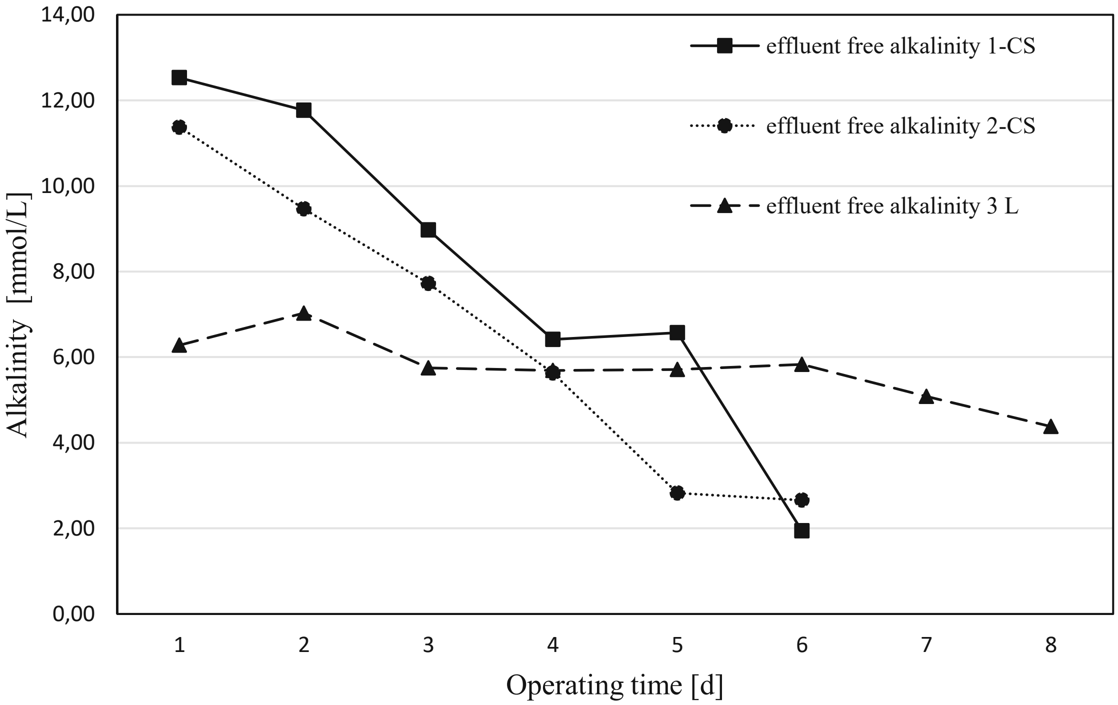


Figure 22. Free alkalinity [mmol/L] in the effluent of the treated WW during the alkalinity limitation experiment:1-CS; ■-solid line, 2-CS; ●-dotted line and 3L reactor; ▲-dashed line

There was a steep decrease of the free alkalinity present in the effluent of both semi-scale pilot plants, but not in the case of the 3 L reactor (Figure 22). Free alkalinity is given by the amount of acid consumed until pH 8, using phenolphthalein as an indicator. The free alkalinity was reduced much faster in 1-CS and 2-CS than in the 3 L reactor.

On day 3 of the experiment, the free alkalinity in 1-CS and 2-CS was reduced by 48.8% and 50.5% respectively, while in the 3L reactor only by 9.4%.

Anammox bacteria are susceptible to changes in pH, hence a low buffering system does not provide optimal conditions for biomass growth. According to Brito (2015), the PN/A reactor with simultaneous partial nitritation and anammox process was shown to be sensitive to small variations of pH which lead to losses on ammonia removal efficiency occurred when pH values dropped lower than 6.71 ± 0.01.

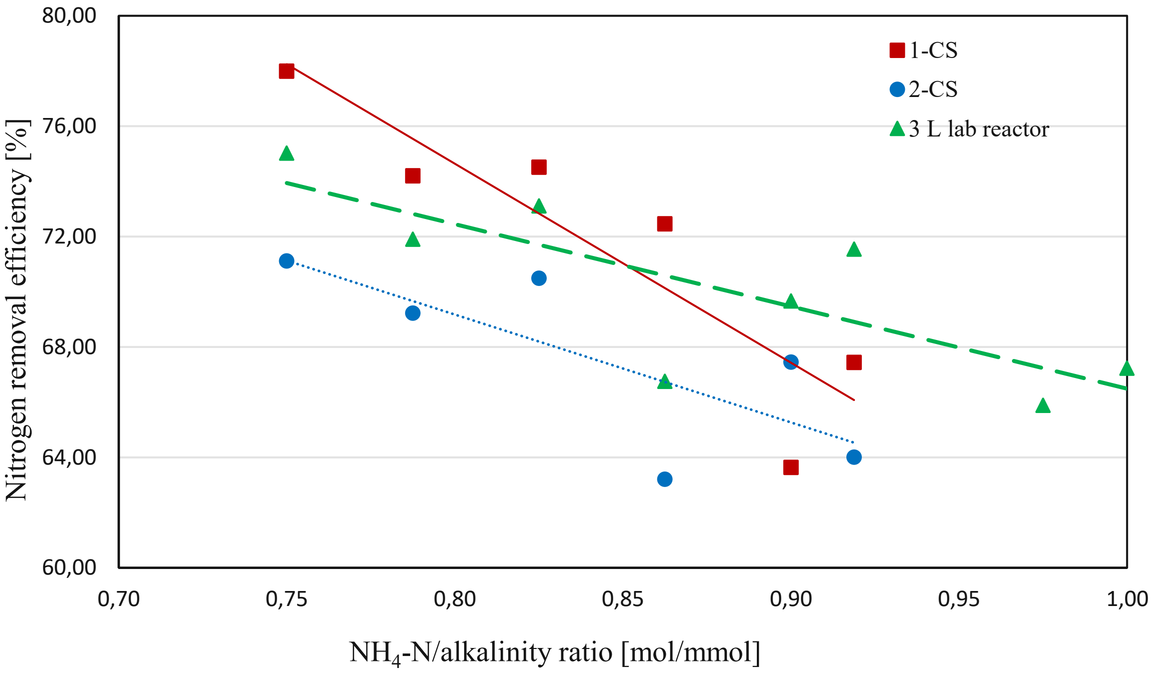


Figure 23. NH_4-N/alkalinity ratio [mol/mmol] in regard to the nitrogen removal efficiency; ■-1-CS SBR; ● 2-CS continuous;▲ SBR 3 L lab scale reactor.

The results showed that the one-compartment pilot plant was more prone to suffer from alkalinity limitation than the two-compartment pilot plant and the 3 L lab scale reactor was the least susceptible. The sensitivity to alkalinity limitation was retrieved by the steepness of the slope (Figure 23 and Table 14)

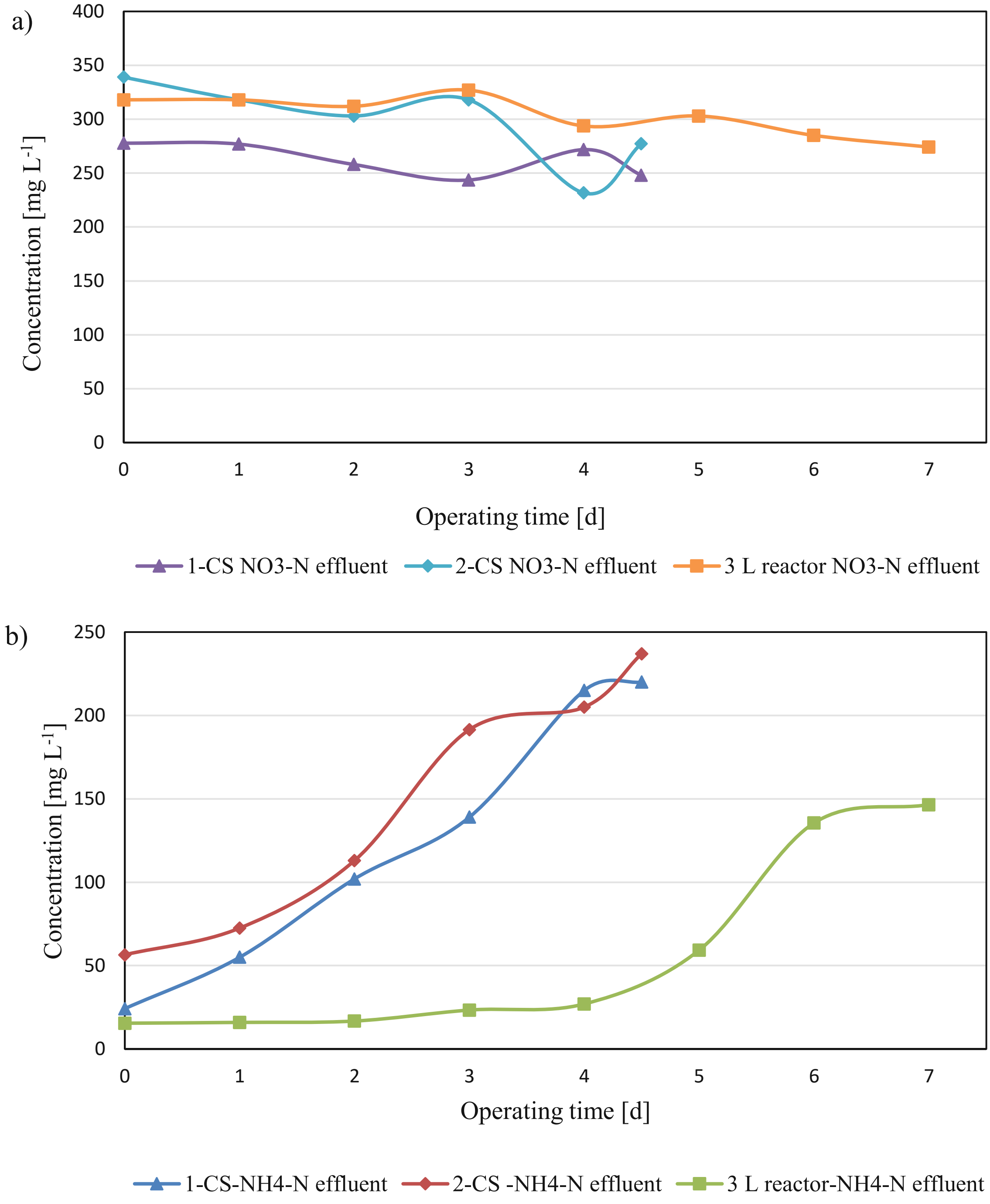


Figure 24. a) NH_4-N and b) NO_3-N concentration [mg L^{-1}] in the effluent wastewater during the alkalinity experiment.

As seen in Figure 24, NO_3-N in all three systems followed the same pattern, while this was not the case with NH_4-N. The effluent NH_4-N in the small scale required longer time to increase.

Table 14. Characteristics of the 1-CS SBR, 2-CS continuous and SBR 3 L lab reactor during the alkalinity limitation investigation.

Parameter	1-CS SBR	2-CS continuous	3L SBR
Sensitivity to alkalinity limitation	72	39	29
Average N removal pro m^3 aeration [kg/m^3]	0.0125	0.0180	0.0024
Average pH	7.4292	7.3848	7.4330
Average volumetric loading rate [$N/m^3/d$]	0.65	0.61	0.58

The semi-scale reactors were 1.5 m deep, an attempt to avoid or at least reduce any possible alkalinity loss through stripping of CO_2. Due to the small size, the 3 L lab reactor should have been more inclined to lose alkalinity due to stripping effects. Measurements of CO_2 emissions may elucidate this.

The 3L SBR reactor showed resilience regarding alkalinity limitation in comparison to the two semi-scale pilot plants. The NOB suppression was managed better? Nevertheless, the reasons why particularly the small-scale reactor stood out should be subject of further investigation.

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6. Interpretation

The importance of a sufficient amount of alkalinity in PN/A process to achieve satisfactory levels of nitrogen removal is mentioned before (Klein et al., 2012), showing that an increase in influent alkalinity improved the nitrogen removal efficiency.

The incremental accumulation of nitrates in effluent wastewater due to extensive aeration may cause also more nitrous oxide production and release of emissions in the atmosphere. High nitrates in effluent wastewater were correlated with nitrous oxide production reported by Sambola (2012).

It is interesting that all three systems had increased values of nitrates in the effluent wastewater, yet the 3 L lab scale outperformed the semi-scale pilot plants during the alkalinity investigation. Furthermore, the N-compound determination in the bioreactor would have given an insight into the NH4-N/NO2-N ratio, this way the functionality of AOB in regard to being a limiting factor or not would have been clearer.

During the particle measurement there were less particles measured in the 2-CS system, this could be due to the biomass loss a month prior to when the actual particle measurement took place, but also continuous operation with the mesh leads to a particle selection, as smaller particles are removed from the system. The N removal efficiency in the month November for 1-CS and 2-CS was 75.7% and 69%, respectively. Based on the results of other months it is very likely that the N removal efficiency of the 2-CS would have been higher if it was not for a part of the biomass landing in the effluent wastewater. Furthermore, there was a modest decrease in the N removal efficiency in the month of December and this came as a result of the alkalinity limitation experiment. Nevertheless, statistically there was no significant difference in N removal efficiency between the 1-CS and 2-CS.

Furthermore, systems using physical separator in the form of a screen to out-select NOBs has been implemented for mainstream sewage treatment (Han et al., 2016). Since the 2-CS in continuous mode is only in one side of the bioreactor aerated, and according to Yang et al.,(2016) over 90% of nitrous oxide emissions occurred in the aerated period and less than 8% in the non-aerated period in the full-scale, it enfolds the potential for larger scale application with less nitrous oxide emissions.

7. Conclusion and Outlook

The partial nitritation and anammox process was successfully started in the two-semi-scale pilot plants. At the beginning it was not stable enough as variations in the influent alkalinity may have presented difficulties in the start-up of the PN/A process treating municipal reject water. Due to the supplemental addition of $NaHCO_3$ the stability of the two semi-scale pilot plants improved. The nitrite concentration in effluent was low which indicated a well-functioning PN/A process. On the other hand, there was an accumulation of nitrates in the effluent probably due to the excessive aeration. In the two semi-scale pilot plants the average ratio of NH_4-N/alkalinity before addition of $NaHCO_3$ was 0.81±0.09 and after addition of $NaHCO_3$ decreased to 0.75±0.06.

Scaling up or down remains a difficult task to tackle while keeping the parameters constant for all different scaled systems. These findings do suggest a scale dependency in the influent NH_4-N/alkalinity ratio between the semi-scale pilot plants and the lab scale bioreactor. The 3 L lab bioreactor was the least susceptible to the alkalinity during the stepwise reduction of the influent alkalinity in WW. Moreover, the small-scale reactor was comparable to the two semi-scale pilot plants in regard to the N removal efficiency, by reaching on average 0.0024 kg N removal pro m^3 aeration. Minor modifications set up in the configuration of the WWTP influenced the outcome variables. The 2-CS had an integrated mesh to do the separation of the nitritation side and anammox side. However, this modification did not lead to enhanced N removal efficiency.

The statistical results, retrieved by conducting an independent samples t-test did not deliver any significant difference in N removal efficiency between 1-CS and 2-CS. Additionally the mesh in the 2-CS did help retain larger particles. The 2-CS had 33.9% particles in range of 50-150µm and 27.15% of 10-50µm while 1-CS had it inversely, particles of size 50-150µm were 39.85% and particles of size 10-50µm were 43.27%. Nevertheless, there was a higher number of particles measured in 1-CS than 2-CS. SAA measured values, along the operating time, corresponded to the N removal efficiency, therefore it became dispensable as a biomass monitoring parameter.

The sludge conditioning deserves more attention and further investigation on the reject WW coming from it. There is a possibility that the reject WW might be disturbed with substances which subsequently might be responsible for the impairing interference during the start-up. The improvement of the algorithm for intermittent aeration, precisely fine-tuning in response the pH and DO set points would assist the promotion of the biology involved and augment the performance of the system.

8. Summary

The general increase of population and its tendency to concentrate disproportionately high in urban settings have risen challenges in treating the municipal discharged water. The way of living has changed rapidly and become more modern. This has subsequently brought alterations in consumption and waste. The load of pollutants like nitrogen and phosphorus in discharged waters has intensified, that WW without prior treatment cannot be released into the natural water bodies, without the risk of causing eutrophication. Various strategies can be implemented to treat the WW rich in ammonia. The conventional approaches demand more resources, for that reason the biological method of treating WW is seen as more convenient in terms of costs and sustainability. The employment of anammox bacteria is an alternative option that has been applied during the last two decades to assist in N removal from WW such as municipal reject water.

The objective of this study was to investigate the influence of scale and configuration on partial nitritation and anammox process while the alkalinity is decreased gradually. The nitrogen removal efficiency was assessed, while the NH_4-N/ alkalinity ratio increased.

In order to evaluate the scale dependency, two semi-scale bioreactor and a 3L lab scale bioreactor were used to carry out the experiment. Furthermore, with the aim to examine if minor changes in configuration will make a difference on the overall performance of the PN/A process, a mesh was integrated into one of the two semi-scale bioreactors, creating two compartments. The aeration took place only in one side of the bioreactor, in the nitritation part. To evaluate the effect the mesh had in the biomass retention, a particle measurement was conducted. Moreover, specific anammox activity was determined to characterize the fitness of biomass.

The two semi-scale pilot plants were successfully started. The stability improved after supplemental feed of bicarbonate alkalinity. Before the $NaHCO_3$ feed, the average NH_4-N/alkalinity ratio was 0.81±0.09, while after addition 0.75±0.06. VSS comprised in the range between 74-89% of TSS. After additional alkalinity was brought into the reactors the average VSS [g L^{-1}] in 1-CS was 3.97±0.80, whereas in the 2-CS-AN and 2-CS-NI was 3.28±1.07 and 4.67±2.05 respectively. Nitrogen compounds in the effluent indicated in a successfully run PN/A. Nitrites maintained a low value in the effluent wastewater. The average concentration of nitrites in 1-CS and 2-CS was 4.0±7.9 mg L^{-1} and 6.1±23.3 mg L^{-1}, respectively. The incremental accumulation of nitrates was present in both systems, also in the 3L reactor, during the alkalinity limitation experiment. The average SAA value in 1-CS was 0,197± 0,197 [g N_2–N g VSS^{-1} d^{-1}] and in 2-CS in anammox side and nitritation side were 0,199±0,179 and 0,205±0,145 respectively. Particle measurement evaluation revealed that there were larger anammox granules in the 2-CS than 1-CS. Whereas, the 1-CS NI side contained more particles measured than 1-CS AN as expected. The average nitrogen removal efficiency after alkalinity addition in 1-CS and 2-CS was 73%±6.5 and 69%±6.8 respectively. The 3L lab scale bioreactor showed the most resilience regarding alkalinity limitation. Even though in all three systems the effluent nitrates followed the same pattern. The reason (s) why the small-scale scale demonstrated robustness more than the two semi-scale bioreactors should be subject of further studies.

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10. List of abbreviations

1-CS: one-compartment system

2-CS: two-compartment system

2-CS-AN: two-compartment system Anammox side

2-CS-NI: two-compartment system Nitritation side

Anammox: Anaerobic Ammonium Oxidation

AOB: ammonia oxidizing bacteria

CANON: completely autographic nitrogen removal over nitrite

CH_4: methane

COD: Chemical Oxygen Demand

d: day

DO: Dissolved Oxygen

FA: Free ammonia

FNA: Free nitrous acid

GHG: Greenhouse Gas emissions

IC_{50}: half maximal Inhibitory Concentration

MBBR: Moving Bed Biofilm Reactor

N: Nitrogen

N_2H_4: hydrazine

N_2O: nitrous oxide

NH_4-N: Ammonium Nitrogen

NO: nitric oxide

NO_2-N: Nitrite Nitrogen

NO_3-N: Nitrate Nitrogen

NOB: nitrite oxidizing bacteria

OM: organic matter

PN/A: Partial nitritation/Anammox

SAA: Specific Anammox Activity

SBR: sequencing batch reactor

SHARON: single reactor system for high ammonia removal over nitrite

SNAP: Single-stage Nitrogen removal using the Anammox and Partial Nitritation

SRT: sludge retention time

TOC: Total Organic Carbon

TSS: Total Suspended Solids

UASB: Upflow Anaerobic Sludge Blanket

VSS: Volatile Suspended Solids

WW: Wastewater

WWTP: Wastewater Treatment Plant

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