INHIBITORY IMPACT OF NITRITE ON THE ANAEROBIC AMMONIUM OXIDIZING (ANAMMOX) BACTERIA: INHIBITION MECHANISMS AND STRATEGIES TO IMPROVE THE RELIABILITY OF THE ANAMMOX PROCESS AS A N-REMOVAL TECHNOLOGY

by

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DEDICATION

A mi abuelo Arroyo
a mis padres y mi hermana

a Lila
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ABSTRACT

The anaerobic oxidation of ammonium (anammox) with nitrite as electron acceptor is a microbial process that generates nitrogen gas as main final product. After being discovered in the Netherlands in the 1990s, anammox has been applied in state-of-the-art biotechnologies for the removal of N pollution from ammonium rich wastewaters. The anammox process offers significant advantages over traditional nitrification-denitrification based processes. Since anammox does not need elemental oxygen, it allows for important savings in aeration. Furthermore, due to the autotrophic nature of the bacteria, anammox does not require external addition of electron donor, often needed in systems with post-denitrification. Although the anammox bacteria have high specific activity, they are slow growing, with doubling times that can range from 10 to 25 d. Therefore, in case of a toxic event causing the death of the biomass, a long recovery period will be required to reestablish full treatment capacity.

The purpose of this work is to investigate the inhibition of anammox bacteria by compounds commonly found in wastewaters, including substrates, intermediates and products of the anammox reaction. Among common wastewater constituents, sulfide was shown to be especially harmful, causing complete inhibition of anammox activity at concentrations as low as 11 mg H₂S L⁻¹. Dissolved oxygen was moderately toxic with a
50% inhibiting concentration of 2.3 and 3.8 mg L\(^{-1}\) to granular and suspended anammox cultures, respectively. Among the various compounds involved in the anammox reaction, special attention was paid to nitrite. Numerous literature reports have indicated inhibition of anammox bacteria by its terminal electron acceptor. However to date, there is no consensus explanation as to the mechanism of nitrite inhibition nor on how the inhibition is impacted by variations in the physiological status of anammox cells. The mechanisms of anammox inhibition by nitrite were thoroughly investigated in batch and continuous experiments of this dissertation. The results of this work demonstrate that conditions hindering generation of metabolic energy have a detrimental effect on the tolerance of anammox cells to toxic levels of nitrite. The absence of ammonium during events of nitrite exposure was shown to exacerbate its toxic effect. As a result of nitrite inhibition, nitric oxide, an intermediate of the anammox reaction, accumulated in the head space of the batch experiments. Moreover, nitrite inhibition was enhanced at the lowest range of pH tested (6.4-7.2), while same nitrite concentrations caused no inhibition under mildly alkaline conditions (7.5-7.8). Although other authors have relied on the classic concept that undissociated nitrous acid is the species responsible for the inhibition, the results in this work indicate that the pH affects the inhibitory effect of nitrite, irrespective of the free nitrous acid concentration.

Nitrite stress triggered an active response of the anammox bacteria, which temporarily increased their ATP content to mitigate the inhibition. Additionally,
starvation of anammox microorganisms, caused during storage or by sustained underloading of bioreactors, was found to limit the capacity of the bacteria to tolerate exposure to nitrite.

The results of this dissertation indicate that the tolerance of anammox bacteria to NO$_2^-$ inhibition relies on limiting its accumulation in sensitive regions of the cell. Active metabolism in presence of NH$_4^+$ allows for active consumption of NO$_2^-$, avoiding accumulation of toxic intracellular NO$_2^-$ concentrations. Furthermore, secondary active transport proteins may be used by anammox bacteria to translocate nitrite to non-sensitive compartments. Nitrite active transport relies on a proton motive force. Therefore, conditions such as low pH (below 7.4) or absence of energy sources, which may disturb the maintenance of the intracellular proton gradient, will increase the sensitivity of anammox cells to NO$_2^-$ inhibition. Strategies for the operation and control of anammox bioreactors must be designed to avoid exposure of the biomass to nitrite under the absence of ammonium, low pH or after periods of starvation.
CHAPTER 1

INTRODUCTION

1.1 Nitrogen pollution

Nitrogen (N) is a necessary element for all living beings. It is a constituent in DNA, proteins, chlorophyll, etc. In bacterial cells, N accounts for 12% of the dry weight (Metcalf et al., 2003). Although N is the most abundant element in the atmosphere, most of it is not available to living organisms since it is present as the inert N\(_2\) gas. Only a few specialized bacteria are able to fix N transforming it to reactive nitrogen (Nr) (Fig. 1.1). In pre-industrial times, N cycling resulted in a relatively constant global pool of readily available N. However, the global N cycle has been significantly altered, especially since 1960s, due to human activity (Galloway et al., 2003). In particular, the invention of the Haber Bosch process, a widely applied method that allows the chemical synthesis of ammonia from hydrogen (H\(_2\)) and N\(_2\) gas (Smil, 2001), has had an enormous impact on
the anthropogenic input of Nr. The amount of Nr fixed has increased from 15 Tg Nr yr⁻¹ in 1860, to 187 Tg Nr yr⁻¹ in 2005, when the Haber Bosch process generated 121 Tg Nr (Galloway et al., 2008). Although part of the anthropogenic N pollution is dispersed (from the application of fertilizers for agriculture, combustion of fossil fuels, etc.), a large share of the fixed N ends up in concentrated streams. Ammonium (NH₄⁺), generated by mineralization of organic matter, is an important contaminant in a variety of wastewaters, e.g. domestic wastewater, animal wastes (Driscoll et al., 2003), or landfill leachate (Berge et al., 2005). Some of this concentrated Nr is discharged in watersheds, i.e. wastewaters contribute 36-81% of the Nr load to all the estuaries in the north east of the US (Driscoll et al., 2003). The concentration of nitrate (NO₃⁻), from fertilizers and oxidation of NH₄⁺ pollution, in major rivers in Northeastern U.S. has increased by 3- to 10-fold since records are available (Vitousek et al., 1997). N-contamination of groundwater has also been observed in agricultural regions around the globe. Although the groundwater contamination problem is probably less significant than in surface waters, the long residence time of water in aquifers means that N contamination is likely to last for a long time (Howarth et al., 1996).

Since N is a limiting nutrient in uncontaminated water bodies, pollution of water bodies generates excessive growth of primary producers, also called eutrophication. When nutrients become limiting and water is too turbid to allow passage of sunlight, the algae die and degrade, leading to depletion of dissolved oxygen, bad odor and, ultimately,
death of fish and loss of biological diversity (Howarth, 1991). NH$_4^+$ is toxic to aquatic life (Randall, Tsui, 2002), and can be oxidized to nitrite (NO$_2^-$) and NO$_3^-$, consuming the dissolved oxygen (Karrman and Jonsson, 2001) and causing acidification (Schindler et al., 1985). The depletion of dissolved oxygen (DO) caused by N pollution has caused the generation of oxygen-minimum zones like in the Chesapeake Bay, the Baltic and Black Seas, and the northern Gulf of Mexico (Camargo, Alonso, 2006).

Moreover, NO$_2^-$ and NO$_3^-$ pose a threat for human health at concentrations higher than 10 mg·L$^{-1}$ (U.S. Public Health Service standard). NO$_3^-$ in drinking water is transformed to NO$_2^-$ in the stomach, which substitutes oxygen in hemoglobin, disturbing the transport of oxygen in the blood. This phenomenon is known as “methemoglobinemia” and it can cause death in infants (Lee, 1970).

1.2 Regulations

Given the serious threat that nitrogen pollution poses on environment and water resources legislative measures have been taken in different countries to minimize discharge of N polluted effluents to water bodies.
Table 1.1. Secondary treatment standards for municipal wastewater in the US.

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<th>7 day Average</th>
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<td>BOD₅</td>
<td>30 mg L⁻¹</td>
<td>45 mg L⁻¹</td>
</tr>
<tr>
<td>TSS</td>
<td>30 mg L⁻¹</td>
<td>45 mg L⁻¹</td>
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<tr>
<td>Removal efficiency</td>
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</tbody>
</table>

In the United States, there is not a federal law limiting nitrogen discharges to surface water. The Federal water pollution control act (1972), requires secondary treatment for municipal wastewaters (or treatment with comparable effluent quality), providing effluents which comply with the secondary treatment standards (Table 1.1). The Clean Water Act (1977) enables states and municipalities to regulate the discharge of polluted effluents to “waters of the United States”. The regulation of N polluted wastewaters is applied through the national permit discharge elimination system (NPDES). The NPDES targets point sources, including publicly owned treatment plants (public sewage treatment plants), industrial wastewaters, process and cooling water, as well as stormwater discharges, including both stormwater sewer systems and industrial sources. Due to the toxicity of \( \text{NO}_2^- \) and \( \text{NO}_3^- \) to humans, the EPA has set a maximum contaminant level of 10 mg N L⁻¹ (\( \text{NO}_2^- + \text{NO}_3^- \)) in drinking water (National Primary Drinking Water Regulations), with a maximum \( \text{NO}_2^- \) level of 1 mg N L⁻¹.
Table 1.2. Requirements for discharges from urban wastewater treatment plants to “sensitive areas” subject to eutrophication in the EU.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Concentration</th>
<th>Minimum percentage reduction of total influent load</th>
<th>Reference method measurement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total N</td>
<td>15 mg N L⁻¹ (10⁴-10⁵ p.e.)</td>
<td>80</td>
<td>Molecular absorption spectrophotometry</td>
</tr>
<tr>
<td></td>
<td>10 mg N L⁻¹ &gt;(10⁵ p.e.)</td>
<td>70-80</td>
<td></td>
</tr>
</tbody>
</table>


In countries of the European Union, regulation is provided by the Council Directive on Urban Waste-water Treatment (91/271/EEC). The law sets requirements for discharges of urban wastewaters and industrial wastewaters. It discriminates between discharge to “sensitive areas” which are subject to eutrophication (Table 1.2) and discharge to “less sensitive areas”. The directive requires secondary treatment (or equivalent) for all wastewater entering collection systems, although for wastewaters discharged in “less sensitive coastal areas”, primary treatment may be sufficient.

1.3 Current biotechnologies for nitrogen removal

Existing biotechnologies allow for nitrogen removal from wastewaters by taking advantage of N biotransformation reactions in the microbial nitrogen cycle (Fig. 1.1).
Figure 1.1. Microbial nitrogen cycle. *Most of the nitrogen in biomass is in the form of ammunes, nevertheless, due to contribution of nitrogen from nucleotides, the average oxidation state of nitrogen in biomass is higher than –III. Adapted from Colasanti, 2011. DNRA: Dissimilatory nitrate reduction to ammonium. Assimilative processes not included.

Even though nitrogen removal has been traditionally accomplished through the well-known process of nitrification-denitrification, in the last years novel technologies have been developed which are more cost-effective and allow for treatment of higher N loading rates than the traditional process. Both, traditional and novel technologies will be introduced in this chapter.
1.3.1 Conventional nitrification-denitrification processes

The nitrification-denitrification process is carried out in two metabolic steps, first \( \text{NH}_4^+ \) is oxidized to \( \text{NO}_3^- \) in a strictly aerobic process, and \( \text{NO}_3^- \) is then converted to \( \text{N}_2 \) gas. Nitrifying bacteria and archaea play a key role in the microbial cycling of fixed nitrogen, in seas, estuaries and soils (Mosier et al., 2008)

1.3.1.1 Nitrification

Nitrification is the chemolitoautotrophic aerobic oxidation to \( \text{NO}_3^- \) of nitrogenous inorganic compounds, such as \( \text{NH}_4^+ \), hydroxylamine (\( \text{NH}_2\text{OH} \)), and \( \text{NO}_2^- \) (Wong, 2003). Complete nitrification occurs in two stages. First, \( \text{NH}_4^+ \) is oxidized to \( \text{NO}_2^- \), and nitrite is then transformed into nitrate \( \text{NO}_3^- \). Each conversion is carried out by different genus of bacteria. Ammonia oxidation is carried out by \textit{Nitrosomonas}, \textit{Nitrosococcus}, \textit{Nitrosopira}, \textit{Nitrosovibrio} and \textit{Nitrosobolus} (Wong, 2003), also called ammonia oxidizing bacteria (AOB), with \( \text{NH}_2\text{OH} \) as reaction intermediate (Güven, Schmidt, 2009). The equation for ammonia oxidation by \textit{Nitrosomonas}, assuming a cell yield of 0.15 g cells/g \( \text{NH}_4^+ \)-N, is as follows:
\[ \text{NH}_4^+ + 1.3818 \text{O}_2 + 0.0909 \text{HCO}_3^- \rightarrow \]
\[ \rightarrow 0.0182 \text{C}_5\text{H}_7\text{NO}_2^- + 0.9818 \text{NO}_2^- + 1.0364 \text{H}_2\text{O} + 1.89 \text{H}_2\text{CO}_3 \]  

(1.1)

Nitrite oxidation to nitrate is carried out by a diverse group of alpha Proteobacteria called nitrite oxidizing bacteria (NOB), including *Nitrospira, Nitrospina, Nitrosococcus*, and *Nitrocystis*, however, the most recognized genus is *Nitrobacter* (Wong, 2003), whose nitrite metabolism for a cell yield of 0.02 g cells/g NO\textsubscript{2}\textsuperscript{−}-N follows the reaction:

\[ \text{NO}_2^- + 0.0025 \text{NH}_4^+ + 0.01 \text{HCO}_3^- + 0.01 \text{H}^+ + 0.4875 \text{O}_2 \rightarrow \]
\[ \rightarrow 0.0025 \text{C}_5\text{H}_7\text{NO}_2^- + 0.0075 \text{H}_2\text{O} + \text{NO}_3^- \]  

(1.2)

By addition of the two processes including cell synthesis, the overall equation is represented as follows:

\[ \text{NH}_4^+ + 1.83 \text{O}_2 + 1.98 \text{HCO}_3^- \rightarrow 0.021 \text{C}_5\text{H}_7\text{NO}_2^- + \]
\[ + 0.98 \text{NO}_3^- + 1.041 \text{H}_2\text{O} + 1.88 \text{H}_2\text{CO}_3 \]  

(1.3)

The process is pH dependent, with declining activity below pH 7.0, and consumes alkalinity, thus, extra CaCO\textsubscript{3} is commonly supplemented to avoid pH inhibition (Metcalf
et al., 2003). Due to the low growth rate, typical sludge retention time (SRT) in activated sludge systems is between 10-20 d (Metcalf et al., 2003).

1.3.1.2 Denitrification

Denitrification is the biological reduction of NO$_3^-$ and NO$_2^-$ to N$_2$ gas, generally carried out throughout a heterotrophic process under anoxic-anaerobic conditions (Wong, 2003). Although the complete reduction involves successive reductions with NO$_2^-$, nitric oxide (NO), and nitrous oxide (N$_2$O) as intermediates, it can be performed by a single kind of bacteria. Different gram-negative Proteobacteria are able to use NO$_3^-$ and NO$_2^-$ as electron acceptors and a wide range of organic compounds as electron donor and carbon source, to obtain energy, yielding N$_2$ gas as main product. Due to the metabolic diversity of denitrifying bacteria, they occupy very diverse niches. In the process, alkalinity is generated as shown in the following equation, assuming a cell yield of 0.45 g cells/g NO$_3^-$-N, with methanol as electron donor:

\[
\begin{align*}
NO_3^- + 1.08CH_3OH + 0.24H_2CO_3 \rightarrow 0.056C_5H_7NO_2^- + \\
+ 0.47N_2 + 1.68H_2O + HCO_3^- 
\end{align*}
\] (1.4)
Some examples of heterotrophic denitrifiers are *Pseudomonas, Alcaligenes, Paracoccus,* and *Thiobacillus.*

1.3.1.3 N removal technologies based on nitrification-denitrification

Different technologies based on the combination of nitrification and denitrification have been developed to treat nitrogen from wastewaters. Different configurations of suspended growth systems are widely used (Fig. 1.2). These processes combine N removal with elimination of organic matter, and they alternate aerobic tanks, where nitrification occurs, with anoxic tanks or zones where the \( \text{NO}_3^- \) produced in aerated zones is consumed by denitrifiers using organic matter. In systems with pre-denitrification, the organic carbon of the wastewater is used as electron donor. On the other hand, in systems with post-denitrification, externally added electron donor is often needed. Bioaugmentation can be used to enhance nitrification in highly loaded plants, e.g. Bioaugmentation batch-enhanced (BABE). This configuration allows for improved denitrification since the size of the aerobic zone can be reduced (Salem et al., 2002, Salem et al., 2004).

In schemes where nitrogen treatment efficiency is correlated to internal recycle (e.g. Modified Ludzack Ettinger, Bardenpho), high removal rates are associated to high reactor volumes and, therefore, to high immobilized costs (Baeza et al., 2004). When
post-denitrification is required, additional electron donor may be needed (Zhu et al., 2008). Furthermore, high oxygen inputs are required for complete nitrification (BOD$_{\text{NH}_4^+}$ = 4.57 kg O$_2$/kg NH$_4^+$-N). Conventional plants are not conceived for nitrogen removal and they are not cost effective for wastewaters with low C/N ratio where high costs are related to electron donor supply (Tam et al., 1992), and additional cost may be associated to high sludge production rates.

Combined processes where nitrogen removal is accomplished in a single reactor have been developed. Nitrification and denitrification can be achieved in sequencing batch reactors (SBR) alternating aeration with anoxic periods (Keller et al., 1997). The biomass is allowed to settle at the end of each cycle and just a fraction of the supernatant is extracted in each cycle. The system selects for high settleability biomass, which allows for higher concentrations of volatile suspended solids (VSS), higher volumetric removal rates, and generates an effluent with low total suspended solids (TSS), making unnecessary a secondary clarifier (Munch et al., 1996). Although it is a very reliable system, the SBR configuration is not suitable for large plants (Zhu et al., 2008).
Figure 1.2. Simplified schemes of suspended growth systems for N removal. RAS: Return Activated Sludge, AD: Anaerobic Digester.
Simultaneous nitrification and denitrification (SND) can be performed in a single reactor with granular sludge (Beun et al., 2001, de Kreuk et al., 2005). Application of low DO concentration allows for formation of gradients in granules enabling for the generation of aerobic and anoxic microniches suitable for the coexistence of nitrifiers and denitrifiers in the granules (Pochana, Keller, 1999, Third et al., 2003) (Fig 1.3). The process has significant advantages since an anoxic tank is not needed and extra carbon addition can be saved (Zhu et al., 2008).

Figure 1.3. Simultaneous nitrification and denitrification in microniches of granular sludge in an aerobic reactor.
A more refined process was developed in the Netherlands in which NH$_4^+$ is partially oxidized to NO$_2^-$ instead of NO$_3^-$ and the denitrification occurs with NO$_2^-$ as electron acceptor (Hellinga et al., 1998). The process was originally called single reactor system for high ammonia removal over nitrite (SHARON), although later on, SHARON has been referred to the process of partial nitritation to NO$_2^-$, excluding the step of denitrification. In order to avoid the generation of NO$_3^-$, the activity of NOB needs to be suppressed (Picireanu et al., 1997). The concentration of free ammonia (NH$_3$), short sludge retention time, high temperature and limited alkalinity are key parameters that favor the out-competition of NOB by AOB (Anthonisen et al., 1976, Balmelle et al., 1992, Hellinga et al., 1998). DO is another important parameter. Although several works have reported that AOB have higher affinity for O$_2$ than NOB (Picireanu et al., 1997, Schramm et al., 2000), this does not seem to be universal, and the selection of the concentration of dissolved oxygen seems to be case specific (Wett et al., 2013). Compared to processes involving complete nitrification and denitrification over NO$_3^-$, SHARON saves 25% oxygen in nitrification, and 40% of the methanol that is often needed to complete denitrification (Notenboom, 2002).

In the last two decades, the discovery of bacteria able to oxidize NH$_4^+$ anaerobically has transformed the perspective of N removal. Utilizing these new bacteria, novel and more sustainable processes have been developed which will allow the
The anaerobic oxidation of $\text{NH}_4^+$ (anammox) with $\text{NO}_2^-$ as terminal electron acceptor, yielding $\text{N}_2$ and $\text{NO}_3^-$ as main products, is the recently discovered missing link in the N-cycle (Strous et al., 2006). $\text{NH}_4^+$ was thought to undergo oxidation exclusively under aerobic conditions until the thermodynamic feasibility of the anaerobic oxidation of $\text{NH}_4^+$ was predicted in 1977 (Broda, 1977):

$$\text{NH}_4^+ + \text{NO}_2^- \rightarrow \text{N}_2 + 2\text{H}_2\text{O} \quad \Delta G^o = -359 \text{ kJ} \cdot (\text{mol NH}_4^+)^{-1} \quad (1.5)$$

The occurrence of the process was observed years later in a denitrifying pilot plant in Rotterdam (The Netherlands) (Mulder et al., 1995), and the biological nature of the reaction was confirmed by Van de Graaf et al., in 1995.

The anammox process is carried out by several chemolithoautotrophic bacteria, related to *Planctomycetes*. Five “Candidatus” genera have been studied, *Brocadia* (Strous et al., 2010a).
et al., 1999a), Kuenenia (Schmid et al., 2001), Scalindua (Kuypers et al., 2003), Anammoxoglobus (Kartal et al., 2007b) and Jettenia (Quan et al., 2008). Anammox bacteria are being found worldwide in fresh water ecosystems (Schubert et al., 2006, Zhang et al., 2007), marine sediments (Rich et al., 2008, Schmid et al., 2007), and wastewater treatment plants (Mulder et al., 1995), and they are responsible for up to 50% of the oceanic N losses (Kuypers et al., 2005).

The doubling time of anammox bacteria generally range from 11 to 30 d (Strous et al., 1998, van de Graaf et al., 1996) although some punctual works have reported doubling times as low as 1.8 d (Isaka et al., 2006) and 3 d (van der Star et al., 2008). The low growth rate leads to a low cell yield, as shown by the overall stoichiometry (Strous et al., 1998):

\[
\begin{align*}
NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ & \rightarrow \\
1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O
\end{align*}
\]

Due to the high specific activity of about 0.8 kg N kg dry weight (DW)^{-1} d^{-1} (Kartal et al., 2004), the anammox process is emergently used in the treatment of N-rich wastewaters, especially in cases where C/N ratio is low. In these cases, anammox presents several advantages over conventional N-removal processes. Due to the autotrophy of the process, 100% savings are made in organic carbon addition for NO_2^-.
and NO$_3^-$ reduction. Since only half of the NH$_4^+$ needs to be partially oxidized to NO$_2^-$, up to 63% of the oxygen supply are saved when compared with the complete nitrification-denitrification process.

1.4.1 *Morphology and metabolism:*

The cytoplasm in anammox bacteria is divided in three compartments as shown in Fig. 1.2. The cell wall is a peptidoglycan lacking, proteinaceous membrane; the first compartment is called paryphoplasm and it is separated from the riboplasm, where the nucleoid and ribosomes are, by the intracytoplasmic membrane. Inside the riboplasm, there is a third and unique, compartment called anammoxosome (van Niftrik et al., 2008b).

The anammox metabolism occurs in the anammoxosome (van Niftrik et al., 2004). NO$_2^-$ is reduced to nitric oxide NO by a NO$_2^-$-oxidoreductase (NirS), then hydrazine (N$_2$H$_4$) is formed from NH$_4^+$ and NO by a N$_2$H$_4$-synthase enzyme (HZS). Finally N$_2$H$_4$ is oxidized to produce N$_2$ gas by a N$_2$H$_4$-dehydrogenase (HDH) (Kartal et al., 2011). A NO$_2^-$-oxidoreductase oxidizes NO$_2^-$ to NO$_3^-$, generating the electrons necessary for carbon assimilation (Kuenen, 2008).
The anammoxosome membrane is unique among microorganisms, since it contains a very particular type of lipids, called ladderanes (van Niftrik et al., 2004). This lipids have concatenated cyclobutane rings in the alkyl chain, bound to the glycerol by ether or ester bonds. The ladderane lipids confer the anammoxosome membrane higher density and a lower permeability to solutes (Boumann et al., 2009, Damste et al., 2002).

The main function of the anammoxosome is related to generation (Jetten et al., 2009, Lindsay et al., 2001) and conservation of energy (van Niftrik, 2013). The oxidation
of N$_2$H$_4$ in the anammoxosome, generates four high energy electrons shuttled to an electron transport chain (Jetten et al., 2009, Kartal et al., 2011). The energy released by these electrons is used to generate an intracellular proton gradient between both sides of the anammoxosome membrane that energizes the production of ATP in the riboplasm (van der Star et al., 2010). The low permeability of the anammoxosome membrane would confer the anammox bacteria a better efficiency in energy conservation, necessary in such slow growing microorganisms (van Niftrik et al., 2004). Furthermore, it has been suggested to serve as defensive barrier against highly toxic reaction intermediates (e.g. N$_2$H$_4$ and NO) (van Niftrik et al., 2004), or against toxic free nitrous acid (HNO$_2$) (Lotti et al., 2012). The import or export of substrates (NH$_4^+$, NO$_2^-$) and product (NO$_3^-$) into or from the anammoxosome is regulated by membrane proteins found in its genome, i.e., amtB, NirC/focA, NarK (Gori et al., 2011, Medema et al., 2010, van de Vossenberg et al., 2013).

Additionally, anammox bacteria accumulate glycogen in the riboplasm (van Niftrik et al., 2008a), which the bacteria would use for cell maintenance during starving conditions. Moreover, iron particles have been found in the anammoxosome (van Niftrik et al., 2008a), which could serve as iron reservoir to produce the high amount of heme proteins necessary for anammox metabolism, or as an alternative electron acceptor when formate is available as electron donor (Strous et al., 2006).
Indeed anammox bacteria have shown metabolically diverse. They can use electron donors different from NO$_2^-$ (i.e., NO$_3^-$, Fe$^{3+}$, Mn$^{4+}$) (Strous et al., 2006). Moreover anammox bacteria are able to oxidize propionate (Guven et al., 2005, Kartal et al., 2007b), acetate (Kartal et al., 2008), and formate (Strous et al., 2006) as electron donors for dissimilative NO$_3^-$ reduction to NH$_4^+$ (Kartal et al., 2007a). The ability of anammox bacteria to use organic acids expands the perspective of future applications. Anammox bacteria do not incorporate organic C into cell biomass; therefore the application of anammox to degradation of C-BOD would be very advantageous over heterotrophic processes since the sludge production would be significantly reduced. The application of anammox for C-BOD removal has been already demonstrated at a lab-scale nitritation-anammox SBR fed with a synthetic wastewater with CH$_3$COOH-COD/NH$_4^+$-N of 0.5 (Winkler et al., 2012). Although the results are promising, and the future applications of anammox may include treatment of sewage (Kartal et al., 2010a) current applications of the anammox process are limited to N removal.

1.4.2 Application and technologies based on Anammox process

As previously mentioned, the anammox process is cost-efficient compared to other N-removal processes based on nitrification and denitrification. Anammox applications are currently restricted to treatment of NH$_4^+$ rich wastewaters containing low
BOD under mesophilic conditions. It has been applied to treatment of effluents from a wide variety of sources (Vlaeminck et al., 2012): wastewaters from the food industry - fish canning (Dapena-Mora et al., 2006), potato processing effluent (Abma et al., 2010, Mulder et al., 2012), fermentation and distilleries effluents (Vlaeminck et al., 2012), glutamate wastewaters (Hu et al., 2013b), fertilizer manufacturing industry (Keluskar et al., 2013), tannery (Abma et al., 2007), semiconductor manufacturing (Tokutomi et al., 2011), coking effluents (Li et al., 2010), domestic wastewater and digested sludge liquor (Joss et al., 2009), urine (Udert et al., 2008), digested black water (Vlaeminck et al., 2009), and landfill leachate (Hippen et al., 1997).

The application of the anammox process requires a mixture of NH$_4^+$ and NO$_2^-$ of approximately 1:1 molar. Since most of N polluted effluents contain NH$_4^+$ as the major N compound, a partial nitritation step is required where half of the NH$_4^+$ is aerobically oxidized to NO$_2^-$ by AOB. Both processes –partial nitritation and anammox– can be carried out in separate units or in a single reactor. Both options have distinct advantages and disadvantages. The two stage partial nitritation-anammox enables the independent optimization of each step, the risk of anammox bacteria being outcompeted by heterotrophic denitrifiers is lower than in the single reactor configuration (Hu et al., 2013a). Furthermore the anammox reaction is inhibited by DO (Egli et al., 2001), which can be avoided by ensuring anaerobic conditions in the separate anammox reactor. On the other hand, the immobilized costs and the C-footprint derived from greenhouse gas
emissions of the single reactor system are considerably lower (Kampschreur et al., 2008, Kampschreur et al., 2009). So far, about 40 full scale installations utilize the anammox process (Hu et al., 2013a), and only four have separate nitritation and anammox reactors (Desloover et al., 2011, Tokutomi et al., 2011, van der Star et al., 2007).

The industry has adopted the name of deammonification, although other equivalent terminologies exist for the single stage process, with disregard of the configuration utilized: Completely autotrophic nitrogen removal over nitrite (CANON) and oxygen limited autotrophic nitrification-denitrification (OLAND) (Kuai, Verstraete, 1998, Third et al., 2001).

Furthermore, different technologies utilize different commercial terminologies, namely based on the type of biomass used, and the place where they were developed (Hu et al., 2013b). Due to the slow growth of anammox bacteria, efficient retention of the anammox biomass is of utmost importance. The kind of biomass utilized, *i.e.*, biofilm (attached or granular), suspended, or hybrid, determines the configuration of the reactor, as well as the control strategy. In all the systems where nitrification needs to be interrupted in NO$_2^-$ without further oxidation to NO$_3^-$, the repression NOB represents a challenge (Vlaeminck et al., 2012). Each technology deploys different strategies to avoid NO$_2^-$ oxidation, namely based on type of biomass utilized.
1.4.2.1 Biofilm based systems

**Granular sludge:** Granular biomass is utilized both, in two reactor systems, or in single reactor systems.

*Separate SHARON – Anammox:* When the partial nitritation and anammox are physically separated, nitritation by AOBs is performed in an aerated tank (SHARON), without sludge recycle. Under mesophilic conditions, the doubling time of AOB is shorter than that of NOB, and short retention time is utilized to avoid NO\textsubscript{2} oxidation (Hellinga et al., 1998). The effluent of the SHARON reactor is decanted in order to minimize contamination of the anammox reactor with nitrifying biomass. In order to meet the NH\textsubscript{4}\textsuperscript{+}:NO\textsubscript{2}\textsuperscript{−} requirement for anammox, the alkalinity in the SHARON reactor must be just enough to allow for oxidation of just half of the NH\textsubscript{4}\textsuperscript{+} (van Dongen et al., 2001). The anammox reaction takes place in an upflow anaerobic sludge bed reactor. High upflow velocity is desired to favor granulation and avoid the washout of the slow growing anammox bacteria. High volumetric loading rates in the anammox reactor ranging 7.1-9.5 kg N m\textsuperscript{−3} d\textsuperscript{−1} have been demonstrated in a full scale plant, with total N removal of up to 90% and NO\textsubscript{2}\textsuperscript{−} effluent concentrations lower than 10 mg N L\textsuperscript{−1}(van der Star et al., 2007).

*Single stage partial nitritation-anammox with granular sludge:* Partial nitritation and anammox can be accomplished in a single aerobic air lift- or sequencing batch- reactor,
with granular sludge (Kampschreur et al., 2009, Sliekers et al., 2002, Third et al., 2001). The creation of aerobic and anoxic microniches allows for growth of AOB on the surface and anammox bacteria in the core of the granules (Fig. 1.5).

![Diagram](image_url)

**Figure 1.5.** Partial nitritation and anammox occurring in microniches of a granule.

The limitation to DO transfer, and consumption of DO by AOB in the outer layer of the granules allows the maintenance high DO concentrations (5 mg L$^{-1}$) in the bulk liquid without causing inhibition to the anammox bacteria (Kampschreur et al., 2009). Volumetric loading rates of 1.7-2.0 kg N m$^{-3}$ d$^{-1}$ with 75% total nitrogen removal, have been maintained in a full scale plant treating wastewater from a potato processing facility (Kampschreur et al., 2009).
**Attached growth biomass:** Different technologies utilize plastic carriers as support for attached growth of anammox biomass (Christensson et al., 2013, Rosenwinkel, Cornelius, 2005). The dominant configuration for attached growth systems is moving bed biofilm reactor (MBBR). The diffusional limitations in the biofilm enables for the generation of anoxic zones were anammox bacteria thrive. Due to the thinness of the biofilm, low DO concentrations are maintained. NOB repression is achieved by application of a control loop with online measurement of the ratio between NO$_3^-$ generated to NH$_4^+$ consumed. When this ratio is higher than 11% aeration is interrupted to avoid NOB from utilizing excess DO (Christensson et al., 2013).

1.4.2.2 Suspended growth:

In Switzerland, several wastewater treatment plants utilize anammox to treat digested sludge centrate. In those plants partial nitritation and anammox occur in single SBR reactors with continuous aeration. The aeration rate is controlled based on DO (lower than 1 mg L$^{-1}$) and NO$_2^-$ concentration (always lower than 5 mg NO$_2^-$-N L$^{-1}$). The length of the aeration phase is controlled by online NH$_4^+$ measurement (Joss et al., 2009).

Another plant in the Netherlands, treating wastewater from a potato processing factory, utilizes anammox in a four step N removal process. In this case anammox takes place in a separate reactor under anoxic conditions, following a partial nitritation reactor.
The effluent of the anammox reactor is further polished in two downstream reactors performing nitrification and denitrification. The performance of the plant is controlled by the SRT (46 d), provided by a settler installed at the end of the biological treatment train. The plant operates at a nitrogen loading rate (NLR) of 0.054-0.066 kg N m$^{-3}$ d$^{-1}$ (total volume) producing a dischargeable effluent (Desloover et al., 2011).

1.4.2.3 Hybrid growth systems:

Deammonification can be carried out in a single stage SBR with suspended biomass (Wett, 2007). The process is called DEMON®. In order to guarantee appropriate retention of the anammox biomass hydrocyclones are used to select for high density granules, enriched in anammox bacteria, which are recycled to the reactor. The reactor is intermittently aerated. The length of aeration and anoxic periods is controlled based on a tight pH control. The intermittent aeration causes a metabolic lag-phase to NOB, which are outcompeted by anammox bacteria (Wett et al., 2010).

Other alternative configurations have been explored in laboratory scale including rotating biological contactors (Vlaeminck et al., 2012), anammox biomass embedded in gel carriers (Furukawa et al., 2006) or membrane bioreactors (van der Star et al., 2008).
1.4.3 *Inhibition of the anammox process and consequences*

Due to the slow growth of anammox microorganisms, very long start up periods are needed. Initial times of 60 d (Dapena-Mora et al., 2004), 58 d (Liao et al., 2007) have been reported for SBR configurations using activated sludge and methanogenic granular sludge as inocula. In full scale installations doubling times of 11 d (van der Star et al., 2007) to 27 d (Joss et al., 2009) have been measured. Strategies including submerged hollow fiber membrane bioreactors (van der Star et al., 2008) or favoring biofilm formation (Fernandez et al., 2008) have been reported in the literature aiming to reduce start-up periods. Nevertheless, exposure to inhibitory conditions could lead to complete failure of the reactor regardless of the strategy used to retain the biomass. If biomass is lost due to the presence of toxicants in the wastewater, recovery of the full treatment capacity would take too long. Little information is available about the susceptibility of anammox bacteria to compounds commonly found in wastewaters (Dapena-Mora et al., 2007). Among compounds causing inhibition of anammox bacteria, NO$_2^-$, is of special relevance since it is a necessary substrate of the reaction (Table 1.3). Although numerous studies have reported inhibition of ANAMMOX bacteria by NO$_2^-$, the mechanisms responsible for NO$_2^-$ inhibition as well as the operational conditions leading to an increased risk of failure due to NO$_2^-$ inhibition are unknown.
In this dissertation, the inhibition of the anammox process by compounds commonly present in wastewaters is investigated. Furthermore, the inhibition of anammox by NO\textsubscript{2}\textsuperscript{-} has been thoroughly studied, and insight on the conditions to be avoided during operation of anammox reactors is provided.

### Table 1.3. Reported data about NO\textsubscript{2}\textsuperscript{-} inhibition of the ANAMMOX process.

<table>
<thead>
<tr>
<th>NO\textsubscript{2}\textsuperscript{-} Conc. (mg N L\textsuperscript{-1})</th>
<th>Effect</th>
<th>Mode</th>
<th>Conditions</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>Complete Inhibition</td>
<td>batch</td>
<td>7-7.8</td>
<td>(Strous et al., 1999b)</td>
</tr>
<tr>
<td>185</td>
<td>Complete inhibition</td>
<td>batch</td>
<td>7</td>
<td>(Egli et al., 2001)</td>
</tr>
<tr>
<td>350</td>
<td>50% Inhibition</td>
<td>batch</td>
<td>7.8</td>
<td>(Dapena-Mora et al., 2007)</td>
</tr>
<tr>
<td>102</td>
<td>Inhibition</td>
<td>SBR</td>
<td>7.5-8.2</td>
<td>(Lopez et al., 2008)</td>
</tr>
<tr>
<td>430\textsuperscript{a,b}</td>
<td>37% Inhibition</td>
<td>batch</td>
<td>NR</td>
<td>(Kimura et al., 2010)</td>
</tr>
<tr>
<td>60</td>
<td>Inhibition</td>
<td>SBR</td>
<td>7.5</td>
<td>(Fux et al., 2002)</td>
</tr>
<tr>
<td>278*</td>
<td>50% Inhibition</td>
<td>batch</td>
<td>7.8</td>
<td>(Fernandez et al., 2012)</td>
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<td>500</td>
<td>35% Inhibition</td>
<td>batch</td>
<td>7.6-7.7</td>
<td>(Scaglione et al., 2012)</td>
</tr>
<tr>
<td>171-173\textsuperscript{a}</td>
<td>50% Inhibition</td>
<td>batch</td>
<td>7.6-7.7</td>
<td>(Scaglione et al., 2012)</td>
</tr>
<tr>
<td>400</td>
<td>50% Inhibition</td>
<td>batch</td>
<td>7.5</td>
<td>(Lotti et al., 2012)</td>
</tr>
<tr>
<td>-</td>
<td>Decrease in N removal efficiency</td>
<td>Full-scale gas lift</td>
<td>7-8</td>
<td>(van der Star et al., 2007)</td>
</tr>
<tr>
<td>4.8</td>
<td>Decrease in Activity</td>
<td>Full-scale Floc-based SBR</td>
<td>-</td>
<td>(Wett, 2007)</td>
</tr>
</tbody>
</table>

\textsuperscript{*}Calculated from reported data: 11 µg HNO\textsubscript{2}-N L\textsuperscript{-1} at pH 7.8; \textsuperscript{a} 24 h pre-exposure-no wash; \textsuperscript{b} biomass washed after exposure

NR: not reported
1.5 Acronym list

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>AD</td>
<td>Anaerobic Digester</td>
</tr>
<tr>
<td>AOB</td>
<td>Ammonium Oxidizing Bacteria</td>
</tr>
<tr>
<td>BABE</td>
<td>Bioaugmentation Batch Enhanced</td>
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<tr>
<td>BOD</td>
<td>Biological Oxygen Demmand</td>
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<td>CANON</td>
<td>Completely Autotrophic Nitrogen Removal Over Nitrite</td>
</tr>
<tr>
<td>C-BOD</td>
<td>Carbonaceous Biological Oxygen Demand</td>
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<td>CWA</td>
<td>Clean Water Act</td>
</tr>
<tr>
<td>DNRA</td>
<td>Dissimilatory Nitrate Reduction to Ammonia</td>
</tr>
<tr>
<td>DO</td>
<td>Dissolved Oxygen</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
</tr>
<tr>
<td>FWPCA</td>
<td>Federal Water Polution Control Act</td>
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<td>MBBR</td>
<td>Moving Bed Biofilm Reactor</td>
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<td>N</td>
<td>Nitrogen</td>
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<td>NOB</td>
<td>Nitrite Oxidizing Bacteria</td>
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<tr>
<td>NPDES</td>
<td>National Pollution Discharge Elimination System</td>
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<tr>
<td>Nr</td>
<td>Reactive nitrogen</td>
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<td>OLAND</td>
<td>Oxygen Limited Autotrophic Nitrification-Denitrification</td>
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<td>SBR</td>
<td>Sequencing Batch Reactor</td>
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<td>SHARON</td>
<td>Single system for High Ammonium Removal Over Nitrite</td>
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<td>SND</td>
<td>Simultaneous Nitrification-Denitrification</td>
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<td>SRT</td>
<td>Solid Retention Time</td>
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<td>TSS</td>
<td>Total Suspended Solids</td>
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<td>VSS</td>
<td>Volatile Suspended Solids</td>
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CHAPTER 2

OBJECTIVES

2.1 Aim

The objective of this research is to investigate the inhibition of the anammox bacteria by common wastewater constituents, with special attention to nitrite, the terminal electron acceptor of anammox.

2.1.1 Specific Objectives

1. Evaluate the inhibition of the anammox process by substrates, metabolites and common wastewater constituents on two different anammox enrichment cultures
2. Study the inhibitory effect of nitrite on metabolically active- and resting anammox bacteria

3. Investigate the role of the pH on the tolerance of anammox bacteria to nitrite exposure under active and resting conditions.

4. Investigate the effect of starvation on the tolerance of anammox bacteria to nitrite inhibition.
3.1 Abstract

Anaerobic ammonium oxidation (anammox) is an emerging technology for nitrogen removal that provides a more environmentally sustainable and cost effective alternative compared to conventional biological treatment methods. The objective of this study was to investigate the inhibitory impact of anammox substrates, metabolites and common wastewater constituents on the microbial activity of two different anammox enrichment cultures (suspended and granular), both dominated by bacteria from the genus Brocadia. Inhibition was evaluated in batch assays by comparing the N₂ production rates in the absence or presence of each compound supplied in a range of concentrations. The
optimal pH was 7.5 and 7.3 for the suspended and granular enrichment cultures, respectively. Among the substrates or products, ammonium and nitrate caused low to moderate inhibition, whereas nitrite caused almost complete inhibition at concentrations higher than 15 mM. The intermediate, hydrazine, either stimulated or caused low inhibition of anammox activity up to 3 mM. Of the common constituents in wastewater, hydrogen sulfide was the most severe inhibitor, with 50% inhibitory concentrations (IC$_{50}$) as low as 0.03 mM undissociated H$_2$S. Dissolved O$_2$ showed moderate inhibition (IC$_{50}$ = 2.3 to 3.8 mg L$^{-1}$). In contrast, phosphate and salinity (NaCl) posed very low inhibition. The suspended- and granular anammox enrichment cultures had similar patterns of response to the various inhibitory stresses with the exception of phosphate. The findings of this study provide comprehensive insights on the tolerance of the anammox process to a wide variety of potential inhibiting compounds.

3.2 Introduction

Since anaerobic ammonium oxidation (anammox) was discovered in the early 1990s, it has gained attention due to its importance in the global nitrogen cycle (den Camp et al., 2006). Anammox is catalyzed by chemolithoautotrophic bacteria (Strous et al., 1999a) belonging to five genera Brocadia, Kuenenia, Anammoxoglobus, Jettenia and Scalindua of the phylum Planctomycetes (Harhangi et al., 2011). The anammox reaction
involves the oxidation of ammonium (NH$_4^+$) coupled to the reduction of nitrite (NO$_2^-$) to produce the main product, N$_2$ and a minor product, nitrate (NO$_3^-$) (Eq. 1), under anaerobic conditions (van der Star et al., 2007; Kuena, 2008).

\[
\begin{align*}
NH_4^+ + 1.32NO_2^- + 0.66HCO_3^- + 0.13H^+ & \rightarrow \\
1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O
\end{align*}
\]

(4.1)

The postulated reaction mechanism involves the conversion of NH$_4^+$ and NO$_2^-$ to the intermediate hydrazine (N$_2$H$_4$), which finally leads to the production of N$_2$. Recent studies propose nitric oxide (NO) as one of the intermediates of the anammox reaction (Kartal et al., 2011).

Due to ever-increasing stricter nutrient-nitrogen discharge limits, there is a great need to improve wastewater treatment processes. Compared to the conventional nitrification-denitrification process, anammox provides a more environmentally sustainable and cost-effective alternative for nitrogen removal in wastewaters with low C/N ratio (Renou et al., 2008; Kartal et al., 2010b). However, waste streams commonly contain compounds that might pose inhibitory effects on anammox activity (AA). Additionally, anammox bacteria have a slow growth rate with reported doubling times around 10 to 12 d (Strous et al., 1998; van der Star et al., 2007). Thus, the impact of a
severe toxic event, killing the biomass, would be particularly problematic for anammox due to the long recovery periods needed. Understanding of the factors influencing the activity of anammox bacteria is essential to improving its applicability, including the identification of potential toxic compounds present in specific wastewaters.

The objective of this study was to investigate the inhibitory impact of commonly occurring compounds on two different anammox enrichment cultures dominated by bacteria from the genus *Brocadia*. The compounds tested consisted of anammox substrates (NO$_2^-$ and NH$_4^+$), metabolites (NO$_3^-$ and N$_2$H$_4$) and constituents frequently encountered in wastewater (H$_2$S, O$_2$, NaCl, PO$_4^{3-}$). Additionally the anammox toxicity of two commonly utilized nitrification inhibitors was tested. The inhibition was evaluated in batch assays by comparing the N$_2$ production rates in the absence or presence of each compound supplied in a range of concentrations.

### 3.3 Material and methods

#### 3.3.1 Microorganisms

Two enriched anammox cultures (granular and suspended) dominated by Candidatus *Brocadia* were used as inoculum. The suspended enrichment culture (SEC)
was washed and centrifuged in a NaCl (1%) solution, and re-suspended with basal medium before transferring into batch assays. This anammox enrichment was originally developed from a returned activated sludge (Sun et al., 2011) and maintained in a membrane bioreactor. The granular anammox enrichment (GEC) was obtained from a full-scale anammox bioreactor operated by Paques BV, Balk, The Netherlands, and maintained in an expanded granular sludge bed reactor. The volatile suspended solids (VSS) content of the GEC sludge was 5.69 ±0.04% of the wet weight. The average size of the Anammox granules was 2.4±0.6 mm. This value was calculated by image analysis of a photograph of a granular sludge sample using the software ImageJ.

3.3.2 Batch bioassays

Batch assays were performed in serum flasks (160 ml), and incubated on a shaker (115 rpm) in the dark at 30±2 °C. The anammox biomass was added to the assays at 5 vol% for the anammox SEC and 0.6 g VSS L\(^{-1}\) for the GEC. Flasks were supplied with 100 ml basal mineral medium (pH 7.4-7.5) (described in the APPENDIX A), containing bicarbonate (2.5 g L\(^{-1}\)) as the only added carbon. The medium was also supplemented with a stoichiometric mixture (1.32:1, mol NO\(_2^-\):mol NH\(_4^+\)) of NO\(_2^-\) (3.57 mM) and NH\(_4^+\) (2.71 mM), unless otherwise described. Abiotic controls were prepared by excluding the
addition of microbial inoculum. Controls lacking NH$_4^+$ were included to measure background endogenous (denitrification) consumption of NO$_2^-$.

The flasks were sealed with rubber stoppers, and then the medium and the headspace were purged with He/CO$_2$ (80/20, v/v) to exclude oxygen from the assays. All assays were conducted in duplicate. Headspace samples were analyzed periodically for N$_2$ gas content to monitor the AA. Liquid samples were extracted at the beginning and at the end of each experiment and analyzed for NO$_2^-$, NO$_3^-$ and NH$_4^+$. Experiments testing the inhibitory effect of phosphate were supplemented with phosphate concentrations ranging 5-50 mM (supplied as NaH$_2$PO$_4$•H$_2$O/ Na$_2$HPO$_4$•7H$_2$O at a molar ratio of 0.23). The corresponding controls contained 0.42 mM phosphate.

3.3.3 **Assessment of anammox activity and inhibition**

The anammox activity (AA) was measured based on the N$_2$ production rate and expressed as mmol N$_2$-N L$_{\text{liquid}}^{-1}$ h$^{-1}$. The AA was calculated from the maximum slope of the time course of the N$_2$-N concentration in the headspace as follows: AA= $\Delta$N$_2$/$\Delta$t (mmol N$_2$ L$_{\text{liquid}}^{-1}$ h$^{-1}$). The only exception was for the inhibitory effect of dissolved oxygen (DO) and N$_2$H$_4$, which was assessed based on the NO$_2^-$ consumption rate (mM h$^{-1}$). The inhibition was expressed as the relative AA (RAA) (%)= [AA$_{\text{inhibitor}}$/AA$_{\text{reference}}$] ×
100. In the experiment assessing the effect of different initial pH values, the AA at the optimal pH was chosen as the AA\textsubscript{reference} value. The measurements of liquid concentrations of NO\textsubscript{2}\textsuperscript{−}, NO\textsubscript{3}\textsuperscript{−} and NH\textsubscript{4}\textsuperscript{+} were used to confirm the anammox reaction. Consumption of NO\textsubscript{2}\textsuperscript{−} and NH\textsubscript{4}\textsuperscript{+} as well as formation of NO\textsubscript{3}\textsuperscript{−} (data not shown) corresponded to the N\textsubscript{2} production according to the stoichiometry of anammox reaction in all batch assays (Eq. 1). The final pH value was measured to confirm that the optimal range was maintained.

3.3.4 Analytical methods

NO\textsubscript{3}\textsuperscript{−} and NO\textsubscript{2}\textsuperscript{−} were analyzed by suppressed conductivity ion chromatography, N\textsubscript{2} and O\textsubscript{2} by gas chromatography with thermal conductivity detector, and NH\textsubscript{4}\textsuperscript{+} using a NH\textsubscript{4}\textsuperscript{+} ion selective electrode (Sun et al., 2011). Liquid samples for sulfide or hydrazine determination were collected prior to addition of NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{2}\textsuperscript{−}. Sulfide was analyzed spectrophotometrically by the methylene blue method (Truper and Schlegel et al., 1964). Hydrazine was analyzed according to Van der Star (2008). All other measurements (pH and VSS) were according to Standard Methods (APHA, 2005). More information about analytical procedures is provided in the SM section.
3.3.5  *Molecular methods*

Anammox bacteria in SEC and GEC were characterized by generating a clone library as described in the APPENDIX A.

3.4  **Results and discussion**

3.4.1  *Characterization of enrichment cultures*

The two different inocula were characterized by different anammox species. One unique anammox phylotype was found in each of the enrichment cultures, both showing very high similarity with the 16S rRNA gene of the genus *Brocadia* (> 99.5%). The anammox strain in the SEC was most closely related to *Brocadia caroliniensis*, whereas the GEC was enriched in *Brocadia fulgida* (Fig. A1). The maximum specific AA of the SEC and GEC cultures was 0.48±0.06 and 0.19±0.02 mmol N$_2$ g$^{-1}$ VSS d$^{-1}$, respectively. The pH optima of these cultures were characterized in assays incubated at pH values ranging from 6.5 to 8.3 (based on final pH measurements). The highest AA was achieved at pH 7.5 and 7.3 for SEC and GEC, respectively (Fig. 3.1). Both cultures had sharp pH optima, and important losses in activity exceeding 20% were evident as the pH shifted more than 0.3 units from the optimal pH values. These results showed some disagreement.
with a previous study (Egli et al., 2001) reporting a wider optimal pH range (7.5-8.0). However, a recent study indicated a sharp decrease of AA at pH below pH 7.2 (van der Star et al., 2007). The impact of pH extremes has been rationalized based on the existence of an energy yielding intracellular proton gradient over the anammoxosome membrane (van der Star et al., 2010). Long-term alteration of the pH of the medium could lead to disruption of the proton motive force and thus affecting the associated energy generation. Inhibition has also been attributed to the pH-dependent, unionized and presumably more toxic forms of the substrates, HNO$_2$ and NH$_3$ (Anthonisen et al., 1976; Fernandez et al., 2012).

![Figure 3.1. Effect of pH on the anammox activity of a granular enrichment culture (●) and a suspended enrichment culture (○).]
3.4.2 Effects of anammox substrates and metabolites

To evaluate ammonium toxicity, the enrichment cultures were incubated at different concentrations of NH$_4^+$ (2.7-44.2 mM) while keeping the NO$_2^-$ concentration constant at 3.6 mM. The AA slightly decreased with increasing NH$_4^+$ concentration (Fig. 3.2a). At the highest NH$_4^+$ concentration tested (44 mM), the inhibition observed for GEC and SEC was only 16 and 34%, respectively. The results demonstrated low levels of inhibition caused by NH$_4^+$, which agrees with previous reports (Strous et al., 1999b; Dapena-Mora et al., 2007). Free ammonia is believed to be the actual inhibitor of anammox (Waki et al., 2007; Fernandez et al., 2012; Aktan et al., 2012). In contrast with ammonium, undissociated ammonia is expected to diffuse over the microbial cell membrane. At the pH level tested in this study (7.2), the maximum concentration of free ammonia in the experiments was 6.5 mg L$^{-1}$. This concentration is significantly lower compared to inhibitory values reported for free ammonia (50% inhibition at 46 mg L$^{-1}$) (Fernandez et al., 2012).
Figure 3.2. Effect of anammox reaction substrates and products on the anammox activity of a granular enrichment culture (■) and a suspended enrichment culture (□) exposed to: (a) NH$_4^+$, (b) NO$_2^-$, (c) NO$_3^-$, and (d) N$_2$H$_4$.

To examine the inhibitory effect of NO$_2^-$, batch assays with different initial NO$_2^-$ concentrations (3.6-53.9 mM) were performed at the same concentration of NH$_4^+$ (2.7 mM) (Fig. 3.2b). No significant inhibition was observed for NO$_2^-$ concentrations lower than 7.4 mM. However, NO$_2^-$ concentrations of 15.1 mM or higher dramatically impacted the AA. At this concentration, no N$_2$ production was detected in assays with SEC, while
only 16.4% RAA was observed in the GEC. The IC$_{50}$ of NO$_2^-$ was 10.8±0.05 mM and 13.2±0.13 mM for the SEC and GEC, respectively. These results coincided well with previously reported inhibitory concentrations (Egli et al., 2001; Dapena-Mora et al., 2007), although in one study 7 mM NO$_2^-$ was required to completely inhibit an anammox enrichment culture from a sequencing batch reactor (Strous et al., 1999b). Several authors have attributed the inhibitory effect of nitrite on anammox bacteria to the undissociated species, nitrous acid (HNO$_2$) (Jin et al., 2012, Fernandez et al., 2012). However, a recent study has demonstrated that NO$_2^-$ is the actual inhibitor and that nitrous acid is not responsible for inhibition (Lotti et al., 2012). The latter study hypothesized that the presence of ladderane membrane lipids may hinder diffusion of HNO$_2$, preventing changes in pH that can lead to loss of the proton motive force.

The inhibitory impact of NO$_3^-$ (5.5-100 mM) was evaluated since as a soluble product of anammox, it can potentially accumulate. The RAA of the enrichments decreased by 15-20% (SEC) or 40-50% (GEC) when exposed to NO$_3^-$ concentrations ranging from 5.5 to 20.5 mM (Fig. 3.2c). Inhibition was much more severe when the NO$_3^-$ concentrations were 50 mM or higher.

N$_2$H$_4$ is an intermediate of the anammox reaction (Schalk et al., 1998; Kartal et al., 2011). Bioassays were conducted to evaluate the impact of hydrazine (0.02-2.6 mM)
on AA. A 40% increase of the NO$_2^-$-consumption rate was observed for GEC (Fig. 3.2d). In the case of SEC, no significant stimulation was observed and modest inhibition was observed at 2.7 mM. Accumulation of ammonium occurred in tests with high concentration of hydrazine, and final N$_2$ production was higher than that of the control lacking hydrazine. Addition of N$_2$H$_4$ at concentration of 0.1 mM has been reported to enhance the full recovery of AA from cultures previously inhibited by NO$_2^-$ (Strous et al., 1999b). In our tests, either stimulation or moderate inhibition was observed at concentrations higher than 0.3 mM. However at the lowest concentration tested (0.03 mM), slight stimulation was observed in both enrichments (RAA of 103 and 113% for SEC and GEC, respectively). In test supplied with 2.5 mM hydrazine as the sole nitrogen source, the two cultures had similar hydrazine removal rates, ranging from 1.79 to 1.99 nmol min$^{-1}$ mg$^{-1}$ VSS. The similarity in rates despite large differences in specific AA might be attributed to the different capability to perform the disproportionation of hydrazine by different anammox bacteria (Schalk et al., 1998).

3.4.3 Effects of common wastewater constituents

Sulfide is commonly found in anaerobic reactors as a product of mineralization of organic matter or sulfate reduction. The effect of sulfide on AA was investigated in assays supplied with 0.1-1.0 mM Na$_2$S. The toxicity of sulfide has often been associated
with its unionized form (H₂S), therefore the results are expressed in terms of undissociated H₂S rather than total sulfide added. The equation used to calculate the dissolved undissociated concentration based on pH, total S added and headspace to liquid volume ratio is provided in the SM. Undissociated H₂S caused serious inhibition of the AA with IC₅₀ values of 0.03 and 0.11 mM for the SEC and GEC, respectively (Fig. 3.3). A concentration of unionized H₂S as low as 0.32 mM caused complete inhibition of the SEC. The GEC was also highly inhibited but it was able to conserve a small RAA (24±4%) at higher concentrations (0.9 mM). No H₂S consumption was observed in either abiotic or biological treatments during the course of the experiment (data not shown). H₂S has been previously shown to cause complete anammox inhibition at concentrations of 0.65 mM (Dapena-Mora et al., 2007). In another study, application of pulses of sulfide of 2 mM caused stimulation of ammonium consumption in a fluidized bed anammox reactor (van de Graaf et al., 1996). This phenomenon was explained by the reduction of nitrate by sulfide, producing nitrite, which is the preferred electron acceptor of the anammox process. Our study shows that anammox biomass is severely inhibited at very low sulfide concentrations, not previously reported in the literature. The strong effect of sulfide could be related to the high dependence of anammox process on heme proteins (Kuenen, 2008). Sulfide has been reported to interact with heme centers of cytochrome oxidase as well as to cause reduction of the heme iron in cytochrome c (Pietri et al., 2011), which could potentially lead to disruption of anammox metabolism.
Figure 3.3. Effect of H₂S on the anammox activity of a granular enrichment culture (♦) and a suspended enrichment culture (◊).

Anammox is considered to be strongly inhibited by O₂ (Strous et al., 1997), yet there are a number of processes in which DO is present while nitritation and anammox are simultaneously taking place in the same bioreactor (Strous et al., 1997; Wett, 2007). To better understand the tolerance of anammox cultures to DO exposure, different amounts of O₂ were added to the anaerobic headspace to achieve DO equilibrium concentrations ranging 1-8 mg L⁻¹ (0.03-0.25 mM). The two enrichment cultures had similar inhibitory responses (Fig. 3.4). At low DO of 1 mg L⁻¹ (0.03 mM), only partial inhibition of less than 20% was observed. However, at ambient saturation of DO (8 mg·L⁻¹), the AA was severely inhibited. The IC₅₀ value determined for DO was 3.8±0.6
mg L\(^{-1}\) (0.12±0.02 mM) and 2.3±0.03 mg L\(^{-1}\) (0.07±0.001 mM) for SEC and GEC; respectively. No nitrification was observed in tests inoculated with the SEC and only 12.5% ammonium was nitrified in GEC controls with 20% O\(_2\) in the headspace, fed with ammonium only (data not shown). Exposure to oxygen has been reported to cause different levels of anammox inhibition (Jetten et al., 1998; Strous et al., 1998; Egli et al., 2001). For example, Strous et al., (1998) reported a 90% inhibition on the AA with DO concentration of 0.25 mM. Egli et al., (2001) reported that exposure to low DO concentrations (0.25-1% O\(_2\) vol. in the gas phase) caused reversible inhibition of AA, but irreversible inhibition was observed at higher DO concentrations (18% O\(_2\)). As shown in this study, considerable AA is observed at relatively high DO concentrations. In fact, although ammonium consumption was observed in GEC controls fed with just NH\(_4^+\) in the presence of 20% O\(_2\) in the headspace, NO\(_2^-\) accumulation was not observed; instead simultaneous N\(_2\) gas production was measured. The data suggest that NO\(_2^-\), slowly produced by nitrification, was readily utilized by anammox bacteria even at the high DO concentrations.
Figure 3.4. Effect of O\textsubscript{2} on the anammox activity of a granular enrichment culture (●) and a suspended enrichment culture (○).

Phosphate is commonly found in wastewaters due to its use in detergents and fertilizers (Alamdari and Rohani, 2007). The impact of phosphate at concentrations ranging from 5.5 to 50 mM on the AA was investigated. The results (data not shown) demonstrated that SEC exposure to phosphate caused a modest decrease of RAA with increasing phosphate concentration (IC\textsubscript{50} = 25.3±5.9 mM). On the other hand, phosphate stimulated the RAA of GEC by 60% at concentrations ranging 10-50 mM. Van Graaf et al., (1996) observed complete inhibition of a suspended anammox enrichment exposed to 5 mM phosphate. Dapena-Mora et al., (2007) reported a higher tolerance of anammox biofilms from a gas-lift reactor to phosphate (IC\textsubscript{50} = 20 mM). These results suggest that
the impact of phosphate on AA is highly dependent on the aggregation degree of the biomass. Anammox biofilms are more tolerant to high phosphate concentrations compared with suspended biomass.

A variety of industrial effluents have a high salt content, therefore the impact of salinity on AA is a concern. The SEC was incubated with different NaCl concentrations (50 - 300 mM). The RAA decreased with increasing NaCl levels, and the calculated IC$_{50}$ was 93±4 mM. At concentration of 200 mM and higher, the AA was completely inhibited. The inhibitory behavior demonstrated a linear relationship with NaCl concentration ($R^2 = 0.995$, data not shown). The genera *Candidatus Kuenenia* and *Candidatus Scalindua* have been shown to be relatively tolerant to high salinity with observable activity up to 1.54 M in previously adapted cultures (Kartal et al., 2006; Dapena-Mora et al., 2007). Our results suggest that the genera *Brocadia* may be less suitable for treating high salinity effluents.

3.4.4 Effects of nitrification inhibitors

The effect of nitrification inhibitors was evaluated since inhibitors could potentially be used in studies evaluating contribution of anammox in microaerophilic environments (Table 1). Compounds such as allylthiourea (ATU), or nitrapyrine are
commonly used to suppress enzyme ammonium monooxygenase for ammonium oxidation (Robertson et al., 1989). Chlorate (ClO$_3^-$) has been reported to selectively inhibit nitrite oxidation in soil samples (Belser and Mays, 1980). ATU did not inhibit GEC at 0.034 mM, but caused 40% inhibition of the SEC activity at 0.043 mM. This is in disagreement with a previous study (Dapena-Mora et al., 2007), which tested much higher ATU concentrations (8.6 mM) without observing any appreciable effect. Nitrapyrine caused a slight stimulation of N$_2$ production rate at low concentrations (0.011 - 0.024 mM), but was inhibitory at concentrations higher than 0.023 mM, with an IC$_{50}$ of 0.061±0.002 mM. In contrast to the other nitrification inhibitors, ClO$_3^-$ caused a severe and irreversible inhibition on AA of SEC with an IC$_{50}$ of 0.04±0.002 mM. This is the first report of ClO$_3^-$ inhibition to anammox.
Table 3.1. Effects of various common wastewater contaminants and nitrification inhibitors on the activity of a suspended- and a granular anammox enrichment culture

| Contaminants                        | Tested concentrations (mM) | IC₅₀ (mM) |  |  |
|-------------------------------------|----------------------------|-----------|  |  |
|                                     |                            | Suspension culture | Granular culture |  |
| **Substrates and products**         |                            |           |  |  |
| NH₄⁺                                | 2.99-44.15                 | GMC*      | GMC |  |
| NO₂⁻                                | 4.18-53.92                 | 10.76±0.05 | 13.22±0.13 |  |
| NO₃⁻                                | 5.5-100                    | 32.00±0.39 | 21.23±1.22 |  |
| N₂H₄                                 | 0.02-2.65                  | GMC       | GMC |  |
| **Wastewater Constituents**         |                            |           |  |  |
| Phosphate                           | 0.42-100                   | 25.29±5.88 | GMC |  |
| O₂                                  | 0-0.625                    | 0.12±0.02  | 0.071±0.001 |  |
| Salinity as NaCl                    | 0-300                      | 92.69±4.26 | N/A  |  |
| H₂S                                 | 0.028-3.19                 | 0.03±0.001 | 0.096±0.014 |  |
| **Nitrification inhibitors**        |                            |           |  |  |
| Allylthioure &                       | 0-0.09                     | 0.04±0.004 | GMC |  |
| Nitrapyrin &                        | 0-21                       | N/A       | 14.17±0.51 |  |
| ClO₃⁻                               | 0-0.6                      | 0.04±0.002 | N/A |  |

*The molecular weights of allylthiourea and nitrapyrin are 116.19 g·mol⁻¹ and 230.91 g·mol⁻¹, respectively.

* GMC, greater than the maximum concentration tested.

* N/A means not available.
3.5 Implications

The anammox process is a promising tool for the treatment of wastewaters with high NH$_4^+$ and low C content. Due to the slow growth of anammox bacteria, exposure of the microorganisms to inhibitory compounds should be avoided in order to ensure a fast start-up and guarantee stable process operation.

Two different inocula, a granular and a SEC characterized by two different anammox strains, were exposed to various toxicants. Both cultures showed similar levels of inhibition by each toxicant applied, with exception of phosphate that caused stimulation of the granular sludge and inhibited the activity of the suspended culture at concentrations from 20 to 100 mM. Among the wastewater constituents studied in this work, H$_2$S and DO are of major importance. Sulfide is generated by decay of biomass and sulfate reduction in anaerobic environments, and it has been shown to cause complete inhibition of the anammox reaction at concentrations as low as 0.3 mM unionized H$_2$S (Fig. 3.3). In effluents where sulfide is present, measures should be taken to remove it prior to anammox treatment, e.g. by addition of iron (III) to precipitate sulfide. Although anammox microorganisms are inhibited in the presence of DO, considerable AA was observed at relatively high DO concentrations, which makes the combination of the
anammox process with partial nitritation feasible to accomplish complete nitrogen removal in a single reactor.

The AA is very dependent on the pH level. A sharp decrease of AA occurred when the pH level shifted 0.3 pH units from the optimum, thus pH control is a critical parameter for the operation of the anammox process.
CHAPTER 4

PRE-EXPOSURE TO NITRITE IN THE ABSENCE OF AMMONIUM STRONGLY INHIBITS ANAMMOX

4.1 Abstract

Anaerobic ammonium oxidizing bacteria (Anammox) are known to be inhibited by their substrate, nitrite. However, the mechanism of inhibition and the physiological conditions under which nitrite impacts the performance of anammox bioreactors are still unknown. This study investigates the role of pre-exposing anammox bacteria to nitrite alone on their subsequent activity and metabolism after ammonium has been added. Batch experiments were carried out with anammox granular biofilm pre-exposed to nitrite over a range of concentrations and durations in the absence of ammonium. The effect of pre-exposure to nitrite alone compared to nitrite simultaneously fed with ammonium was evaluated by measuring the anammox activity and the accumulation of
the intermediate, nitric oxide. The results show that the inhibitory effect was more
dramatic when bacteria were pre-exposed to nitrite in absence of ammonium, as revealed
by the lower activity and the higher accumulation of nitric oxide. The nitrite
concentration causing 50% inhibition was 53 and 384 mg N L$^{-1}$ in the absence or the
presence of ammonium, respectively. The nitrite inhibition was thus 7.2-fold more severe
in the absence of ammonium. Biomass exposure to nitrite (25 mg N L$^{-1}$), in absence of
ammonium, led to accumulation of nitric oxide. On the other hand when the biomass was
exposed to nitrite in presence of ammonium, accumulation of nitric oxide was only
observed at much higher nitrite concentrations (500 mg N L$^{-1}$). The inhibitory effect of
nitrite in the absence of ammonium was very rapid. With 74% loss in activity during the
first 30 min. The results taken as a whole suggest that nitrite inhibition is more acute
when anammox cells are not actively metabolizing. Accumulation of nitric oxide in the
headspace most likely indicates disruption of the anammox biochemistry by nitrite
inhibition, caused by an interruption of the hydrazine synthesis step.

4.2 Introduction

The anaerobic oxidation of ammonium (NH$_4^+$) (anammox) is a novel technology
for the removal of nitrogen pollution from wastewaters. The anammox process is
catalyzed by chemolithoautotrophic bacteria of the phylum Planctomycetes that use
nitrite (NO$_2^-$) as terminal electron acceptor and NH$_4^+$ as an electron donor, allowing for NH$_4^+$ removal in the absence of oxygen (Strous et al., 1999a). Anammox is advantageous over the traditional nitrification-denitrification process for nutrient-N containing effluents since oxygen needs are decreased by up to 57%, and no additional electron donor is needed as would otherwise be the case for denitrification. Unlike other prokaryotes, anammox bacteria have a complex internal compartmentalization. The central organelle, called anammoxosome is the locus of the anammox metabolism (Kartal et al., 2011). The catabolism of anammox bacteria involves the reduction of NO$_2^-$ to nitric oxide (NO) by a nitrite oxidoreductase (NirS). Subsequently, hydrazine synthase enzyme (HZS) forms hydrazine (N$_2$H$_4$) by combining NH$_4^+$ with NO. Lastly, N$_2$H$_4$ is oxidized to dinitrogen gas (N$_2$) by hydrazine dehydrogenase (HDH) (Kartal et al., 2011). The oxidation of N$_2$H$_4$ produces four high energy electrons that are used to generate an intracellular proton gradient which energizes the production of ATP (van der Star et al., 2010).

Inhibition of anammox microorganisms by substrates and intermediates has been extensively studied. NH$_4^+$ has been found to cause low inhibition corresponding to a 50% inhibiting concentration (IC$_{50}$) of 770 mg NH$_4^+$-N L$^{-1}$ (Dapena-Mora et al., 2007). Similarly, the intermediates NO and N$_2$H$_4$ cause little or no inhibition to anammox (Carvajal-Arroyo et al., 2013a, Schalk et al., 1998). On the other hand, different levels of anammox inhibition by NO$_2^-$ have been reported in batch and continuous reactors. Strous et al., (1999b) found complete inhibition of the anammox activity at NO$_2^-$ concentration
of 100 mg N L\(^{-1}\), while other authors have reported higher tolerance to NO\(_2^-\) with IC\(_{50}\) values of 350 mg N L\(^{-1}\) (Dapena-Mora et al., 2007) and 400 mg N L\(^{-1}\) (Lotti et al., 2012). Decreases in nitrogen removal efficiency, due to NO\(_2^-\) overload in a full scale anammox reactor, have also been reported (van der Star et al., 2007). The undissociated species, free nitrous acid (FNA), has been suggested to be responsible for the inhibitory effect of NO\(_2^-\) to anammox bacteria (Fernandez et al., 2012, Jaroszynski et al., 2011). On the other hand other researchers claim that the inhibition is only dependent on the total NO\(_2^-\) concentration (Lotti et al., 2012).

NO\(_2^-\) is known to cause toxicity in a wide variety of microorganisms (Philips et al., 2002). FNA, acting as a protonophore, inhibits the production of adenosine triphosphate (ATP) by disrupting bacterial transmembrane proton gradients (Sijbesma et al., 1996). Inhibition of different enzymes by NO\(_2^-\) has been reported (He et al., 2006, Titov and Petrenko 2003). In some cases NO\(_2^-\) radicals or reactive derivatives are responsible for the toxicity (Hurst and Lymar 1997). The reactive nitrogen species can bind to biomolecules such as the well-known formation of nitrotyrosine from reaction with tyrosine moieties (Monzani et al., 2004).

The mechanisms controlling the inhibitory impact of NO\(_2^-\) on anammox bacteria and the conditions under which NO\(_2^-\) impacts the performance of the anammox process
are still unclear. Therefore, control of NO\(_2^-\) inhibition remains a difficulty in the application of anammox reactors. In this work, the impact of pre-exposing anammox bacteria in granular biofilms to NO\(_2^-\) alone was compared with exposure to NO\(_2^-\) during active metabolism (when NO\(_2^-\) is added simultaneously with NH\(_4^+\)). Moreover, the potential generation of toxic by-products during NO\(_2^-\) exposure was evaluated. The inhibitory effect of NO\(_2^-\) was evaluated in batch assays by comparison of the anammox activity and accumulation of the intermediate NO in anammox cultures previously pre-exposed to NO\(_2^-\) in the presence or absence of NH\(_4^+\).

4.3 Materials and Methods

4.3.1 Origin of the biomass

All the experiments were inoculated with anammox granular sludge cultivated and maintained in a laboratory-scale expanded granular sludge bed (3 L) fed with a synthetic medium at a loading rate of 3.7 g N L\(^{-1}\) d\(^{-1}\). The reactor was originally inoculated with anammox granular sludge provided by Paques BV (Balk, The Netherlands) from a full-scale anammox wastewater treatment plant in The Netherlands. This inoculum was used to start up the reactor which was operated for one year before carrying out the experiments. The volatile suspended solids (VSS) content of the biomass
from the laboratory reactor was 5.69 ±0.04% of the wet weight. The average size of the anammox granules was 2.4±0.6 mm (calculated by image analysis of a photograph of the granular sludge sample using the software ImageJ). Bacteria from the genus *Brocadia* were the dominant anammox microorganisms in the sludge granules (Carvajal-Arroyo et al., 2013a).

4.3.2 **Batch bioassays**

Batch assays were performed in duplicate and incubated on an orbital shaker (160 rpm) in the dark at 30±2°C. Serum flasks (160 mL) were supplied with basal mineral medium (100 mL) and anammox biomass (0.71 g VSS L⁻¹). The mineral medium was prepared using ultrapure water (Milli-Q system; Millipore) and contained the following compounds (mg L⁻¹): NaH₂PO₄·H₂O (57.5), CaCl₂·2H₂O (100), MgSO₄·7H₂O (200), and 1.0 mL L⁻¹ of two trace element solutions. Trace element **solution 1** contained (in mg L⁻¹): FeSO₄ (5,000), and ethylenediamine-tetraacetic acid (EDTA) (5,000). Trace element **solution 2** contained (in mg L⁻¹): EDTA (1,500), ZnSO₄·7H₂O (430), CoCl₂·6H₂O (240), MnCl₂ (629), CuSO₄·5H₂O (250), Na₂MoO₄·2H₂O (220), NiCl₂·6H₂O (190), Na₂SeO₄·10H₂O (210), H₃BO₃ (14), and NaWO₄·2H₂O (50). Either NaHCO₃ (47.6 mM), phosphate (30 mM) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (25 mM) were utilized as buffer systems, as described below. The serum flasks were sealed
with rubber stoppers and aluminum crimp seals. When NaHCO₃ buffer was used, the liquid and the headspace were purged with a gaseous mixture of He/CO₂ (80/20, v/v), resulting in a final pH of 7.1-7.2. In the case of using HEPES buffer, the medium was supplemented with NaHCO₃ (50 mg L⁻¹) as carbon source, and the pH was adjusted to 7.2 with NaOH. Liquid and the headspace was purged with ultra-high purity He. When phosphate buffer was used, NaH₂PO₄ and Na₂HPO₄ were added at a molar ratio of 0.46 and the medium was also supplemented with NaHCO₃ (50 mg L⁻¹). The pH in these experiments was 7.3.

Table 4.1 summarizes the test conditions utilized in the various experiments. The addition of NO₂⁻ and NH₄⁺ to the bioassays was performed following the protocols described below and depicted in Fig. 4.1.

Protocol 1: NH₄⁺ and NO₂⁻ were added simultaneously (“simultaneous exposure”). The experiments were carried out in a mineral medium with NaHCO₃/CO₂ as buffer system.

Protocol 2: Bioassays were supplemented with NO₂⁻ and pre-incubated for different time periods ranging up to 24 h (“NO₂⁻-pre-exposure”). After the pre-exposure period, bioassays were spiked with NH₄⁺. In treatments where the residual concentration
of NO$_2^-$ was lower than 50 mg N L$^{-1}$, NO$_2^-$ was supplemented to attain 50 mg N L$^{-1}$. The experiments were carried out in a mineral medium with NaHCO$_3$/CO$_2$ as a buffering system.

*Protocol 3*: The biomass was pre-exposed to NO$_2^-$ for 24 h. After that period, the biomass was allowed to settle and the liquid was decanted and replaced by 100 mL of mineral medium containing no N-compounds. This process was repeated twice to ensure that no NO$_2^-$ remained in the granules (“washed granules”). Subsequently, the bottles were closed and flushed with He, and NH$_4^+$ and NO$_2^-$ were added simultaneously. In order to avoid pH variations during the manipulation of the flasks, these experiments were carried out in HEPES buffer and in phosphate buffer.

In assays performed according to protocols 2 and 3, controls were included where the biomass was pre-exposed to NH$_4^+$ (76 mg N L$^{-1}$) (in absence of NO$_2^-$) during the “pre-exposure period.” The controls were supplemented with NO$_2^-$ (100 mg N L$^{-1}$) after 24 h of incubation. Likewise, controls were included in which no N-containing substrates were added during the pre-exposure period. These controls were supplemented with NO$_2^-$ and NH$_4^+$ after 24 h of incubation.
Protocol 4: In these experiments, fresh anammox biomass was incubated with anaerobically decanted liquid medium obtained from a nitrite pre-exposure assay. Afterwards the medium was supplemented with NH$_4^+$, the bottles were sealed and purged with He. The experiments were carried out in HEPES- or in phosphate-buffered medium.

Table 4.1. Summary of conditions applied to each experiment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Protocol</th>
<th>Pre-exposure Period</th>
<th>Monitoring Period</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>NO$_2^-$ (mg N L$^{-1}$)</td>
<td>Time (h)</td>
</tr>
<tr>
<td>NO$_2^-$ inhibition in presence of NH$_4^+$</td>
<td>Protocol 1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NO$_2^-$ inhibition in absence of NH$_4^+$</td>
<td>Protocol 2</td>
<td>0-100</td>
<td>24</td>
</tr>
<tr>
<td>Effect of the length of the pre-exposure period</td>
<td>Protocol 2</td>
<td>100</td>
<td>0-12</td>
</tr>
<tr>
<td>Washing Effect</td>
<td>Protocol 3</td>
<td>100</td>
<td>24</td>
</tr>
<tr>
<td>Toxicity of pre-incubated medium</td>
<td>Protocol 4</td>
<td>100</td>
<td>24</td>
</tr>
</tbody>
</table>
In all the cases, samples of the headspace were analyzed for \( \text{N}_2 \) and \( \text{NO} \) at the beginning and at the end of the pre-exposure period, and periodically, after addition of \( \text{NH}_4^+ \). Liquid was sampled after addition of the substrates and at the end of the experiments, for analysis of \( \text{NH}_4^+ \), \( \text{NO}_2^- \) and \( \text{NO}_3^- \).

**Figure 4.1.** Experimental protocols for addition of substrates in batch bioassays.
4.3.3 Analytical methods

Nitrate (NO$_3^-$) and NO$_2^-$ were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a Dionex IonPac AS18 analytical column (4 × 250 mm) and an AG18 guard column (4 × 50 mm). During each run, the eluent (15 mM KOH) was used for 20 min. NH$_4^+$ was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH$_4^+$ electrode (Mettler Toledo, Columbus, OH, USA). N$_2$ was analyzed using a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA, USA) fitted with a Carboxen 1010 Plot column (30 m x 0.32 mm) and a thermal conductivity detector. The temperatures of the column, the injector port and the detector were 220, 110 and 100°C, respectively. Helium was used as the carrier gas and the injection volume was 100 µL. NO was analyzed using a chemiluminescence detector model NOA 280i (General Electric, Fairfield, CT, USA). The VSS content was analyzed according to Standard Methods (APHA, 2005).

4.3.4 Assessment of specific anammox activity and inhibition

The specific anammox activity (SAA) was measured based on the N$_2$ production rate and expressed as g N g VSS$^{-1}$ d$^{-1}$. The SAA was calculated from the maximum slope
of the time course of the $N_2$ concentration in the headspace as follows: $(\text{SAA}) = \Delta N_2 (g \text{ VSS} \Delta t)^{-1}$. The activity of each experiment was normalized with respect to the activity of a control not subjected to inhibitory conditions, normalized anammox activity ($\text{nAA, \%}$) $= (\text{SAA}_{\text{inhibited}}/\text{SAA}_{\text{control}}) \times 100$. The concentration of $\text{NO}_2^-$ causing 50% inhibition ($\text{IC}_{50}$) was calculated by interpolation in the graphs plotting the nAA as a function of the $\text{NO}_2^-$ concentration.

4.4 Results

4.4.1 Inhibition by $\text{NO}_2^-$ in the presence or absence of $\text{NH}_4^+$

Since $\text{NO}_2^-$ is a substrate and inhibitor of anammox bacteria, its impact on the anammox process may be different depending on whether it is being actively metabolized or not. Thus the presence of $\text{NH}_4^+$ may affect the tolerance of anammox bacteria to $\text{NO}_2^-$ inhibition. The effect of $\text{NO}_2^-$ on the nAA of anammox granular sludge was evaluated over a range of $\text{NO}_2^-$ concentrations in the presence of $\text{NH}_4^+$ and compared to the residual nAA of biomass pre-exposed to $\text{NO}_2^-$ in absence of $\text{NH}_4^+$ during 24 h. The inhibitory effect of $\text{NO}_2^-$ was greatly enhanced in the absence of $\text{NH}_4^+$. Fig. 4.2A compares the time course of $N_2$ formation with and without pre-exposure to $\text{NO}_2^-$ (100 mg N L$^{-1}$). In experiments where $\text{NH}_4^+$ and $\text{NO}_2^-$ were fed simultaneously from the beginning, rapid
production of N₂ occurred. However, in experiments where the biomass was first pre-exposed to NO₂⁻ for 24 h prior to the NH₄⁺ addition, there was essentially no N₂ production for 22 h after subsequent addition of NH₄⁺. Fig. 4.2B shows the nAA as a function of NO₂⁻ concentration with and without NO₂⁻ pre-exposure for 24 h. The graph clearly illustrates there is large difference in the impact of NO₂⁻ depending on whether it was pre-exposed or fed simultaneously with NH₄⁺. The IC₅₀ values were 53 and 384 mg NO₂⁻-N L⁻¹ for incubations pre-exposed to NO₂⁻ and simultaneous incubations, respectively. Complete inhibition was observed at 100 and 500 NO₂⁻-N L⁻¹, respectively. Based on the IC₅₀ values, the NO₂⁻ was approximately 7.2-fold more inhibitory when pre-exposed compared to simultaneous feeding. Controls pre-exposed to NH₄⁺ (instead of NO₂⁻) caused no detrimental effect nor did starving the anammox biomass of both NO₂⁻ and NH₄⁺ have any negative impact (Fig. B1; APPENDIX B). Thus it is the exposure to NO₂⁻ alone rather than a short starvation period that was responsible for the impact.
Figure 4.2. Impact of NO$_2^-$ pre-exposure on NO$_2^-$ inhibition. 

A, Time course of N$_2$ production with NO$_2^-$ pre-exposure for 24 h (□) and with simultaneous NO$_2^-$ and NH$_4^+$ feeding (■). The NO$_2^-$ and NH$_4^+$ concentrations used were 100 and 38 mg N L$^{-1}$, respectively. 

B, The effect of NO$_2^-$ concentration on the nSAA after pre-exposure for 24 h to NO$_2^-$ alone (□) or when simultaneously fed with NH$_4^+$ and NO$_2^-$ (■). The maximum SAA in simultaneously fed treatments and pre-exposed treatments, was 0.33±0.02 and 0.40±0.02 g N g VSS$^{-1}$ d$^{-1}$, respectively.
4.4.2 Effect of the duration of the pre-exposure period

A separate experiment was designed in order to determine how rapidly the NO$_2^-$ pre-exposure inflicts full impact. Anammox granular sludge was pre-exposed to NO$_2^-$ (100 mg N-NO$_2^-$ L$^{-1}$) for different periods of time prior to the addition of NH$_4^+$ in order to test the role of pre-exposure time on inhibition of anammox metabolism (Table 4.1, Figure 3). The results obtained show that anammox inactivation by NO$_2^-$ occurred quickly. After only 30 min of pre-exposure to NO$_2^-$ the inhibition was 74%. When the pre-exposure was continued up to 12 h, the observed inhibition approached 100%. (Fig 4.3). Thereafter, further increments in the inhibition required more time to impart further losses in activity.

![Figure 4.3. Effect of the time of pre-exposure to 100 mg NO$_2^-$-N L$^{-1}$ on nAA of anammox sludge in absence of NH$_4^+$. The SAA of the non-pre-exposed control was 0.98±0.02 g N g VSS$^{-1}$ d$^{-1}$.](image-url)
4.4.3 Role of the liquid medium pre-incubated with NO$_2^-$

The strong inhibition observed following exposure of anammox bacteria to NO$_2^-$ pre-exposure (Fig. 4.2) could be due to the formation of toxic byproducts during pre-incubation. To test this hypothesis, anammox bacteria were exposed to decanted culture media obtained from bioassays pre-exposed to NO$_2^-$ for 24 h. The procedure followed in these assays was according to protocol 4 (Fig. 4.1). Likewise, the medium of anammox bacteria pre-exposed to NO$_2^-$ for 24 h was decanted; the biomass was washed and the assays were replaced with fresh medium to determine if the washing reversed the toxicity or if the anammox cells were damaged by the NO$_2^-$ pre-exposure. The experimental procedure followed in these assays was protocol 3 (Fig. 4.1).

Fig. 4.4 compares the nAA of biomass simultaneously fed with NH$_4^+$ and NO$_2^-$ (A), biomass pre-exposed to NO$_2^-$ (B), biomass washed after pre-exposure to NO$_2^-$ (C), and healthy biomass exposed to a medium decanted from a 24 h NO$_2^-$ pre-exposure treatment (D). These bioassays were conducted both in HEPES and phosphate buffer. Washing the biomass after the pre-exposure period caused a moderate relief in the inhibition. The observed nAA after recovery was higher in HEPES buffer (42%) than in phosphate buffer (20%). The use of a medium pre-incubated with NO$_2^-$ caused inhibition of healthy biomass (22% in HEPES buffer, and 39% in phosphate buffer). These results
Figure 4.4. The nAA of simultaneously fed biomass (A), biomass pre-exposed to NO₂⁻ (B), biomass washed after biomass pre-exposure (C), and healthy biomass exposed to a medium decanted from another NO₂⁻ pre-exposed assay (D). Bars indicate the buffer system used in each experiment: HEPES buffer (■), phosphate buffer (□). The SAA of the simultaneous fed controls (A) were 0.80±0.03 and 0.92±0.04 g N g VSS⁻¹ d⁻¹ in HEPES and phosphate buffer, respectively.
4.4.4 *Nitric oxide accumulation*

Accumulation of NO gas, an anammox intermediate (Kartal et al., 2011), was observed in the headspace of bioassays inhibited by exposure to NO$_2^-$ . In experiments where the biomass was exposed simultaneously to NO$_2^-$ and NH$_4^+$ , accumulation of NO was only observed in treatments with very high NO$_2^-$ levels (500 mg NO$_2^-$-N L$^{-1}$) coinciding with conditions in which N$_2$ production was completely inhibited (Fig. 4.5). In contrast, NO gas was detected at much lower NO$_2^-$ concentrations if the biomass was pre-exposed to NO$_2^-$ (Fig. 4.6). Such conditions occurred when the NO$_2^-$ concentration was equal or higher than 25 mg NO$_2^-$-N L$^{-1}$. As shown in Fig. 4.6, the concentration of NO in the headspace of these assays increased with the increasing concentrations of NO$_2^-$ in the pre-exposure period. At 500 mg NO$_2^-$-N L$^{-1}$, the 24 h production of NO in simultaneously fed cultures (Fig. 4.5) was the same as that of 100 NO$_2^-$-N L$^{-1}$ in NO$_2^-$ pre-exposed cultures (Fig. 4.5) suggesting that a 5-fold lower NO$_2^-$ concentration was needed to achieve the same impact on NO production in the pre-exposed cultures.
Figure 4.5. - Time course of N$_2$ production (closed symbols) and NO accumulation (open symbols) at initial NO$_2^-$ concentrations of 57 (diamonds), and 500 (squares) mg NO$_2^-$-N L$^{-1}$ when a simultaneous feeding protocol of NO$_2^-$ and NH$_4^+$ was utilized.

The impact of the duration of NO$_2^-$ pre-exposure (100 mg NO$_2^-$-N L$^{-1}$) on anammox activity and NO accumulation was also evaluated (Fig. 4.7). The increasing durations of the pre-exposure had increasing inhibitory impacts on the anammox activity and caused parallel increases in the NO accumulation. By comparison, NO levels in the control were below the detection limit. At the end of the pre-exposure period, the levels of NO in the headspace were considerably higher in treatments exposed to NO$_2^-$ for longer periods. After addition of NH$_4^+$ (76 mg N L$^{-1}$), the concentration of NO in the gas
phase continued to increase with time in all the pre-exposed treatments suggesting that the anammox bacterial cells were damaged. However, NO levels decreased gradually after 2 to 6 h of incubation after NH$_4^+$ addition, which occurred in synchrony with the moment there was partial recovery in the anammox activity.

![Figure 4.6. NO gas accumulation after 24 h of pre-exposing the anammox biomass to different concentrations of NO$_2^-$ in the absence of NH$_4^+$.](image-url)
Figure 4.7. Time course of N\textsubscript{2} (A) and NO (B) produced after pre-exposing the anammox biomass to 100 mg NO\textsubscript{2}-N L\textsuperscript{-1} for 0 min (●), 15 min (♦), 1h (▲) and 12 h (■).
4.5 Discussion

4.5.1 Influence of physiological state on NO$_2^-$ inhibition of anammox bacteria

The results obtained indicate that the inhibitory effect of NO$_2^-$ to anammox is greatly enhanced in the absence of NH$_4^+$ (Fig. 4.2). The IC$_{50}$ value determined for NO$_2^-$ was 7.2 times lower when the anammox culture was pre-exposed to NO$_2^-$ (non-metabolizing) versus simultaneous exposure to NH$_4^+$ that enables active metabolism. Although a NO$_2^-$ concentration of 100 mg N L$^{-1}$ has been reported to be a safe limit for operation of anammox bioreactors (Jin et al., 2012), the results in this work show that under non-metabolizing conditions, considerable inhibition can occur following pre-exposure to lower NO$_2^-$ concentrations.

Non-metabolic pre-exposure to NO$_2^-$ was shown to cause detrimental impact very quickly, with 74% of the full impact occurring within 30 min of incubation (Fig. 4.3). The results in Fig. 4.3 suggest that in the absence of NH$_4^+$ the initial toxic effect is occurs immediately as NO$_2^-$ contacts the cells. After 1 h of pre-exposure, further contact with the NO$_2^-$ does not cause a proportional decrease in activity. This could be due to two reasons. The first may be an active mechanism of detoxification carried out by cells. The second may be due to additional time required to extensively damage biomolecules. Other
authors have suggested that mass transfer limitation may impact the extent of the inhibition caused by NO$_2^-$.

Suspended biomass has been shown to be more sensitive to NO$_2^-$ than granular sludge (Cho et al., 2010), and the highest tolerance to NO$_2^-$ inhibition, reported in the literature, was observed in anammox biomass embedded in a gel carrier (Kimura et al., 2010).

In order to determine if the toxicity caused by pre-exposure of anammox cells to NO$_2^-$ is irreversible, the cells were washed prior to addition of NH$_4^+$ and NO$_2^-$. The washing caused a modest recovery of the SAA, when compared to the non-washed biomass (Fig. 4.4). This indicates that the NO$_2^-$ toxicity is only partially reversible by washing, confirming that a large portion of the toxicity observed was due to a lasting damage to cells. The low recovery of activity after biomass washing obtained in this work contrasts with the high recovery rates previously reported in the literature. For example, Scaglione (2012) observed almost complete recovery of SAA after pre-exposure to 100 mg NO$_2^-$-N L$^{-1}$ for 48 h, and Lotti (2012) observed that the SAA decrease after exposure to 500 mg N L$^{-1}$ was not higher than 35%. In these two cases the NO$_2^-$ concentration applied after the washing was lowered to 50 mg N L$^{-1}$, while in our case the level of NO$_2^-$ was the same as in the pre-exposure period (100 mg N L$^{-1}$). Other factors such as pH differences may have also contributed to the divergence. The medium pH is known to have a marked influence on NO$_2^-$ inhibition to anammox activity (Jaroszynski et al., 2011). The medium pH used in the current study was 7.2-7.3; whereas
the previous studies applying washing used pH values ranging from 7.5-7.7 (Lotti et al., 2012, Scaglione et al., 2012).

Additionally, activity tests performed with liquid medium recovered from the pre-incubation with NO$_2^-$ (100 mg N L$^{-1}$) showed that this medium inhibited healthy anammox cells (38.8 and 21.5% in phosphate and HEPES buffers, respectively) (Fig. 4.4). These findings indicate that a toxic by-product may have been formed during the pre-incubation period. The difference in the results obtained in HEPES buffer and phosphate buffer may be related to the chemistry of the formation of the toxic by-product, favored in phosphate buffered medium.

The formation of toxic NO$_2^-$ derivatives in biological medium has been previously reported (Philips et al., 2002). NO and intermediates produced by NO$_2^-$ reduction can potentially generate other toxic products like nitrogen dioxide or peroxynitrite anion with high reactivity against biomolecules, including DNA, lipids, or proteins (e.g., tyrosine residues) (Mehl et al., 1999). The reaction products of NO$_2^-$ and different amino acids were shown to be more toxic to activated sludge, than NO$_2^-$ itself (Philips et al., 2002).

4.5.2 *NO accumulation, evidence of disrupted anammox cells*

NO gas accumulated in experiments where NO$_2^-$ inhibition occurred. Five times more NO$_2^-$ were needed to cause NO gas accumulation in experiments with simultaneous
exposure than in NO$_2^-$ pre-exposed treatments. The amount of NO accumulated in the headspace depended on the NO$_2^-$ concentration as well as on the length of the pre-exposure period (Figs. 4.6 and 4.7B).

In anaerobic environments, NO can be generated chemically or, from biological reduction of NO$_2^-$ by denitrification or anammox. NO can be generated chemically from the reaction of Fe$^{2+}$ in the medium and NO$_2^-$ (Kampschreur et al., 2011). The observed dependence of the NO production on the NO$_2^-$ concentration could suggest a chemical reaction as the source of the NO in our assays. Nevertheless, chemical formation of NO was discarded from evidence in abiotic controls containing 100 mg NO$_2^-$-N L$^{-1}$, in which NO could not be detected. Another possible source of NO gas is endogenous denitrification. Experiments where the granular sludge was amended with NO$_2^-$ (100 mg N L$^{-1}$), and hydrogen or methanol at stoichiometric concentrations, were monitored for longer than 24 h, and denitrifying activity could not be detected (Fig. B2; APPENDIX B). Therefore, anammox seems to be the source of the NO. The low amount of NO detected is consistent with the use of an endogenous source of electrons by anammox bacteria. As shown in Fig. 4.7, the accumulation of NO did not cause a complete halt in the N$_2$ production, and therefore the accumulation of NO seems to be an indicator of disruption of anammox metabolism by NO$_2^-$ inhibition, rather than the cause of the inhibition (Kartal et al., 2010b). NO and NH$_4^+$ are the substrates of the enzyme hydrazine synthase which produces N$_2$H$_4$, later oxidized to N$_2$. The accumulation of NO under
conditions of NO$_2^-$ inhibition suggests that this step of the anammox catabolism may be interrupted.

4.5.3 *Hypothesis for NO$_2^-$ inhibition*

The absence of NH$_4^+$ enhances the toxic effect of NO$_2^-$. The big difference in the extent of the inhibition observed under metabolizing conditions or under non-metabolizing conditions (in absence of NH$_4^+$) suggests that the inhibition does not depend only on the NO$_2^-$ concentration but also in the physiological status of the cells.

Three different phenomena could explain this behavior. Firstly, active anammox metabolism is only made feasible in the presence of NH$_4^+$, which provides a sink for NO$_2^-$, lowering its concentration to non-toxic levels. Clearly the accumulation of NO$_2^-$ in the anammoxosome is due in part to this first phenomena since without NH$_4^+$ there will be no metabolism of NO$_2^-$. Consequently it will accumulate and potentially inhibit HZS and as a consequence NO will accumulate as was witnessed in this study. The NO accumulation and anammox inhibition linger even after adding NH$_4^+$.

Secondly, mechanisms of NO$_2^-$ detoxification are probably dependent upon the availability of metabolic energy to pump NO$_2^-$ out of sensitive regions of the cell. If cells
are non-metabolizing, NO\textsubscript{2}\textsuperscript{-}-pumps will not be active. Consequently NO\textsubscript{2}\textsuperscript{-} may not be adequately pumped out of sensitive areas of the cell (e.g. riboplasm, anammoxosome) where lasting damage to biomolecules can potentially be imparted. Anammox catabolism leads to the generation of an intracellular proton gradient between both sides of the anammoxosome membrane (van der Star et al., 2010). NO\textsubscript{2}\textsuperscript{-} active transport proteins (NirC) have been found in the anammox genome (van de Vossenberg et al., 2013), which are H\textsuperscript{+} and NO\textsubscript{2}\textsuperscript{-} symporters dependent on a transmembrane proton motive force. Therefore, the capability of anammox bacteria to actively metabolize NH\textsubscript{4}\textsuperscript{+} and NO\textsubscript{2}\textsuperscript{-}, and maintain the proton gradient, will directly affect the active transport of NO\textsubscript{2}\textsuperscript{-} between the anammoxosome and the other compartments (e.g. riboplasm). Some authors have suggested that the intracellular proton gradient is positive inside the anammoxosome (van der Star et al., 2010, van Niftrik and Jetten 2012), therefore NirC could be involved in NO\textsubscript{2}\textsuperscript{-} detoxification, translocating NO\textsubscript{2}\textsuperscript{-}. In order to validate this mechanism, the role of NO\textsubscript{2}\textsuperscript{-} transport proteins in anammox bacteria, as well as the effect of the pH on NO\textsubscript{2}\textsuperscript{-} inhibition need to be further investigated.

Thirdly NH\textsubscript{4}\textsuperscript{+} may act as a reductant for the proper turn-over of enzymes and their cofactors. Inactivated oxidized enzyme cofactor requires electron equivalents to properly turn over. There is ample evidence that NO\textsubscript{2}\textsuperscript{-} inhibited cells can be rapidly recovered using highly reduced substrates such N\textsubscript{2}H\textsubscript{4} or hydroxyl amine (NH\textsubscript{2}OH) (Bettazzi et al., 2010, Strous et al., 1999b).
The higher permeability of biological membranes to undissociated compounds has led to the belief that free nitrous acid and not the NO$_2^-$ anion causes inhibition of anammox bacteria (Fernandez et al., 2012, Jaroszynski et al., 2011). Nevertheless, the uniqueness of the ladderane anammoxosome membrane (Fuerst et al., 2006) has been suggested to be a barrier for FNA passage (Lotti et al., 2012), and therefore the mechanism of NO$_2^-$ accumulation in the anammoxosome would not depend only on the bulk concentration of free nitrous acid.

4.6 Implications

The anammox process can be inhibited by nitrite. NO$_2^-$ inhibition is not only dependent on the bulk NO$_2^-$ concentration, but also on the physiological status of the cells. The susceptibility of anammox bacteria to inhibition by NO$_2^-$ is higher when NH$_4^+$ is not available. On the other hand, when NH$_4^+$ is actively being metabolized, anammox bacteria have a higher resistance to NO$_2^-$ inhibition. The inhibitory effect of NO$_2^-$ in absence of NH$_4^+$ occurs very quickly, impacting the activity of the cells in a matter of minutes. The detrimental effect of NO$_2^-$ can be partially reverted by washing of the cells. The anammox process is applied in combination with a previous step, of partial nitrification, were approximately half of the NH$_4^+$ is oxidized to NO$_2^-$. This can be done in different configurations (i.e., CANON, SHARON, sequencing batch reactor). This
work shows that the operation of the nitritation step is critical for the safe application of the anammox process. An event resulting in complete oxidation of \( \text{NH}_4^+ \) to \( \text{NO}_2^- \), during the nitritation step, could lead to failure of the anammox process. Or if \( \text{NH}_4^+ \) and \( \text{NO}_2^- \) are being pumped into an anammox reactor from two different sources, a failure of the \( \text{NH}_4^+ \) delivery pump could have a serious inhibitory impact. Strategies must be followed to avoid such events and, in the case that they occur, measures need to be in place to minimize the duration of the disturbance.
CHAPTER 5

THE ROLE OF pH ON THE RESISTANCE OF RESTING- AND ACTIVE ANAMMOX BACTERIA TO NO$_2^-$ INHIBITION

5.1 Abstract

The anaerobic oxidation of ammonium (anammox) uses nitrite as terminal electron acceptor. The nitrite can cause inhibition to the bacteria that catalyze the anammox reaction. Currently there is no consensus on whether free nitrous acid or ionized nitrite is responsible for the toxic effect. This work investigated the effect of the pH and the concentration of nitrite on the activity and metabolism of anammox granular sludge under different physiological conditions. Batch activity tests in a range of pH values were carried out in which either actively metabolizing cells or resting cells were exposed to nitrite in the presence or absence of the electron donating substrate ammonium, respectively. The response of the bacteria was evaluated by analyzing the
specific anammox activity, the accumulation of nitric oxide, and the evolution of the ATP content in the biomass. Additionally, the effect of the pH on the tolerance of the biomass to single substrate feeding interruptions was evaluated in continuous anammox bioreactors. The results show that the concentration of free nitrous acid alone cannot be used to predict the inhibition of actively metabolizing anammox bacteria. At pH higher than 7, the ionized NO$_2^-$ concentration is more predictive of the inhibition. The exposure of resting cells to NO$_2^-$ (100 mg N L$^{-1}$) at pH values below 7.2 caused complete inhibition of the anammox activity. The inhibition was accompanied by accumulation of the intermediate, nitric oxide, in the gas phase. In contrast, just mild inhibition was observed for resting cells exposed to the same NO$_2^-$ concentration at pH values higher than 7.5 or any of the pH values tested in assays with actively metabolizing cells. ATP initially increased and subsequently decreased in time after resting cells were exposed to NO$_2^-$ suggesting an active response of the cells to nitrite stress. Furthermore, bioreactors operated at pH lower than 6.8 had greater sensitivity to NO$_2^-$ during an ammonium feed interruption than a bioreactor operated at pH 7.1. The results suggest that actively metabolizing biomass is resistant to nitrite toxicity over a wide range of pH values; whereas the ability of resting cells to tolerate NO$_2^-$ inhibition is seriously impeded at mildly acidic pH values.
5.2 Introduction

Anaerobic ammonium oxidation (anammox) is novel technology for biological nitrogen removal in \( \text{NH}_4^+ \)-rich, carbon-poor wastewaters. The anammox reaction is catalyzed by chemolithoautotrophic bacteria of the phylum *Planctomycetes* that use ammonium (\( \text{NH}_4^+ \)) as electron donor and nitrite (\( \text{NO}_2^- \)) as terminal electron acceptor (Strous et al., 1999a). The anammox process is advantageous over traditional nitrification-denitrification process since it allows for significant savings in aeration, electron donor used for denitrification is not needed, and, due to their low cell yield, the sludge production is low (Strous et al., 1999a). Anammox cells are divided in different compartments separated by lipidic membranes. The metabolism of anammox bacteria occurs in the anammoxosome (Kartal et al., 2011), the central organelle that is surrounded by a unique ladderane lipid membrane (Fuerst et al., 2006). In this organelle, \( \text{NO}_2^- \) is first reduced to nitric oxide (NO) which is later combined with \( \text{NH}_4^+ \) yielding hydrazine (\( \text{N}_2\text{H}_4 \)). Finally, \( \text{N}_2\text{H}_4 \) is oxidized to dinitrogen gas (\( \text{N}_2 \)). Upon \( \text{N}_2\text{H}_4 \) oxidation, a pH gradient is generated over the anammoxosome membrane that fuels the production of ATP (van der Star et al., 2010).

Among the compounds involved in the anammox reaction, \( \text{NO}_2^- \) has gained attention due to its potential to cause inhibition of anammox bacteria. Evidence of \( \text{NO}_2^- \)
inhibition can be found in various literature reports. In batch experiments, complete inhibition at 100 mg NO$_2^{-}$-N L$^{-1}$ was reported by Strous (1999), while other authors observed 50% inhibition at nitrite concentrations ranging from 350 to 400 mg NO$_2^{-}$-N L$^{-1}$ (Dapena-Mora et al., 2007, Lotti et al., 2012). Similarly to the results obtained with nitrifying bacteria (Anthonisen et al., 1976), NO$_2^{-}$ inhibition of anammox bacteria has frequently been attributed to the undissociated species, free nitrous acid, HNO$_2$ (FNA) (Fernandez et al., 2012). On the other hand, some authors concluded that inhibitory impact of nitrite was caused by the NO$_2^{-}$ anion (Lotti et al., 2012). Anammox activity has been found in a wide pH range (7-9) (Egli et al., 2001) with the optimum at pH 7.2-7.4 (Carvajal-Arroyo et al., 2013a). The pH impacts directly the speciation of HNO$_2$/NO$_2^{-}$, but it can also affect anammox bacteria by altering other metabolic processes which rely on the pH gradients, e.g., energy generation by ATPases or pH dependent active transport proteins, some of which are NO$_2^{-}$ transporters (Lu et al., 2013, van de Vossenberg et al., 2013). Some studies have reported enhanced inhibition of anammox bacteria when NO$_2^{-}$ was supplied alone, in a pre-exposure period, prior to addition of NH$_4^+$ (Carvajal-Arroyo et al., 2013b, Scaglione et al., 2012), suggesting that factors other than the FNA concentration may be implied in the mechanism of NO$_2^{-}$ inhibition.

The conditions affecting the tolerance of anammox bacteria to NO$_2^{-}$ inhibition are not well understood. There is no consensus on the contribution of the unionized FNA and the dissociated NO$_2^{-}$ to the inhibition of the anammox bacteria. In this work, batch and
continuous experiments were carried out to study the influence of the pH on the response
of anammox granular sludge when pre-exposed to NO$_2^-$ alone (resting cells) and when
simultaneously supplied with NH$_4^+$ (metabolically active cells). The effect of NO$_2^-$ was
evaluated by comparing the anammox activity, the accumulation of the intermediate NO
gas, and the ATP content of the sludge under different pH conditions.

5.3 Materials and Methods

5.3.1 Origin of the biomass

Anammox granular sludge was used in all the experiments. The sludge was
cultivated and maintained in a 3-L laboratory-scale expanded granular sludge bed
(EGSB) reactor fed with synthetic medium at a loading rate of 3.7 g N L$^{-1}$ d$^{-1}$. The reactor
was originally inoculated with anammox granular sludge provided by Paques BV (Balk,
The Netherlands) from a full-scale anammox wastewater treatment plant in The
Netherlands. The average size of the granules was 2.4±0.6 mm (calculated by image
analysis using the software ImageJ). The volatile suspended solids (VSS) content of the
biomass from the nursing reactor was 5.69±0.04% of the wet weight.
5.3.2 Batch bioassays

Batch activity tests were performed in duplicate and incubated in an orbital shaker (160 rpm) in a dark climate controlled room at 30±2 °C. The serum flasks (160 mL) were supplied with basal mineral medium (100 mL) and inoculated with 0.71 g VSS L⁻¹ of anammox granules. The mineral medium was prepared using ultrapure water (Milli-Q system; Millipore, Billerica, MA, USA) according to the following recipe (mg L⁻¹): NaH₂PO₄•H₂O (57.5), CaCl₂•2H₂O (100), MgSO₄•7H₂O (200), and 1.0 mL L⁻¹ of two trace element solutions. Trace element solution 1 contained (in mg L⁻¹): FeSO₄ (5,000), and ethylenediamine-tetraacetic acid (EDTA) (5,000). Trace element solution 2 contained (in mg L⁻¹): EDTA (1,500), ZnSO₄•7H₂O (430), CoCl₂•6H₂O (240), MnCl₂ (629), CuSO₄•5H₂O (250), Na₂MoO₄•2H₂O (220), NiCl₂•6H₂O (190), Na₂SeO₄•10H₂O (210), H₃BO₃ (14), and NaWO₄•2H₂O (50). Either NaHCO₃, or 4-(2-hydroxyethyl)-1-piperazinethanesulfonic acid (HEPES) were utilized as buffer systems, as described below. The serum flasks were sealed with rubber stoppers and aluminum crimp seals. In bicarbonate buffered experiments, the amount of NaHCO₃ added (21.2-190.5 mM) was selected to obtain the target pH (6.8-7.8) after purging the liquid and the headspace with a gaseous mixture of He/CO₂ (80/20, v/v). For high pH experiments (8.0-8.2) the liquid and headspace were flushed with ultrahigh purity He. In the case of using HEPES buffer, the medium was supplemented with NaHCO₃ (50 mg L⁻¹) as carbon source, and the pH
was adjusted to 7.2 with a concentrated solution of NaOH. Liquid and the headspace were purged with ultra-high purity He.

The substrates were added by injection of concentrated solutions of NaNO₂ and NH₄HCO₃. In pre-exposure experiments, the bottles were supplemented with either NO₂⁻ or NH₄⁺, and incubated for a “pre-exposure period” of 24 h prior to addition of the missing substrate. In simultaneous exposure experiments, both substrates were fed together to the concentration desired in each experiment.

5.3.3 Continuous bioreactors

Three laboratory-scale UASB reactors (500 mL) were operated in parallel. Each reactor was inoculated with 1.24 g VSS L⁻¹ of anammox granular sludge and incubated in a dark climate controlled room at 30±2 °C. The reactors were fed with a basal mineral medium (described above), at a hydraulic retention time of 0.25 d. The medium was supplemented with NH₄⁺ and NO₂⁻ at 108 and 129 mg N L⁻¹, respectively. For operation at pH values of 6.5 and 7.2, the concentration of NaHCO₃ was 0.5 and 2.5 g L⁻¹ respectively, and the medium was flushed with He/CO₂ (80/20, v/v). During operation at pH 8.3, the medium was supplemented with NaHCO₃ (2.5 g L⁻¹) and flushed with ultrahigh purity He.
The three reactors were operated during 8 days at pH 7.2. Subsequently, the pH of the reactors R1 and R3 was manipulated as described in Table 5.1. In order to study the effect of substrate accumulation in the reactors, the feeding of NH$_4^+$ or NO$_2^-$ to the reactors was interrupted on day 18 and 32 for periods of 48 h.

**Table 5.1.** pH of the influent of the anammox bioreactors during the different operation periods.

<table>
<thead>
<tr>
<th>Reactor</th>
<th>Operation period</th>
<th>Period I 0-8 d</th>
<th>Period II 9-40 d</th>
<th>Period III 40-end</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td></td>
<td>7.2</td>
<td>6.5</td>
<td>7.2</td>
</tr>
<tr>
<td>R2</td>
<td></td>
<td>7.2</td>
<td>7.2</td>
<td>7.2</td>
</tr>
<tr>
<td>R3</td>
<td></td>
<td>7.2</td>
<td>8.3</td>
<td>-</td>
</tr>
</tbody>
</table>

The N$_2$ production was measured by liquid displacement using a 2% (w/v) NaOH solution to scrub the CO$_2$ out of the biogas. The performance of the reactors was monitored by measuring the N$_2$ generation, as well as the pH value and NH$_4^+$, NO$_2^-$ and NO$_3^-$ concentration in the influent and effluent.
5.3.4 ATP extraction and quantification

The granules (0.10-0.12 g wet weight) were disintegrated with a syringe and suspended in 9 mL of boiling Tris-EDTA (20-2 mM) buffer. The closed vials were incubated in boiling water for 2 min and then submerged in an iced-water bath. The ATP in the extract was quantified according to the manufacturer’s instructions using a commercial ATP determination kit (Life Technologies, Grand Island, NY, USA). Bioluminescence was analyzed in a fluorescence spectrometer (Model LS-55, Perkin Elmer, USA), see APPENDIX C. The VSS content of the wet granules in each sample was quantified.

5.3.5 Analytical methods

Nitrate (NO\textsubscript{3}\textsuperscript{-}) and NO\textsubscript{2}\textsuperscript{-} were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Dionex, USA) fitted with a Dionex IonPac AS18 analytical column (4 × 250 mm) and an AG18 guard column (4 × 50 mm). During each run, the eluent (15 mM KOH) was used for 20 min. The flowrate was 1 mL min\textsuperscript{-1}. NH\textsubscript{4}\textsuperscript{+} was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH\textsubscript{4}\textsuperscript{+} electrode (Mettler Toledo, USA). N\textsubscript{2} was analyzed using a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, USA) fitted
with a Carboxen 1010 Plot column (30 m x 0.32 mm) and a thermal conductivity detector. The temperatures of the column, the injector port and the detector were 220, 110 and 100°C, respectively. Helium was used as the carrier gas and the injection volume was 100 µL. NO was analyzed using a chemiluminescence detector model NOA 280i (General Electric, USA). The VSS content was analyzed according to Standard Methods (APHA 2005).

5.3.6 Assessment of specific anammox activity and inhibition

The specific anammox activity (SAA) was measured based on the N₂ production rate and expressed as g N g VSS⁻¹ d⁻¹. The SAA was calculated from the maximum slope of the time course of the N₂ concentration in the headspace as follows: (SAA) = ∆N₂ (g VSS ∆t)⁻¹. The anammox activity in each assay was normalized with respect to the activity of a control not subjected to inhibitory conditions, normalized anammox activity (nAA, %) = (SAA_{inhibited}/SAA_{control}) x 100. The concentration of NO₂⁻ causing 50% inhibition (IC₅₀) was calculated by interpolation in the graphs plotting the nAA as a function of the NO₂⁻ concentration.
5.4 Results

5.4.1 The role of FNA on the inhibition of actively metabolizing anammox bacteria

NO$_2^-$ inhibition of the anammox process has been commonly attributed to FNA due to its higher ability to diffuse through cell membranes (Anthonisen et al., 1976). A set of batch experiments was carried out to explore the effect of NO$_2^-$ and FNA on the activity of actively metabolizing anammox bacteria simultaneously fed NH$_4^+$ and NO$_2^-$. Different concentrations of FNA were applied by alternating combinations of total NO$_2^-$ concentrations (60-800 mg N L$^{-1}$) and pH values (6.6-7.8) in HEPES buffered medium. Figure 5.1A shows that both total NO$_2^-$ and pH affected the SAA. The maximum SAA was observed at a NO$_2^-$ concentration of 160 mg N L$^{-1}$ but higher concentrations caused a linear decrease in the activity of the biomass. The inhibition did not occur instantaneously. Inhibited treatments showed a gradual decrease of the SAA during the first 2.4 h (Fig. C1; APPENDIX C). The pH also influenced the inhibitory effect of NO$_2^-$. When inhibitory levels of NO$_2^-$ were applied, lower IC$_{50}$ values were observed at lower pH, e.g. the IC$_{50}$ at pH 7.4 was 621 mg NO$_2^-$-N L$^{-1}$, whereas at pH 7 the IC$_{50}$ was 442 mg NO$_2^-$-N L$^{-1}$. The SAA was decreased the most with a combination of high NO$_2^-$ concentration and low pH values, which are conditions expected to correspond to the highest FNA levels. Although these findings could potentially be interpreted to mean that
FNA is correlated to anammox inhibition. Figure 5.1B shows that the FNA concentrations are not predictive of the SAA. Very disparate SAA values were observed at equal concentrations of FNA, e.g. no inhibition was observed when FNA was 0.038 mg N L\(^{-1}\) at a pH value of 7, but 71% decrease in activity was observed at 0.039 mg N L\(^{-1}\) when the pH was 7.8. These results show that although in the lower range of pH tested (6.6-7.0) the SAA is well described by the concentration of FNA, at higher pH values, the FNA grossly overestimates the inhibition of the anammox activity.
Figure 5.1. Effect of Total NO$_2^-$ (Panel A) and FNA (Panel B) on the anammox activity at pH $= 6.6$ (□), pH $= 7$ (∗), pH $= 7.3$–$7.4$ (△) and pH $= 7.7$–$7.8$ (○) in metabolically active cells fed NH$_4^+$ and NO$_2^-$ simultaneously.
5.4.2 The role of the pH on the inhibition by NO$_2^-$ pre-exposure (resting cells)

The inhibitory effect of NO$_2^-$ is greatly enhanced when cells are metabolically non-active (resting cells) as observed in experiments where anammox bacteria were pre-exposed to NO$_2^-$ only, prior to the addition of NH$_4^+$ (Carvajal-Arroyo et al., 2013b, Scaglione et al., 2012). In the present study, the effect of pre-exposing the granules to NO$_2^-$ (100 mg N L$^{-1}$) was evaluated at different pH values. After the pre-exposure period, NH$_4^+$ (76 mg N L$^{-1}$) was added, and the pH of each treatment was kept constant. For comparison, the effect of the pH was also evaluated with metabolically active cells where NH$_4^+$ and NO$_2^-$ were supplied simultaneously. The NO$_2^-$ inhibition of resting cells was seriously impacted by the pH. When the medium pH was near 7.1, 100 mg N L$^{-1}$ of NO$_2^-$ caused complete inhibition of anammox resting cells (Fig. 5.2). In contrast, no inhibition occurred if NO$_2^-$ and NH$_4^+$ were fed simultaneously to metabolically active cells at a similar pH. Likewise, no NO$_2^-$-inhibition was observed at pH 7.5, irrespective of whether resting or active cells conditions were used during NO$_2^-$ exposure.
Figure 5.2. Time course of N₂ production by metabolically active anammox cells simultaneously exposed to NH₄⁺ (76 mg N L⁻¹) and NO₂⁻ (100 mg N L⁻¹) at pH 7.03 (▲) and 7.52 (●), and NO₂⁻ pre-exposed resting cells (100 mg N L⁻¹) subsequently supplemented with NH₄⁺ (76 mg N L⁻¹) at pH 7.11 (△) and 7.52 (○).

A series of assays comparing exposure of resting and metabolically active cells to NO₂⁻ (100 mg N L⁻¹) were conducted with over a complete range of pH values. The SAA is plotted as a function of pH for two different buffering systems (NaHCO₃/CO₂ and HEPES) (Fig. 5.3A). Assays inoculated with resting cells pre-exposed to NO₂⁻ were highly inhibited at pH values lower than 7.2. The activity gradually increased with pH, approaching the values in assays simultaneously fed with NH₄⁺ when the pH increased up to the range of 7.5-7.6. This behavior was in stark contrast to the simultaneously fed
treatments which had a high SAA at all pH values including the pH range of 6.8 to 7.2 which was highly inhibitory for the NO_2^- pre-exposed resting cells. The SAA of the simultaneously fed treatments incubated in bicarbonate buffer started to decrease at pH values higher than 7.4, but this was due to the high concentration of NaHCO_3 required (>10 g NaHCO_3 L^{-1}) to maintain mildly alkaline conditions with 20% CO_2 in the flush gas; thereby imposing salt inhibition (Carvajal-Arroyo et al., 2013a). Assays performed in HEPES buffer, on the other hand, provided high SAA activities up to the highest pH value tested of 8.2.

The concentration of NO was measured in the head space of all the experiments. A high concentration of NO gas, up to 1820 ppm, accumulated during the pre-exposure period in the resting cell treatments with pH values lower than 7.4 (Fig. 5.3B). In most treatments, NO accumulation stopped after addition of NH_4^+ with the exception of the treatment with the highest NO level, which continued accumulating NO although at a much lower rate than during the pre-exposure period. Similarly to the SAA, the accumulation of NO resting cell treatments was influenced by the pH. The highest NO concentrations were detected in treatments under pH 7.2, which also showed the highest levels of inhibition. Much lower accumulation of NO was observed as the pH approached 7.4 and higher, corresponding to conditions with the highest SAA activity after the NO_2^- pre-exposure.
Figure 5.3. Effect of the pH on the response of anammox granular sludge to NO$_2^-$ exposure. A, SAA of metabolically active biomass simultaneously exposed to NO$_2^-$ (100 mg N L$^{-1}$) and NH$_4^+$ (76 mg N L$^{-1}$) (closed symbols) and SAA of resting cells pre-exposed for 24h to NO$_2^-$ (100 mg N L$^{-1}$) subsequently supplemented with NH$_4^+$ (76 mg N L$^{-1}$) in bicarbonate buffer (squares) and HEPES buffer (circles). B, Accumulation of
NO gas in the headspace after 24h of pre-exposure to NO$_2^-$ only (100 mg N L$^{-1}$) at different pHs, with HEPES buffer (circles) and bicarbonate buffer (squares). Transient accumulation of trace NO was also observed during incubation of metabolically active cells simultaneously fed NO$_2^-$ and NH$_4^+$ (data not shown).

Trace NO concentrations were also detectable in metabolically active cells, although the maximum NO concentrations observed were two orders of magnitude lower than in resting cells. Instead of accumulation, transitory peaks of NO were observed at pH 7.2 (15 ppm$_v$) and pH 7.0 (27 ppm$_v$), which disappeared after 3 h and 6 h, respectively (data not shown).

Inhibition of anammox bacteria by nitrite may be related to the availability of metabolic energy to the anammox cells, since the inhibition is exasperated when the energy providing NH$_4^+$-substrate is absent in the lower range of pH values suitable for metabolism. Additional experiments were performed to test this hypothesis.

5.4.3 ATP response to NO$_2^-$ stress

The alteration of the metabolism of anammox bacteria due to nitrite- or pH inhibition was studied by measuring the evolution of the ATP content of the biomass in batch tests. In these assays, the bacteria were pre-exposed to 50 mg NO$_2^-$-N L$^{-1}$ under
different pH values (6.6-8.4). For comparison, pre-exposure to NH$_4^+$ (38 mg N L$^{-1}$) in absence of NO$_2^-$ was also evaluated. In both cases, after 24 h of pre-exposure, the stoichiometric concentration of the missing substrate was supplied to all the treatments, and N$_2$ gas production was subsequently monitored.

Figure 5.4 shows the evolution of the ATP content of the biomass together with the time course of N$_2$ production for each treatment. A temporary increase in the ATP content was observed in treatments with NO$_2^-$ pre-exposure having low to circumneutral pH values of 6.6 and 7.1. The ATP content of the biomass peaked after 3.5 h of NO$_2^-$ exposure and subsequently decreased reaching minimum values at the end of the pre-exposure period. The highest ATP peak was measured in the treatments with the pH of 7.1, which showed a maximum SAA of 0.32±0.04 g N g VSS$^{-1}$ d$^{-1}$. Complete inhibition was caused by exposure to NO$_2^-$ at the lowest pH value of 6.6. Although in both treatments the ATP peaked upon addition of NO$_2^-$, at pH 7.1, the ATP content remained at values similar to the initial, whereas in the treatment at pH 6.6, the ATP content remained low after addition of the NH$_4^+$. 
Figure 5.4. Time course of N\textsubscript{2} production (close symbols) and evolution of the ATP content (open symbols) of resting cells pre-exposed to 50 mg NO\textsubscript{2}\textsuperscript{-}-N L\textsuperscript{-1} (A) and 38 mg NH\textsubscript{4}\textsuperscript{+}-N L\textsuperscript{-1} (B) at pH 6.5 (squares), 7.2 (circles) and 8.3 (triangles). The dotted vertical line represents the addition of the missing substrate, in stoichiometric concentration.
In contrast, NH$_4^+$ pre-exposure did not cause an increase in the ATP content of the biomass nor inhibited the anammox bacteria. All the treatments at the highest pH tested (ranging from 8.5-8.6) showed a moderate reduction in the activity, more likely caused by exposure to high pH values rather than nitrite inhibition (Jaroszynski et al., 2011). The results from the batch experiments clearly indicate that exposure of resting cells to NO$_2^-$ alone at slightly acidic pH conditions can potentially be disruptive to the anammox process. Thus such an event should have important consequences during the continuous operation of anammox bioreactors.

5.4.4 Long term effect of pH on the stability of anammox bioreactors

Three continuous lab-scale UASB reactors with anammox granular sludge were utilized to evaluate the role of the pH both during stable operation and during events of substrate interruption. The reactors were operated for 8 days at the same pH (7.2) prior to switching the pH of the influent (Table 5.1). At this point the N$_2$ production (Fig. 5.5A) as well as the removal of NO$_2^-$ and NH$_4^+$ (Fig. 5.6) was similar in the three reactors. After changing the pH of the influent of the reactors R1 and R3 to 6.4-6.8 and 8.1-8.6, respectively, the N$_2$ production of the reactor exposed to high pH values (R3), started to decrease gradually, and approached zero after 8 days. The failure of the reactor could be confirmed by accumulation of NH$_4^+$ and NO$_2^-$ in the effluent as shown in Figure 5.6. In
contrast, applying a low pH to R1 did not affect its N removal capacity, and the reactor showed a N\textsubscript{2} production similar to R2. On day 19 and 20, the reactors were fed with a medium containing just NH\textsubscript{4}\textsuperscript{+} as the only substrate by interrupting the supply of NO\textsubscript{2}\textsuperscript{−}. As a consequence, NH\textsubscript{4}\textsuperscript{+} accumulated in both reactors R1 and R2 (Fig. 5.6A), concomitantly with the disappearance of the N\textsubscript{2} production. The 2-day period of exposure to NH\textsubscript{4}\textsuperscript{+} alone however did not affect the N removal potential of the biomass, in either of the reactors, which both recovered full treatment capacity after restoring NO\textsubscript{2}\textsuperscript{−} in the feeding.
Figure 5.5. Effect of the pH on the performance of UASB reactors subjected to NO2- and NH4+ substrate interruption, respectively. Evolution of the daily N2 production (A) and effluent pH (B) of the reactors R1 (◇), R2(□), R3(●) during different operation periods (I: start up, II: application of different pH to each reactor, III: recovery of optimum pH in R1).
Forcing exposure of the biomass to NO$_2^-$ alone by discontinuing of NH$_4^+$ feed between the days 32-34 caused the N$_2$ production to stop and NO$_2^-$ accumulated up to 128±1 mg N L$^{-1}$ and 119±12 mg N L$^{-1}$ in the effluents of R1 and R2, respectively. These concentrations were approximately the same as those fed to the reactors via the influent. As opposed to sole exposure to NH$_4^+$, the 2-day period of sole exposure to NO$_2^-$ caused irreversible failure of R1, operated at pH values ranging from 6.4-6.8 (Fig. 5.5). Upon reestablishment of NH$_4^+$ in the feeding, both substrates accumulated in the effluent of R1, and no signs of recovery could be observed. Even after the pH of the R1 influent was increased to 7.1 on day 40, the N$_2$ production remained non-detectable, confirming complete disruption of the N removal capacity of the biomass. In contrast R2, which had been operated at pH ranging 7.0-7.4, showed complete removal of NH$_4^+$ and NO$_2^-$ after NH$_4^+$ was supplemented again in the feeding. This immediate recovery of the substrate consumption rate was not immediately reflected in the N$_2$ production, which showed a progressive recovery. Nonetheless full N$_2$ production was restored after 3-4 days.
Figure 5.6. Concentration of NH$_4^+$ (A) and NO$_2^-$ (B) in the influent (close symbols) and effluent (open symbols) of the reactors R1 (diamonds), R2 (squares) and R3 (circles), during different operation periods (I: start up, II: application of different pH to each reactor, III: recovery of optimum pH in R1).
5.5 Discussion

5.5.1 Short term effect of NO$_2^-$ inhibition

The results taken as a whole indicate that FNA alone cannot be used to predict inhibition of anammox activity. As shown in Figure 5.1B, equal concentrations of FNA lead to very different levels of inhibition. On the other hand the inhibitory effect of the NO$_2^-$ was influenced by the pH and higher levels of inhibition were observed at lower pH values (Fig. 5.1A). Other authors have suggested that FNA is responsible for the inhibitory impact of nitrite toward anammox bacteria (Fernandez et al., 2012) on the basis of findings derived from studies with nitrifying and denitrifying bacteria (Anthonisen et al., 1976), but the study did not provide direct evidence to support that hypothesis. The high variability in the response observed under equal concentrations of FNA suggests that the ionized form NO$_2^-$ and the pH affect anammox bacteria independently of the FNA concentration.

Nitrite inhibition is exacerbated when the resting cells are pre-exposed to NO$_2^-$ prior to addition of NH$_4^+$ (Carvajal-Arroyo et al., 2013b). The ability of the resting cells exposed to the same NO$_2^-$ to perform the anammox reaction upon subsequent addition of NH$_4^+$ was very much affected by the pH (Fig. 5.2A). Complete inhibition was observed
when the pre-exposure period took place at pH below 7.2. Some other studies have reported enhanced inhibition caused by pre-exposure to NO$\text{$_2$}^-$ in absence of NH$_4^+$ (Lotti et al., 2012, Scaglione et al., 2012). In these studies the recovery of the activity was generally high (80% and 75%, at 100 and 250 mg NO$\text{$_2$}^-$-N L$^{-1}$, respectively). This can be explained by the relatively high pH applied during the experiments, ranging from 7.5 to 7.8, and a washing of the cells after the pre-exposure which helped in the recovery of the activity. As shown by our results, the pH plays a critical role in the damage caused during pre-exposure of resting cells to NO$\text{$_2$}^-$, and maximum activities occur at pH values over 7.5. Lotti et al., (2012) reported that the level of inhibition during NO$\text{$_2$}^-$ pre-exposure events was not affected by pH values from 6.8-7.8 which seemingly contradicts the findings presented here. Nevertheless, their experiments are not really comparable since their protocol provided a thorough washing of the biomass after the pre-exposure period. Furthermore, both NO$\text{$_2$}^-$ concentration and pH were readjusted to non-inhibitory values (pH = 7.5 and NO$\text{$_2$}^-$ concentration of 50 mg N L$^{-1}$), which allows for evaluating the reversibility of the inhibition rather than analyzing the in situ effect of the cells at the original pH in the medium remaining after pre-exposure.

The inhibitory effect of NO$\text{$_2$}^-$ to resting cells has been shown to occur rapidly, almost in parallel with NO$\text{$_2$}^-$ diffusion through the granules (Carvajal-Arroyo et al., 2013b). In contrast, when NO$\text{$_2$}^-$ is fed together with NH$_4^+$, the inhibition was observed to
occur progressively, as the N₂ production rate decreased during the first 2.4 h, until a steady value for each treatment was reached (Fig. C1).

The occurrence of NO₂⁻ inhibitory conditions resulted in the accumulation of NO gas (Fig. 5.2B). Aside from anammox bacteria, NO gas could also have been formed by chemical reaction of NO₂⁻ and Fe²⁺ (Kampschreur et al., 2011). In the present study, however, chemical production of NO was not detected in abiotic controls. Moreover, NH₄⁺ greatly influenced NO accumulation which would not be expected if NO generation of NO had a chemical origin. NO could also be formed by denitrification or nitrifier denitrification (Kampschreur et al., 2008), but the generation of NO gas was not impacted by supplying H₂ or methanol as electron donors (results not shown). The absence of NO accumulation in the presence of NH₄⁺ further supports the generation of NO by anammox bacteria. The accumulation of NO gas, generated by anammox bacteria under conditions of NO₂⁻ inhibition, suggests an interruption of the metabolic steps following NO₂⁻ reduction, i.e., synthesis of N₂H₄, and/or generation of N₂ gas (Kartal et al., 2011).

The anammox bacteria obtain energy from the transformation of NH₄⁺ and NO₂⁻ into N₂ gas. Therefore, when the anammox reaction is not taking place, a decrease in the ATP content of the biomass could be expected. In contrast, when the resting cells were exposed to NO₂⁻ at pH 6.6 and 7.1, the ATP levels temporarily increased by 1.6 and 1.8
fold, respectively. This response was not observed at high pH or in resting cells pre-exposed to NH$_4^+$. As indicated before the greatest inhibition of resting cells pre-exposed NO$_2^-$ occurred at pH lower than 7.2. These results taken together indicate that the ATP peaks are a response of the cells to NO$_2^-$ stress, which is more evident in the lower range of pH tested. In response to a potentially harmful situation caused by excessive NO$_2^-$, the bacteria may be recruiting energy from food storage for detoxification or for repair of cell damage. As depicted in Figure 5.4A, the ATP content of the biomass pre-exposed to NO$_2^-$ at pH 7.1 remained high after the pre-exposure period, whereas in the inhibited biomass the ATP content became depleted even after addition of NH$_4^+$. This suggests that at low pH values the bacteria need to invest more energy to overcome damage caused by NO$_2^-$.

A similar response to NO$_2^-$ stress was observed in denitrifying poly-phosphate accumulating bacteria (DPAOs) (Zhou et al., 2010). In DPAOs, the application of NO$_2^-$ caused an increase in phosphorus release and in consumption of glycogen, concomitantly with transient increase in the cellular ATP levels. Also in Desulfovibrio vulgaris, NO$_2^-$ stress triggered a series of transcriptional responses, including up-regulation of genes favoring ATP generation by substrate level phosphorylation (He et al., 2006).
5.5.2 Mode of action of $\text{NO}_2^-$ and FNA

$\text{NO}_2^-$ is known to cause toxicity on a wide variety of microorganisms. It has a high affinity for metals in the center of enzymes, and it is very reactive against biomolecules (Philips et al., 2002), causing nitration of moieties in proteins such as the production of nitrotyrosine (Monzani et al., 2004). In some cases it has been found that the reaction products of $\text{NO}_2^-$ (Reactive Nitrogen Species) are even more toxic than nitrite itself (Philips et al., 2002). Furthermore, FNA is hydrophobic and can pass through membranes via passive diffusion (Almeida et al., 1995). Due to this property it has been suggested that FNA could act as a protonophore causing inhibition by disrupting trans-membrane proton gradients in various microorganisms, e.g. denitrifying bacteria (Sijbesma et al., 1996) or even compartmentalized organisms like yeasts (Mortensen et al., 2008).

We have hypothesized that $\text{NO}_2^-$ inhibition of anammox bacteria occurs due to accumulation of $\text{NO}_2^-$ in a sensitive area of the cells (e.g., riboplasm, anammoxosome) (Carvajal-Arroyo et al., 2013b). Accumulation of $\text{NO}_2^-$ can occur through three different mechanisms: i) low $\text{NO}_2^-$ turnover capability in absence of electron donor, ii) inactivation of oxidized enzymes due to the lack of reducing equivalents, and iii) interruption of $\text{NO}_2^-$ detoxification by active pumps dependent on energy originating from trans-membrane
proton motive force. In absence of NH$_4^+$, the NO$_2^-$ is not actively consumed and, therefore, the ability of the cells to avoid inhibition must be controlled by their capability to pump NO$_2^-$ out of the sensitive region. Indeed NO$_2^-$ transport proteins have been found in anammox bacteria (NirC) (van de Vossenberg et al., 2013). This enzyme has been related to detoxification functions in *E. coli* (Lu et al., 2013). Its primary function would be facilitated passive NO$_2^-$ import but, due to the toxicity of NO$_2^-$, it also functions as a facultative secondary active NO$_2^-$ /H$^+$ exporter, keeping low (non-toxic) intracytoplasmic NO$_2^-$ concentrations (Jia et al., 2009). The secondary transporter capability of the enzyme is therefore dependent on the existence of a trans-membrane proton gradient. Thus, environmental conditions reducing the proton motive force will necessarily hinder the NO$_2^-$ export capacity. Interestingly, the presence of toluene was shown to enhance NO$_2^-$ inhibitory effect on anammox bacteria (Hernández et al., 2013), and its role augmenting the permeability of cell membranes was suggested to increase the toxic effect of NO$_2^-$.

As demonstrated by our results, the ability of resting cells to tolerate NO$_2^-$ pre-exposure was very dependent on the pH. Even in the presence of NH$_4^+$, the inhibition was stronger when lower pH values were applied. The activation of the NO$_2^-$ export system by active pumps works at the expense of the proton motive force. While the anammox reaction takes place, the proton motive force is actively maintained (van der Star et al., 2010). Therefore, when the anammox reaction does not occur (e.g. when NH$_4^+$ is absent),
low pH may potentially dissipate residual proton gradients created by endogenous metabolism and, as a consequence, an alternative energy source is needed. Anammox bacteria are known to accumulate glycogen as molecule for energy storage (van Niftrik et al., 2008a). Therefore, glycogen could be used by the cells as source of energy in cases when the anammox reaction is not taking place. This could be enough to overcome mild NO$_2^-$ toxic events, but insufficient when very harsh conditions are imposed.

5.5.3 The effect of NO$_2^-$ and pH during continuous operation

The pH is a critical parameter for the operation of anammox reactors. Both the application of low and high pH values showed advantages and disadvantages. Under low pH conditions, R1 showed a much higher sensitivity to NO$_2^-$ inhibition when that coincided with a 2 d interruption of NH$_4^+$ in the feed and the activity of the biomass could not be recovered afterwards. Just prior to the NH$_4^+$-feed interruption, operation of anammox at pH 6.4-6.8 was suitable for the anammox conversion. The same feed interruption had a very limited impact on the performance of R2 which was operated at pH 7.1. The results indicate that a combination of low pH and a feed interruption of NH$_4^+$ can have grave consequences for anammox bioreactors. Operation of the bioreactors at high pH also caused instability in the anammox bioreactor due to high pH or free ammonia associated with high pH (Jaroszynski et al., 2011).
5.6 Implications

NO$_2^-$ is an inhibitor of anammox bacteria. The inhibitory effect cannot be predicted solely by the concentration of FNA, because ionized NO$_2^-$ may play a role in the observed inhibition, especially when the pH conditions are higher than 7. The sensitivity of the bacteria to NO$_2^-$ inhibition strongly depends on pH, and on the physiological status of the cells. Resting cells are severely inhibited when exposed to NO$_2^-$ at mildly acidic pH values, but they tolerate exposure to NO$_2^-$ when the pH ranges from 7.4 to 7.8. On the other hand, application of pH values above 8 for extended periods of time result in inhibition and ultimate inactivation of the biomass. This information needs to be taken into account during design and operation of anammox bioreactors. In order to avoid failure of the process, the operation must be kept within a fairly narrow window with its optimum near 7.4.
CHAPTER 6

STARVED ANAMMOX CELLS ARE LESS TOLERANT TO NO$_2^-$ INHIBITION

6.1 Abstract

Anaerobic ammonium oxidating (anammox) bacteria can be inhibited by their terminal electron acceptor, nitrite. Serious inhibition of the anammox bacteria by nitrite occurs if the exposure coincides with the absence of the electron donating substrate, ammonium, or mildly acidic conditions. Little is known about the effect of the nitrogen loading rate on the sensitivity of anammox bacteria to nitrite inhibition. Starvation of the biomass may occur during severe underloading in bioreactors or storage of the biomass. This work investigated the effect of starvation on the sensitivity of anammox bacteria to nitrite exposure. Batch activity tests were carried out where anammox biomass subjected to different levels of starvation was exposed to nitrite in the presence and absence of ammonium. The response of the bacteria was evaluated by measuring the specific anammox activity and the evolution of the ATP content in the biomass over time. The
effect of starvation on the tolerance of anammox bacteria to nitrite was further evaluated in continuous bioreactors, by imposing nitrite accumulation (by interrupting the ammonium feeding), after operation at different nitrogen loading rates. The results show that starvation impairs the capacity of anammox cells to tolerate nitrite. The 50% inhibitory concentrations of nitrite in starved- and fresh- resting cells was 7 mg N L\(^{-1}\) and 52 mg N L\(^{-1}\), respectively. Starvation only moderately affected the inhibition caused to active cells, exposed to nitrite and ammonium simultaneously. The ATP content in resting cells increased upon addition of \(\text{NO}_2^-\). The maximum ATP content observed in starved cells was 30% lower than in fresh cells. Moreover, underloading anammox bioreactors decreased their tolerance to nitrite exposure. Accumulation of 107 mg \(\text{NO}_2^-\) - N L\(^{-1}\) after operation at 0.95 g N L\(^{-1}\) d\(^{-1}\) did not cause observable inhibition of the bacteria. On the other hand, relatively similar nitrite levels (101 mg \(\text{NO}_2^-\)-N L\(^{-1}\)) completely disrupted the N removal capacity of the biomass when the reactor was underloaded (0.10 g N L\(^{-1}\) d\(^{-1}\)).

### 6.2 Introduction

The anammox process is the microbial catalyzed oxidation of ammonium (\(\text{NH}_4^+\)) using nitrite (\(\text{NO}_2^-\)) as electron acceptor, generating \(\text{N}_2\) gas as major final product. After its discovery in the early 1990s, the anammox process has been applied to the treatment
of NH₄⁺ rich wastewaters. Due to the chemolithoautotrophic nature of the process and that elemental oxygen (O₂) is not needed, anammox technology is advantageous over conventional on nitrification – denitrification systems, which are costly and energy intensive. Anammox cells have a complex internal organization, with three lipid membranes that divide the cell in several compartments. The central organelle, called anammoxosome, houses the enzymes responsible for the anammox catabolism (Kartal et al., 2011). As a result of their catabolism, anammox bacteria generate a transmembrane proton gradient between both sides of the anammoxosome membrane, which is used for synthesis of ATP (van der Star et al., 2010). Furthermore the anammox bacteria accumulate glycogen, a polymer for energy storage that the anammox bacteria may use for cell maintenance during periods of starvation (van Niftrik et al., 2008).

One of the most intriguing aspects of anammox bacteria is their potential to be inhibited by one of their substrates, NO₂⁻. Although the literature reporting NO₂⁻ inhibition of anammox bacteria is abundant, the mechanism by which it occurs in not known and there is divergence on the threshold levels of NO₂⁻ that cause inhibition (Table 6.1). The physiological status of the cells may affect the resistance of anammox bacteria to NO₂⁻ (Lotti et al., 2012, Scaglione et al., 2012 and Chapter 4), being resting cells more sensitive to NO₂⁻ than metabolically active cells (Chapter 4). Furthermore, the toxic effect caused by NO₂⁻, is enhanced when the pH is low (Chapter 5).
Table 6.1. Reported data about nitrite toxicity on anammox bacteria.

<table>
<thead>
<tr>
<th>Nitrite Concentration (mg NO$_2^{-}$-N L$^{-1}$)</th>
<th>Reduction in activity</th>
<th>Operation Mode</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>100%</td>
<td>SBR*</td>
<td>(Strous et al., 1998)</td>
</tr>
<tr>
<td>350</td>
<td>50%</td>
<td>batch</td>
<td>(Dapena-Mora et al., 2007)</td>
</tr>
<tr>
<td>75</td>
<td>28%</td>
<td>batch</td>
<td>(Bettazzi et al., 2010)</td>
</tr>
<tr>
<td>430</td>
<td>37%</td>
<td>batch, sludge embedded in gel carrier</td>
<td>(Kimura et al., 2010)</td>
</tr>
<tr>
<td>750</td>
<td>100%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>50%</td>
<td>batch</td>
<td>(Lotti et al., 2012)</td>
</tr>
<tr>
<td>185</td>
<td>50%</td>
<td>batch</td>
<td>Chapter 3</td>
</tr>
<tr>
<td>384</td>
<td>50%</td>
<td>batch</td>
<td>Chapter 4</td>
</tr>
</tbody>
</table>

Overload Decrease in nitrogen removal Full scale nitritation-anammox (van der Star et al., 2007)

*SBR: sequencing batch reactor

The available reports on NO$_2^{-}$ inhibition of anammox bacteria are based on batch experiments which utilize biomass from nursing reactors, or observations made on continuous bioreactors, but little is known about the sensitivity of anammox bacteria to NO$_2^{-}$ after being subjected to starving conditions. Starvation can occur in anammox bacteria in underloaded bioreactors or during storage of sludge. Due to the slow growth
of anammox bacteria, new bioreactors are usually started up with enriched biomass from other wastewater treatment plants (Joss et al., 2009, Vlaeminck et al., 2012, Wett, 2006), and the biomass is often stored, remaining inactive during weeks or months. Although studies have been carried out to optimize storage conditions (Vlaeminck et al., 2007), there is no reports on the effect of the starvation on the tolerance of the bacteria to NO$_2^-$.

In this work we evaluated the inhibitory effect of NO$_2^-$ on an anammox enrichment culture subjected to different degrees of starvation. The impact of NO$_2^-$ toxicity and starvation were evaluated by studying the specific anammox activity of fresh and starved biomass after treatments of NO$_2^-$ exposure, as well as the evolution of the ATP content of the biomass during such exposure events. Furthermore, we explored the resilience of continuous anammox bioreactors to events of NO$_2^-$ exposure, operated under different nitrogen loading rates (NLR).
6.3 Materials and Methods

6.3.1 Batch bioassays

Anammox granular sludge (Chapter 5) was used in all the experiments. Batch activity tests were performed in duplicate and incubated in an orbital shaker (160 rpm) in a dark climate controlled room at 30 ± 2 °C. The serum flasks (160 mL) were supplied with 100 mL basal mineral medium (Sun et al., 2011) and inoculated with 0.71 g VSS L\(^{-1}\) of anammox granules. The basal medium was buffered with NaHCO\(_3\) (4 g L\(^{-1}\)). Subsequently the serum flasks were sealed with rubber stoppers and aluminum crimp seals. The liquid and headspace were flushed with a gaseous mixture of He/CO\(_2\) (80/20, v/v), leading to a pH ranging 7.1-7.3.

In experiments with “fresh biomass” the inoculum was withdrawn from the nursing reactor immediately before the preparation of the experiments. In experiments performed with “starved” biomass, the bottles were incubated in absence of N sources for a defined period of time (starvation period), in serum flasks prepared as described above. After the starvation period, the biomass was decanted, washed and replenished with buffered fresh mineral medium. Then the flasks were sealed and purged with He/CO\(_2\) as previously described.
The substrates were added by injection of concentrated solutions of NaNO$_2$ and NH$_4$HCO$_3$. In pre-exposure experiments, the bottles were supplemented with either NO$_2^-$ or NH$_4^+$, and incubated for a “pre-exposure period” of 24 h (resting cells) prior to addition of the missing substrate. In simultaneous exposure experiments (metabolically active cells), both substrates were fed together to the concentration desired in each experiment.

6.3.2 *Continuous bioreactors*

Three laboratory-scale upflow anaerobic sludge blanket (UASB) reactors (500 mL) were operated in parallel. Each reactor was inoculated with 1.43 g VSS L$^{-1}$ of anammox granular sludge and incubated in a dark climate controlled room at 30 ± 2 ºC. The reactors were fed with a basal mineral medium (described above), and operated always at a hydraulic retention time of 0.25 d. The feeding media contained 4 g L$^{-1}$ of NaHCO$_3$, and was flushed with He/CO$_2$ (80:20; v:v) to make it anaerobic and provide a pH of 7.2-7.3. The reactors were operated for 136 days. NO$_2^-$ and NH$_4^+$ were fed to the reactors a molar ratio of 1.2 (NO$_2^-$:NH$_4^+$). The NLR of the reactors was varied in four different stages, 0.95 g N L$^{-1}$ d$^{-1}$ (0-42 d), 0.20 g N L$^{-1}$ d$^{-1}$ (43-82 d), 0.10 g N L$^{-1}$ d$^{-1}$ (83-119 d) and again 0.95 g N L$^{-1}$ d$^{-1}$ (120-136 d). Three times during the operation of the reactors (days 29, 78 and 120), the feeding of either NH$_4^+$ and NO$_2^-$, or both was
interrupted for 48 h. During these interruption events, R1 was fed with medium containing no N compounds; the feed of R2 contained just 129 mg NO$_2^-$ -N L$^{-1}$, but no NH$_4^+$, and the R3 did not receive any NO$_2^-$, and the concentration of NH$_4^+$ in the feeding was 107 mg N L$^{-1}$.

The performance of the reactors was evaluated by monitoring N$_2$ production, and the concentration of NH$_4^+$, NO$_2^-$ and NO$_3^-$ in the influent and effluent of the reactors, as described in (Chapter 5). Furthermore, batch activity tests were periodically carried out with inoculum collected from each reactor. The activity tests were carried out in 25 mL serum flasks, with 14 mL of liquid volume. The preparation of the activity tests was done as previously described.

6.3.3 Analytical methods

Nitrate (NO$_3^-$) and NO$_2^-$ were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Dionex, USA) fitted with a Dionex IonPac AS18 analytical column (4 × 250 mm) and an AG18 guard column (4 × 50 mm). During each run, the eluent (15 mM KOH) was used for 20 min. The flowrate was 1 mL min$^{-1}$. NH$_4^+$ was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH$_4^+$ electrode (Mettler Toledo, USA). N$_2$ was analyzed using
an Agilent 7890 gas chromatograph (Agilent Technologies, USA) fitted with a Carboxen 1010 Plot column (30 m × 0.32 mm) and a thermal conductivity detector. The temperatures of the column, the injector port and the detector were 220, 110 and 100°C, respectively. Helium was used as the carrier gas and the injection volume was 100 µL. The VSS content was analyzed according to Standard Methods (APHA, 2005). The ATP extraction and analysis was performed as in the Chapter 5.

6.3.4 Assessment of specific anammox activity and inhibition

The specific anammox activity (SAA) was measured based on the N\textsubscript{2} production rate and expressed as g N g VSS\textsuperscript{-1} d\textsuperscript{-1}. The SAA was calculated from the maximum slope of the time course of the N\textsubscript{2} concentration in the headspace as follows: (SAA) = \Delta N\textsubscript{2} (g VSS \Delta t)\textsuperscript{-1}. The anammox activity in each assay was normalized with respect to the activity of a control not subjected to inhibitory conditions, normalized anammox activity (nAA, %) = (SAA\textsubscript{inhibited}/SAA\textsubscript{control}) x 100. The concentration of NO\textsubscript{2}\textsuperscript{-} causing 50% inhibition (IC\textsubscript{50}) was calculated by interpolation in the graphs plotting the nAA as a function of the NO\textsubscript{2}\textsuperscript{-} concentration.
6.4 Results and Discussion

6.4.1 Effect of starvation on resistance of anammox resting cells to NO$_2^-$ exposure

The sensitivity of anammox bacteria to the inhibitory effect of NO$_2^-$ has been shown to depend on the physiological status of the cells (Lotti et al., 2012, Scaglione et al., 2012, Chapter 4). Different conditions, such as the absence of NH$_4^+$ (Chapter 4), or the application of low pH in the medium (Chapter 5), which interfere with the mechanisms of generation of the metabolic energy available to the bacteria, have shown to impact the tolerance of the bacteria to NO$_2^-$. Therefore, any factor that can impact the energy status of cells may affect the capacity of the anammox bacteria to tolerate NO$_2^-$. A set of experiments was designed to investigate how starvation could affect the activity of anammox bacteria and their ability to resist inhibition during exposure to NO$_2^-$ in the absence of NH$_4^+$. Figure 6.1 shows the time course of N$_2$ production of fresh and starved anammox biomass, with different modes of substrate feeding including NH$_4^+$ (38 mg N L$^{-1}$) pre-exposure, NO$_2^-$ (50 mg N L$^{-1}$) pre-exposure, and simultaneous feeding of both substrates. Starved cells were highly inhibited by NO$_2^-$ pre-exposure. After addition of NH$_4^+$, the SAA of starved, NO$_2^-$ pre-exposed cells was 0.025 ± 0.010 g N g VSS$^{-1}$ d$^{-1}$. In contrast, NO$_2^-$ pre-exposure did not cause inhibition on fresh cells, which had an SAA
of $0.210 \pm 0.006 \text{ g N g VSS}^{-1} \text{ d}^{-1}$ following NH$_4^+$ addition. The effect of starvation on the SAA to cells simultaneously fed or just pre-exposed to NH$_4^+$ was minor. Therefore, the starvation just compromised the ability of the cells to tolerate NO$_2^-$ in the absence of the energy yielding substrate, NH$_4^+$.

\[\text{Figure 6.1.} \text{ Time course of N}_2 \text{ production of anammox granules pre-exposed for 24 h to 50 mg NO}_2^- \text{ N L}^{-1} \text{ (triangles), 38 mg NH}_4^+ \text{ N L}^{-1} \text{ (squares), or simultaneously fed (circles) after a starvation period of 0 d (close symbols) or 26 d (open symbols).}\]
In order to investigate how fast the bacteria loose their capacity to overcome NO$_2^-$ toxicity under pre-exposure conditions, an experiment was set up after imposing starvation periods from 0-43 d, prior to the pre-exposure treatments. In Figure 6.2, the SAA of simultaneously fed, NH$_4^+$-pre-exposed and NO$_2^-$-pre-exposed granules is plotted against the length of the starvation period applied before the pre-exposure treatments. Anammox cells starved for longer times showed the lowest resistance to NO$_2^-$ inhibition. Most of the ability of the biomass to overcome NO$_2^-$ toxic effect, was lost during the first 10 d of starvation, with a 64% decrease in activity after NO$_2^-$ pre-exposure. The SAA of NO$_2^-$ pre-exposed cells further decreased to 90% after a starvation period of 26 d. On the other hand, the anammox cells that were simultaneously fed or NH$_4^+$-pre-exposed did not lose N conversion capacity after starvation periods of up to 26 d, and their SAA remained fairly constant, in a range 0.18-0.23 g N g VSS$^{-1}$ d$^{-1}$. For the highest starvation period (43 d), the SAA of the biomass in simultaneously fed- and the NH$_4^+$-pre-exposed treatments, decreased by more than 50% when compared to the same experiments performed with fresh biomass. This means that the integrity of the cells was compromised after imposing starving conditions for too long, and therefore part of the inhibition observed under NO$_2^-$ exposure was due to death of cells in the enrichment culture, rather than just NO$_2^-$ effect on activity.
Figure 6.2. SAA biomass pre-exposed to NO$_2^-$ (50 mg N L$^{-1}$) (■), NH$_4^+$ (38 mg N L$^{-1}$) (□) or simultaneously fed with NO$_2^-$ and NH$_4^+$ (■), after different periods of starvation.

Similar results were obtained with *Nitrosomonas europaea*, an NH$_4^+$ oxidizing bacteria. Under NH$_4^+$ limitation, *N. europaea* lost their NH$_4^+$ oxidizing activity, due to NO$_2^-$ toxicity (Stein, Arp, 1998). Moreover the sensitivity of ammonia oxidizers to NO$_2^-$ was shown to be intensified in conditions of NH$_4^+$ starvation (Gerards et al., 1998).
6.4.2 Metabolically active and resting anammox cells respond differently to \( \text{NO}_2^- \) inhibition after starvation

As shown in chapters 4 and 5, anammox bacteria are inhibited by \( \text{NO}_2^- \) through different mechanisms depending on whether \( \text{NH}_4^+ \) is present or absent during the exposure to \( \text{NO}_2^- \). Therefore, the tolerance of metabolically active cells and resting cells to \( \text{NO}_2^- \) inhibition may be impacted differently by starvation. A set of experiments was carried out in which fresh and starved anammox cells were exposed to a range of \( \text{NO}_2^- \) concentrations in the presence of \( \text{NH}_4^+ \) (50-500 mg \( \text{NO}_2^- \cdot \text{N L}^{-1} \)) or in a pre-exposure treatment (0-100 mg \( \text{NO}_2^- \cdot \text{N L}^{-1} \)) prior to addition of the \( \text{NH}_4^+ \). In all these cases, the \( \text{NH}_4^+ \) concentration during the monitoring period was 38 mg \( \text{N L}^{-1} \). In treatments where the concentration of \( \text{NO}_2^- \) during the exposure period was lower than 50 mg \( \text{N L}^{-1} \), additional \( \text{NO}_2^- \) was supplemented at the time of \( \text{NH}_4^+ \) addition to reach 50 mg \( \text{N L}^{-1} \).
Figure 6.3. Effect of starvation on the activity of anammox cells exposed to NO$_2^-$ in presence of NH$_4^+$ (A) or pre-exposed to NO$_2^-$ (B). Closed symbols represent fresh biomass. Open symbols represent biomass starved for 20 d (A) or 14 d (B).
As shown in Figure 6.3A, the ability of starved-resting cells to overcome NO$_2^-$ pre-exposure was very limited when cells were starved. The 50% inhibiting concentration (IC$_{50}$) of NO$_2^-$ to starved-resting cells was $7 \pm 0$ mg NO$_2^-$ -N L$^{-1}$, which is seven times lower than the IC$_{50}$ obtained with fresh (non-starved)-resting granules ($52 \pm 1$ mg NO$_2^-$ -N L$^{-1}$). On the other hand, when the pre-exposure took place in the presence of NH$_4^+$, the IC$_{50}$ of starving cells was only 23% lower than the IC$_{50}$ of fresh granules, which was $384 \pm 0$ mg NO$_2^-$ -N L$^{-1}$ (Fig. 6.3B). The incubation in starving conditions did not affect the integrity of the cells, since the maximum SAA of the biomass was conserved after the respective starvation periods. The dramatic reduction in the resistance of anammox cells to NO$_2^-$ exposure under resting conditions, caused by starvation, suggests that their strategy to mitigate the inhibitory effect of NO$_2^-$ (reduce NO$_2^-$ concentration in sensitive region of the cell) relies on an internal energy source that is depleted during the starvation period. The reduced availability of energy in starved resting cells, would therefore limit their response to NO$_2^-$ toxic concentrations. In contrast, when starved cells are actively metabolizing during NO$_2^-$ exposure, the bacteria are able to tolerate higher NO$_2^-$ levels, given that the N removal capacity is not seriously affected by starvation.
6.4.3 Effect of the starvation on the intensity of the response to NO$_2^-$ stress

In Chapter 5, anammox bacteria were shown to actively respond to NO$_2^-$ stress by increasing intracellular ATP levels. A batch experiment was designed to analyze the effect of starvation on the ATP response to NO$_2^-$ exposure (100 mg N L$^{-1}$) of anammox resting cells. Starved cells produced less ATP than fresh cells (Fig. 6.4). The maximum ATP content of starved cells was 1.54 times higher than initial. The peak was detected 1 h after addition of the NO$_2^-$. Likewise, the ATP content of fresh cells increased after addition of the NO$_2^-$. After 1 h of incubation in the presence of NO$_2^-$, the ATP content of the fresh cells was very similar to that of starving cells, but it kept increasing until reaching a maximum of 2.14 times higher than the initial value after 4 h of NO$_2^-$ exposure. These results are supportive of the idea that anammox cells invest energy to mitigate NO$_2^-$ toxicity. As shown before, starved cells, which generated less ATP, are more sensitive to NO$_2^-$ inhibition than fresh cells. Therefore, the more energy the bacteria are able to recruit for detoxification, the more tolerant they would be to the presence of toxic NO$_2^-$. 

The results indicate that starved biomass is more sensitive to NO$_2^-$ inhibition than fresh biomass. Starvation may happen in continuous reactors, as a consequence of periods
of severe reactor underloading. Therefore, NO$_2^-$ shocks during such periods, may cause serious instability.

**Figure 6.4.** Evolution of the ATP content of anammox cells during a treatment of exposure to 100 mg NO$_2^-$ -N L$^{-1}$, after a starvation period of 0 d (▲) or 19 d (〇).

6.4.4 *Effect of sustained underloading on anammox bioreactors*

Three continuous lab-scale anammox UASB reactors were utilized to evaluate the effect of severe underloading on their ability to recover from NO$_2^-$ exposure events. The three
reactors were initially operated at a NLR of 0.95 g N L\(^{-1}\) d\(^{-1}\). On day 29, the three reactors were subjected to an event of substrate interruption, which lasted 48 h. The influent conditions during this period were as follows: R1 was fed with basal medium containing no N sources, R2 was fed with a medium containing 129 mg NO\(_2\)-N L\(^{-1}\) d\(^{-1}\) as the only N source (Fig. 5), and R3 feeding contained 108 mg NH\(_4\)+-N L\(^{-1}\) d\(^{-1}\) as N source. During these 2 days of substrate interruption no N transformation was observed in any of the reactors and as a consequence, NO\(_2\)\(^{-}\) or NH\(_4\)\(^{+}\) accumulated in the effluent of R2 or R3 respectively. Immediately after reestablishment of the original feeding medium, on day 31, N removal was completely recovered in the three reactors.

On day 43, the NLR of the reactors was reduced to 0.20 g N L\(^{-1}\) d\(^{-1}\) by decreasing the concentration of NO\(_2\)\(^{-}\) and NH\(_4\)\(^{+}\) in the feed. Substrates were again discontinued on day 78, and during this event, 129 mg NO\(_2\)-N L\(^{-1}\) and 108 mg NH\(_4\)+-N L\(^{-1}\) were fed to R2 and R3, respectively. After reestablishment of substrate feeding (0.20 g N L\(^{-1}\) d\(^{-1}\)) on day 80, full NO\(_2\)\(^{-}\) removal was recovered in the three reactors, and only the NH\(_4\)\(^{+}\) excess remained in the respective effluents. The NLR of the three reactors was further reduced to 0.10 g N L\(^{-1}\) d\(^{-1}\) on day 83. The reactors were operated under these conditions for 37 days. Subsequently, a third similar event of substrate interruption was applied on day 120. NO\(_2\)\(^{-}\) (116 mg N L\(^{-1}\)) and NH\(_4\)^{+} (99 mg N L\(^{-1}\)) accumulated in the effluents of R2 and R3 respectively. After 2 days of substrate discontinuing, the original medium (0.95 g N L\(^{-1}\) d\(^{-1}\)) was reestablished in the feeding of the three reactors. The N removal capacity
of R2 was severely reduced due to the exposure to NO$_2^-$ alone during the feed interruption event (Fig. 6.5A). Even after NH$_4^+$ was restored in the feeding of R2, both NH$_4^+$ and NO$_2^-$ accumulated in the effluent, indicating the anammox reaction was severely disrupted. Slow recovery of the N removal could be observed and it attained 42% of the NLR on day 136, when the reactors were stopped.

In contrast, after substrate interruption and subsequent reestablishment of the complete medium, full treatment capacity was recovered in R1 and R3. These results indicate that sustained and severe underloading of the reactors increase the risk of failure due to NO$_2^-$ shocks.

The effect of underloading the reactors was further evaluated by performing periodical batch activity assays with inoculum sampled from the three reactors. The SAA of the biomass of each reactor was measured before and after each event of substrate interruption. As shown in Figure 6.5B, exposure to either NH$_4^+$ or NO$_2^-$, or 2 days of substrate starvation, during operation at 0.95-0.20 g N L$^{-1}$ d$^{-1}$, caused subtle changes in the SAA of the biomass of the reactors. The large standard deviation of the measurements on days 30 and 84 does not allow for further conclusions. Reducing the NLR from 0.95 to 0.20 g N L$^{-1}$ d$^{-1}$, caused a small decrease in the SAA of the granules, but a further reduction in the NLR to 0.10 g N L$^{-1}$ d$^{-1}$ was not translated in an additional decrease of
the SAA. The failure of R2 after the event of the \( \text{NH}_4^+ \) interruption on day 120 could be confirmed by the activity tests, which showed that the biomass was severely inhibited by \( \text{NO}_2^- \). In contrast, the granules in the R1 and R3 were not inhibited by the respective events of substrate interruption (Fig. D1; APPENDIX D), but they benefited from the increase in the NLR on day 120, after which the SAA increased 1.7- and 1.5-fold in R1 and R3, respectively.

The results obtained in the continuous reactors are consistent with the findings from the batch experiments presented before. As shown in Figure 6.5, the sensitivity of the biomass in R2 increased when the reactor was operated at low loading rates, which are conditions expected to cause starvation. The lowest loading rate in R2 ranged 0.07-0.10 kg N kg VSS\(^{-1}\) d\(^{-1}\). Application of similar loading rates in an expanded granular sludge bed reactor was reported to cause starvation of anammox bacteria, as evidenced by a decrease in the heme group content of the biomass (Chen et al., 2013).
Figure 6.5. Influence of NLR on the performance of anammox reactors subjected to events of substrate interruption. **A:** Profiles of $\text{NH}_4^+$ and $\text{NO}_2^-$ concentrations in the influent and effluent of R2. $\text{NO}_2^-_{\text{inf}}$ (solid line), $\text{NH}_4^+_{\text{inf}}$ (dashed line), $\text{NO}_2^-_{\text{eff}}$ (■) and $\text{NH}_4^+_{\text{eff}}$ (○). **B:** Evolution of the SAA of the biomass of the R1 (●), R2 (■) and R3 (▲).
6.4.5 Why does starvation affect the tolerance of anammox cells to NO$_2^-$?

The tolerance of anammox bacteria to NO$_2^-$ is shown impacted by operational conditions such as the absence of NH$_4^+$ (Lotti et al., 2012, Scaglione et al., 2012, Chapter 4) and the application of low pH values (Chapter 5). In absence of NH$_4^+$, the toxic effect of NO$_2^-$ is exacerbated. We have presented two hypotheses to explain the enhanced sensitivity of anammox cells to NO$_2^-$ when NH$_4^+$ is absent. Firstly, during active metabolism the anammox reaction constitutes a continuous sink for NO$_2^-$, avoiding its accumulation inside a sensitive region of the cells, where the inhibition happens (Chapter 4). In the absence of NH$_4^+$ the consumption of NO$_2^-$ is not possible, and therefore NO$_2^-$ accumulates causing toxicity. Secondly, the anammox reaction is used to generate metabolic energy in the form of an intracellular proton gradient between both sides of the anammoxosome (van der Star et al., 2010). The transmembrane proton gradient constitutes the driving force for generation of ATP enabling the proper functioning of active transport proteins located in the anammoxosome membrane. NirC and NarK, are two NO$_2^-$ transporters which have been found in the genome of anammox bacteria (Strous et al., 2006, van de Vossenberg et al., 2013). Although the function of these proteins is not clear, they have been hypothesized to play an important role in the resistance of anammox bacteria to NO$_2^-$ inhibition, as they would actively pump toxic NO$_2^-$ out of the sensitive region of the cells to avoid inhibition (Chapter 4 and 5). The primary energy source fueling active NO$_2^-$ translocation is the anammox reaction, but in
absence of NH$_4^+$, the bacteria may recruit energy from an endogenous source. Anammox bacteria have been reported to store glycogen in the riboplasm (van Niftrik et al., 2008). The glycogen would be used as energy source for cell maintenance during starvation periods, and its role providing energy to maintain the intracellular proton gradient, may be of key importance for the resistance of anammox bacteria to NO$_2^-$ toxic levels. As shown by our results, resting anammox cells responded to NO$_2^-$ stress by promoting the generation of ATP (Fig. 6.4). Moreover, lower peak levels of ATP were observed in starved cells, which were shown to suffer from serious inhibition.

NO$_2^-$ causes inhibition to a wide variety of microorganisms, which have developed different mechanisms of detoxification (Philips et al., 2002). Under conditions of NO$_2^-$ stress, some bacteria have been reported to invest energy to mitigate the inhibitory effect. Denitrifying phosphorus accumulating organisms (DPAOs) and Desulfovibrio spp. have been reported to utilize energy from intracellular energy storage molecules to limit NO$_2^-$ toxicity or mitigate cell damage (He et al., 2006, Zhou et al., 2010). We suggest that when the anammox reaction does not occur, anammox bacteria use the ATP to maintain the intracellular proton gradient, which is a driving force for active NO$_2^-$ transport out of sensitive regions of the cells. This mechanism of defense has been previously described for bacteria suffering inhibition from weak protonophores (weak acids)(Brul, Coote, 1999).
The tolerance of anammox bacteria to NO$_2^-$ is very much influenced by the history of the biomass. This partially explains the high divergence found in literature regarding NO$_2^-$ toxic levels. Anammox cells adapted to treating high loads of N are more resistant to NO$_2^-$ inhibition. On the other hand the ability of starved anammox biomass to withstand NO$_2^-$ shocks is seriously impaired. Therefore strategies must be followed to avoid severe underloading of anammox bioreactors which may occur during dry weather conditions, if bioreactors are oversized or in the treatment of low-strength wastewaters. If an anammox biomass is to be stored due to its application to seasonal wastewaters, or to inoculate new bioreactors, starvation of the inoculum during storage and transportation needs to be minimized. Furthermore, during the start-up, accumulation of NO$_2^-$ in the bioreactors inoculated with starved biomass can cause the failure of the process. The anammox bacteria are specially threatened by NO$_2^-$ when exposure takes place in the absence of NH$_4^+$. Therefore, control strategies need to be implemented to avoid complete oxidation of NH$_4^+$ by nitrifiers, and guarantee a continuous source of NH$_4^+$. 

6.5 Implications
CHAPTER 7  CONCLUSIONS

The anaerobic oxidation of ammonium is a novel and cost-effective biotechnology for the treatment of ammonium rich wastewaters. One of the difficulties inherent to the application of the anammox process to wastewater treatment is that due to the slow growth of anammox bacteria, potential toxic events causing death of biomass will require long recovery periods to reestablish full treatment capacity. Compounds commonly found in wastewaters may pose a threat on the stability of the anammox process. Among the substrates of the anammox reaction, nitrite, the necessary electron acceptor of anammox, is of special concern and may completely inhibit the anammox bacteria under certain conditions. The mechanism of anammox inhibition by nitrite has not been described, nor the conditions under which anammox bacteria are more sensitive to nitrite inhibition.

This research explores the inhibitory effect of several common wastewater constituents on two different enrichment cultures of anammox bacteria. Both sources of inoculum showed similar levels of inhibition by most toxicants studied. On the other hand, PO$_4^{3-}$ stimulated the activity of the granular sludge, although it inhibited the activity of the suspended enrichment culture at concentrations ranging 1.9 to 9.5 g L$^{-1}$. Sulfide, a product of biomass decay and sulfate reduction in anaerobic environments,
caused complete inhibition of anammox bacteria at concentrations as low as 10 mg H_{2}S L^{-1}. Therefore, in effluents where sulfide is present, measures should be taken to remove it prior to anammox treatment, e.g. by addition of iron (III) to precipitate sulfide. Oxygen caused complete inhibition of anammox at 8 mg DO L^{-1}. However, still considerable anammox activity was observed at 2 mg DO L^{-1} which makes it feasible to accomplish complete nitrogen removal in a single reactor combining anammox with partial nitritation.

Among compounds involved in the anammox reaction, only NO_{3}^{-} and nitrite may be found in wastewaters at concentrations potentially harmful to anammox bacteria. NO_{3}^{-} caused moderate inhibition to both enrichment cultures. nitrite was highly inhibitory to both inocula, with IC_{50} values of 151 and 185 mg N L^{-1}, in the suspended and granular cultures, respectively. The inhibitory levels of nitrite are of major concern in systems were nitritation and anammox are carried out in separate units, where nitrite concentrations fed to the anammox reactor are higher than in single reactor systems.

Special attention has been paid to nitrite. Although the inhibition of anammox bacteria by nitrite has been widely reported, there is a great variability on the levels of nitrite causing inhibition. One possible reason is that the tolerance of anammox cells to nitrite, depends on the physiological status of the cells. We have developed a hypothesis
that anammox bacteria invest energy to avoid nitrite inhibition by actively transporting this species away from a sensitive region of the cells. In this dissertation different approaches have been used to disturb the availability of metabolic energy to the anammox bacteria in order to assess its impact on the inhibition response to nitrite exposure. Three approaches were followed. Firstly, the impact of nitrite on the activity of metabolically active anammox cells (simultaneously fed with nitrite and ammonium) was compared to the inhibitory effect caused during exposure to nitrite in the absence of ammonium (resting cells). Secondly, the role of the pH on the resistance of metabolically active- and resting anammox cells to nitrite inhibition was investigated. Thirdly, the inhibitory effect of nitrite was evaluated on starved anammox cells. The findings obtained from batch experiments were further demonstrated in continuous anammox bioreactors.

The nitrite inhibitory effect was enhanced when the exposure took place in the absence of ammonium. The IC$_{50}$ value determined for nitrite was 7.2 times lower in resting cells versus metabolically active cells. The inhibition in resting cells was found to occur very quickly, with 74% loss of activity after only 30 min of exposure to nitrite. The anammox activity was partially recoverable by washing the granules with nitrite free medium, but most of the damage remained in the cells. Moreover, nitrite-containing medium recovered after microbial incubation under ammonium deprivation was found to
cause toxicity to fresh healthy biomass, indicating that a toxic intermediate may be generated during the exposure to nitrite.

The resistance of anammox cells to nitrite inhibition was impacted by the application of low pH values. The inhibitory impact of nitrite on metabolically active anammox cells was moderately enhanced when mildly acidic pH values were applied. The effect of pH on the tolerance of resting cells to nitrite was much more pronounced, e.g., complete inhibition was observed when the exposure to nitrite was conducted at pH 7.1, whereas no activity loss was observed when exposure took place at pH 7.5. Although previous studies have attributed nitrite inhibition to the undissociated free nitrous acid (FNA), the results in this work demonstrate that FNA is poorly predictive of the inhibition. Especially at the higher range of pH tested (7.3-7.8), the resistance of anammox bacteria to nitrite inhibition was a function of the medium pH, irrespective of the FNA concentration.

The inhibitory effect of nitrite was further investigated in starved anammox cells. Starvation reduced the ability of anammox cells to tolerate nitrite. The loss in nitrite tolerance during starvation occurred relatively quickly. Biomass starved for 10 and 26 d showed a 64% and 90% inhibition after being exposed to 50 mg NO$_2^-$-N L$^{-1}$ in the absence of ammonium, respectively. In contrast no inhibition was observed in parallel
experiments carried out with fresh biomass. Metabolically active and resting anammox cells responded differently to nitrite inhibition after the starvation. The IC$_{50}$ of nitrite in resting-starved cells was 7 times lower than in resting-nonstarved cells. On the other hand, the IC$_{50}$ of nitrite in metabolically active cells only decreased 23% due to starvation.

Nitric oxide (NO) gas was observed to accumulate in the headspace of experiments with resting cells subjected to nitrite exposure. The amount of NO recovered increased with the concentration of nitrite in the medium and the length of the nitrite exposure period, and was dependent on the pH of the medium, i.e., the concentration of NO measured in the head space of experiments carried out at pH 7.1 was 70 times higher than at pH 7.5. NO generated during the nitrite exposure period was consumed synchronously with the recovery of the anammox activity after addition of ammonium. Moreover, during nitrite exposure of metabolically active cells, five-fold more nitrite was needed to cause the same impact on NO production than with resting cells. In general, higher NO accumulation corresponded with higher inhibition rates and, therefore, the accumulation of NO gas was interpreted as an evidence of biochemical disruption in the anammox cells.
Anammox bacteria were found to respond actively to nitrite stress. Analysis of the evolution of the ATP content of the biomass showed temporal increase of ATP content in cells after nitrite was supplied in the absence of ammonium. The observation of ATP concentration peaks (with respect to time) were associated with conditions previously shown to cause stress to the bacteria (evidenced by inhibition of the anammox activity and accumulation of NO gas), indicating an active response of the bacteria to overcome nitrite stress. Interestingly, the ATP response measured in starved cells was much lower than in fresh cells, suggesting that the energy source used for the generation of ATP was partially depleted during the incubation under starvation conditions.

The stability of continuous anammox reactors as influenced by the pH and starvation was investigated. A bioreactor operated at pH 7.1 tolerated an event of ammonium feeding interruption causing nitrite accumulation. Under these conditions, the biomass was able to withstand nitrite exposure, and the reactor recovered full treatment capacity as ammonium was reestablished in the feeding. In contrast, a parallel reactor operated at pH ranging 6.4-6.7, was strongly impacted by the nitrite that accumulated after depriving the ammonium feeding. Complete inhibition followed the nitrite exposure, and N removal could not be recovered after ammonium was fed again to the reactor, nor after readjusting the pH to 7.1.
Underloaded anammox bioreactors were shown to be more prone to failure due to nitrite inhibition. When a reactor was operated at a N loading rate of 0.10 g N L$^{-1}$ d$^{-1}$, nitrite exposure due to the absence of ammonium feeding caused inhibition of the biomass in the reactor, which was translated in complete loss of the N removal capacity. Partial recovery of the treatment capacity occurred slowly during the weeks following the toxic event. On the other hand, when the reactor was operated 0.20-0.95 g N L$^{-1}$ d$^{-1}$, a similar event of nitrite exposure was tolerated by the biomass and no signs of instability were observed.

The work in this dissertation revealed different conditions under which anammox bacteria are particularly sensitive to nitrite inhibition, *i.e.* absence of ammonium, mildly acidic pH, and starvation. The mechanisms by which these conditions enhanced nitrite toxicity were discussed. Firstly, ammonium is the electron donor of the anammox reaction, enabling for active consumption of nitrite in actively metabolizing cells, reducing its intracellular concentration to non-toxic levels. Therefore, if ammonium is not available, nitrite will accumulate in sensitive regions of the cells, causing toxicity. Secondly, we hypothesized that anammox bacteria avoid nitrite inhibition by pumping it away from sensitive regions of the cells. Secondary active nitrite transporters - NirC and NarK - have been found in anammox bacteria, which could play an important role in detoxification of nitrite. These transport proteins rely on a proton motive force to actively translocate nitrite. The absence of active metabolism, only possible in the presence of
ammonium, fueling the generation of an intracellular proton gradient, or the application of low pH in the bulk medium, could disrupt the proton gradient that enables active nitrite transport and, therefore, limit the ability of the anammox bacteria to overcome nitrite exposure. When the cells were exposed to nitrite in the absence of active metabolism, the anammox bacteria recruited energy in form of ATP, which could be used to maintain the intracellular proton gradient. When cells were starved, the ATP response was less intense than in fresh cells, indicating that the endogenous energy source supporting the production of ATP was partially depleted. Anammox bacteria have been found to accumulate glycogen to be used for cell maintenance during periods of starvation. Therefore, the depletion of the glycogen pool caused by starvation may compromise the ability of starved cells to overcome nitrite inhibition.

The new insights about the mechanisms of nitrite inhibition, provided in this work, enable the design of operation and control strategies to minimize the risk of failure in anammox bioreactors. In systems where partial nitritation and anammox are physically separated, ammonium feeding to the anammox reactor should be always ensured in order to avoid exposure of the anammox biomass to nitrite in the absence of the electron donor. Operation of the bioreactors at the lower range of pH tested (<7.2) is not recommended, as anammox bacteria have shown more sensitive to nitrite than at mildly alkaline pH values. Additionally, starved cells are less tolerant to nitrite than fresh cells. Therefore when anammox bioreactors are started up with enriched inoculum from other plants, the
length of the storage and shipping period of the inoculum needs to be minimized. Furthermore, the accumulation of nitrite inside anammox bioreactors during the start-up should be avoided to minimize the chances of inhibition of the delicate starved anammox inoculum.
APPENDIX A. Supplementary Data for CHAPTER 3

Basal Medium

The basal mineral medium was prepared using ultrapure water (Milli-Q system; Millipore) and contained the following compounds (mg l\(^{-1}\)): \(\text{NH}_4\text{HCO}_3\) (213.6), \(\text{NaNO}_2\) (246.4), \(\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}\) (57.5), \(\text{CaCl}_2 \cdot 2\text{H}_2\text{O}\) (100), \(\text{MgSO}_4 \cdot 7\text{H}_2\text{O}\) (200), \(\text{NaHCO}_3\) (2,500), and 1.0 mL l\(^{-1}\) of two trace element solutions. Trace element solution 1 contained (in mg l\(^{-1}\)): \(\text{FeSO}_4\) (5,000), and ethylenediamine-tetraacetic acid (EDTA) (5,000). Trace element solution 2 contained (in mg l\(^{-1}\)): EDTA (15,000), \(\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}\) (430), \(\text{CoCl}_2 \cdot 6\text{H}_2\text{O}\) (240), \(\text{MnCl}_2\) (629), \(\text{CuSO}_4 \cdot 5\text{H}_2\text{O}\) (250), \(\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}\) (220), \(\text{NiCl}_2 \cdot 6\text{H}_2\text{O}\) (190), \(\text{Na}_2\text{SeO}_4 \cdot 10\text{H}_2\text{O}\) (210), \(\text{H}_3\text{BO}_3\) (14), and \(\text{NaWO}_4 \cdot 2\text{H}_2\text{O}\) (50).

Analytical methods

Nitrate (\(\text{NO}_3^-\)) and nitrite (\(\text{NO}_2^-\)) were analyzed by suppressed conductivity ion chromatography using a Dionex IC-3000 system (Sunnyvale, CA, USA) fitted with a
Dionex IonPac AS18 analytical column (4×250 mm) and an AG18 guard column (4×50 mm). During each run, the eluent (10 mM KOH) was used for 20 min. NH$_4^+$ was determined using a Mettler Toledo SevenMulti ion selective meter with a Mettler Toledo selective NH$_4^+$ electrode (Mettler Toledo, Columbus, OH, UAS). N$_2$ was analyzed using a Hewlett Packard 5890 Series II gas chromatograph (Agilent Technologies, Palo Alto, CA) fitted with a Carboxen 1010 Plot column (30 m x 0.32 mm) and a thermal conductivity detector. The temperatures of the column, the injector port and the detector were 220, 110 and 100°C, respectively. Helium was used as the carrier gas and the injection volume was 100 µl.

**Molecular characterization of the inocula**

The anammox bacteria in the anammox suspended enrichment culture (SEC) and the anammox granular enrichment culture (GEC) were characterized by generating a clone library. Community genomic DNA was extracted using the FastDNA Spin Kit for Soil (Qbiogene, Inc, Carlsbad, CA) (Sun et al., 2009). The presence of anammox bacteria was confirmed by PCR using specifically designed PCR primer set to target the 16S rRNA gene of anammox bacteria, PLA46F and AMX-820R, which was described in the previous study (Sun et al., 2011). The purified PCR products with primers of PLA46F
and AMX-820R were cloned into plasmid vector pCR 2.1-TOPO using the TOPO TA cloning system (Invitrogen, Carlsbad, CA) to build a clone library. The details of cloning and sequence analysis have been described in the previous study (Sun et al., 2011). The number of clones analyzed for each culture was determined using a rarefaction curve to estimate the diversity as previously reported (Sun et al., 2011). The clones were clustered into phylotypes on the basis of sequence similarity > 99%. Sequence data were aligned with ClustalX, including 16S rRNA gene sequences from reference bacterial strains (GenBank) and unique phylotypes recovered from anammox suspended enrichment and granular sludge, and a tree was constructed using PAUP* version 4.0b10. Two unique phylotypes were identified, one for each enrichment culture, which had a very high similarity (99.5% or higher) with the 16S rRNA gene sequence of species from the genus Brocadia (Fig. A1). The sequences of these clones have been deposited in the GenBank database. The GenBank accession numbers for the sequences used to prepare phylogenetic trees are shown as follows: SEC - Candidatus Brocadia sp. enrichment culture clone MBR-EC-1, accession # JQ691616; GEC - Candidatus Brocadia sp. enrichment culture clone ANA-GR-4, accession # JQ691617; Candidatus Brocadia caroliniensis strain, accession # JF487828; Candidatus Brocadia sp. 40, accession # AM285341; Candidatus Brocadia sp. enrichment culture clone RAS-Ina-1, accession # HM769652; Candidatus Brocadia sp. enrichment culture clone ODS-1, accession # HM769653; Candidatus Brocadia fulgida, accession # DQ459989.
**Figure A1.**- Phylogenetic tree for the anammox bacteria identified detected in the anammox enrichment cultures utilized in this study.

**Calculation of the concentration of unionized H2S**

The concentration of unionized H$_2$S was calculated the equation below:

$$\left[H_2S\right] = \frac{\left[Na_2S\right]_{added}}{\left(H\frac{V_{HS}}{V_L} + \frac{K_{al}}{10^{-pH}} + 1\right)}$$

Where:

- $H$ is the dimensionless Henry’s constant for H$_2$S at 30ºC, (0.4543),
- $V_{HS}$ is the head space volume,
$V_L$ is the liquid volume,

$K_{a1}$ is the equilibrium constant of the first dissociation of H$_2$S, $(1.023 \cdot 10^{-7})$.

References


APPENDIX B. Supplementary data for CHAPTER 4

Effect of pre-exposure to NH$_4^+$ in absence of NO$_2^-$

NO$_2^-$ pre-exposure was studied and discussed in the manuscript. In order to discard side effects, exposure to NH$_4^+$ was also studied. The biomass was incubated for 24h in presence of NH$_4^+$ (76 mg N L$^{-1}$). After the pre-exposure period, NO$_2^-$ was supplemented (up to 100 mg N L$^{-1}$). As shown in Figure B1, neither exposing the anammox granular sludge to NH$_4^+$, nor starving biomass for 24 h had a negative effect on the SAA. Non-pre-incubated control had a SAA of 0.92±0.02 g N g VSS d$^{-1}$ and NH$_4^+$ pre-exposed biomass showed an SAA of 0.90±0.00 g N g VSS d$^{-1}$. The biomass starved for 24 h, showed a SAA of 0.92±0.04 g N g VSS d$^{-1}$. 
Figure B1.- Time course of N\textsubscript{2} production of non-pre-incubated biomass (◆), biomass pre-incubated in absence of N compounds (■), and biomass pre-exposed to NH\textsubscript{4}\textsuperscript{+} for 24 h (○).

**Denitrifying activity of the anammox granular sludge**

The anammox granules were tested for denitrification. Batch experiments were carried out were the biomass was incubated in presence of NO\textsubscript{2}\textsuperscript{-} (100 mg N L\textsuperscript{-1}) and stoichiometric amounts of H\textsubscript{2} gas or methanol, as electron donors for denitrification.
As shown in Figure B2, the production of $N_2$ by the anammox granular sludge was not stimulated in presence of electron donors that could be potentially used by denitrifiers. The $N_2$ production by biomass incubated in presence of $NO_2^-$ only, as well the one incubated in presence of $H_2$ or methanol, was almost inexistent. The NO accumulation was also very similar in the three cases.
APPENDIX C. Supplementary data for CHAPTER 5

Measurement of Bioluminescence

50 µL of ATP extract were added to 450 µL of reagent (ATP determination kit, Life Technologies, USA) and immediately analyzed. Quartz cuvettes of 500 µL were used. Bioluminescence was analyzed in a fluorescence spectrometer (Model LS-55, Perkin Elmer, USA) equipped with a Total Emission mirror. The apparatus was set in bioluminescence mode, (Total Emission Mirror = IN), with excitation source turned off. Delay time = 0 s, Gate time = 180 ms, Cycle time = 200 ms, Flash Count = 1. The slits for excitation (off) and emission were 15 and 20 nm, respectively; Response = 4s, Interval = 1s. The photomultiplier was set to 900V for maximum sensitivity. (See Perkin Elmer, Fluorescence Applications).
**NO$_2^-$ inhibition of active anammox cells**

When NO$_2^-$ and NH$_4^+$ were fed together, the inhibitory effect due to high NO$_2^-$ concentrations did not occur instantaneously, but the N$_2$ production rate decreased progressively during the first 2.4 h (Fig. C1).

**Figure C1.**- Timecourse of N$_2$ production of cells simultaneously fed with NH$_4^+$(76 mg N L$^{-1}$) and NO$_2^-$ (400 mg N L$^{-1}$, circles; 600 mg N L$^{-1}$, squares; 800 mg N L$^{-1}$, triangles) at different pH values (7, close symbols; 7.4, patterned symbols; 7.8, open symbols).
The influence of NLR on the tolerance of anammox bacteria to NO$_2^-$ exposure was studied in anammox upflow bioreactors. Three reactors were operated in parallel with decreasing NLR from 0.95 g N L$^{-1}$ d$^{-1}$ (days 0-43), 0.2 g N L$^{-1}$ d$^{-1}$ (days 43-83) and 0.1 g N L$^{-1}$ d$^{-1}$ (days 83-end). The reactors were subjected to one event of substrate interruption during each of the NLR periods, on days 29, 78 and 121. During these events, which lasted 48 h, R1 was fed with a medium containing no N sources, the medium fed to R2 contained just NO$_2^-$ (129 mg N L$^{-1}$) as N source and R3 was fed with a medium containing just NH$_4^+$ (108 mg N L$^{-1}$). Reactors 1 and 3 were used as control reactors (Fig. D1), to explore whether starvation during the substrate interruption events would hinder the N removal capacity. R1 and R3 were never exposed to NO$_2^-$ in the absence of NH$_4^+$. The removal capacity of the reactors 1 and 3 was not affected by interruption of the substrates. The removal of NO$_2^-$ was near 100% during the complete operation period.
Figure D1.- Profiles of NH$_4^+$ and NO$_2^-$ concentrations in the influent and effluent of control reactors R1 and R3. NO$_2^{\text{inf}}$ (solid line), NH$_4^{+\text{inf}}$ (dashed line), NO$_2^{\text{eff}}$ (■) and NH$_4^{+\text{eff}}$ (○).
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Additional Sources:


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