fermentation

MDPI

Article

Effect of Nitrite and Temperature on Autotrophic Denitrification in Anammox Granular Biomass from a Partial Nitritation–Anammox Reactor

Benjamín Ibarra [1], Yves Lesty [2], Mateo Pastur [3], Celia Castro [4], Chloe Girard [2] and Rolando Chamy [1,*]

[1] Núcleo Biotecnología Curauma, Pontificia Universidad Católica de Valparaíso, Av. Universidad 330, Valparaíso 2373223, Chile; benjamin.ibarra.r@mail.pucv.cl
[2] Aguas Andinas S.A., Presidente Balmaceda 1398, Santiago 8340083, Chile; ylesty@aguasandinas.cl (Y.L.); cgirard@aguasandinas.cl (C.G.)
[3] Cetaqua Chile, Los Pozos 7340, Las Condes 7570475, Chile; mateo-bruno.pastur@cetaqua.com
[4] Cetaqua Barcelona, Ctra. d'Esplugues, Cornellà de Llobregat, 08940 Barcelona, Spain; celia-maria.castro@cetaqua.com
* Correspondence: rchamynbc@gmail.com

Abstract: Anaerobic ammonium oxidation (anammox) is a key process in the removal of nitrogen from wastewater, in which episodes of substrate inhibition may occur. In this study, the effect of nitrite on anammox in the short and long term was investigated using granules from a full-scale SBR reactor in operation. In the short term, maximum activity was achieved at 100 mg $N\text{-}NO_2^-/L$, with higher concentrations being inhibitory. It was determined that the biomass behavior is well interpreted (R^2 = 0.955) by a non-competitive substrate inhibition model (Andrews model), with a K_S of 55.6 mg $N\text{-}NO_2^-/L$ and a K_I of 116.7 mg $N\text{-}NO_2^-/L$, and also well interpreted by the Edwards model (R^2 = 0.957), with a K_S of 36 mg $N\text{-}NO_2^-/L$ and a K_I of 287 mg $N\text{-}NO_2^-/L$. In the long term, the biomass retained its anammox activity at 15 mg $N\text{-}NO_2^-/L$ over a three TRH horizon; however, at 30 mg $N\text{-}NO_2^-/L$, anammox activity decreased by 50% at the end of the experiment. Finally, the effect of temperature on the activity of the anammox granules from a different source was studied, revealing that the activity increases with temperature within the range of 25–35 °C, which can be useful if a rapid increase in activity is desired. Operationally, maintaining nitrite below 30 mg $N\text{-}NO_2^-/L$ ensures stability, while exceeding 100 mg $N\text{-}NO_2^-/L$ causes immediate SAA inhibition and slower recovery.

Keywords: anammox; nitrite; inhibition; temperature; kinetic

Citation: Ibarra, B.; Lesty, Y.; Pastur, M.; Castro, C.; Girard, C.; Chamy, R. Effect of Nitrite and Temperature on Autotrophic Denitrification in Anammox Granular Biomass from a Partial Nitritation–Anammox Reactor. *Fermentation* **2024**, *10*, 637. https://doi.org/10.3390/fermentation10120637

Academic Editors: Minhua Cui and Zechong Guo

Received: 11 November 2024
Revised: 8 December 2024
Accepted: 11 December 2024
Published: 12 December 2024

1. Introduction

The removal of nitrogen in wastewater treatment plants (WWTPs) is essential to mitigate the environmental impacts associated with excess nitrogen, such as eutrophication in aquatic ecosystems. This over-enrichment of water bodies promotes the growth of harmful algal blooms, which can deplete oxygen levels and create hypoxic "dead zones" that devastate aquatic life by causing fish kills and reducing biodiversity. These effects are particularly severe in coastal and freshwater systems, leading to significant ecosystem disruptions [1].

For humans, nitrate contamination in drinking water poses serious health risks. Elevated nitrate levels can lead to methemoglobinemia, or "blue baby syndrome", which is especially dangerous for infants. Furthermore, long-term nitrate exposure has been associated with an increased risk of certain cancers, such as colorectal and thyroid cancer, as well as adverse reproductive outcomes due to the formation of carcinogenic N-nitroso compounds in the body [2].

Traditionally, nitrogen removal has been achieved through a two-stage process. The first stage involves nitrification, where ammonia is converted to nitrite and then to nitrate.

The second stage, known as denitrification, converts nitrate into gaseous nitrogen, allowing it to escape from the liquid phase into the atmosphere, thereby reducing the nitrogen load in wastewater.

In recent years, increasingly stringent nitrogen discharge regulations have necessitated advancements in nitrogen removal technology. These regulations aim to limit the release of harmful nitrogen compounds into the environment, promoting cleaner water systems. Conventional biological methods, such as nitrification and denitrification, have been widely used in both domestic and industrial wastewater treatment facilities to achieve these goals. However, these traditional processes can be energy-intensive due to the need for aeration and additional carbon sources for effective denitrification, leading to higher operational costs [3].

As a response to these challenges, alternative nitrogen removal methods have been explored. Autotrophic denitrification, performed by bacteria that can oxidize ammonia under anaerobic conditions using nitrite as the final electron acceptor, has emerged as a promising solution. This process, known as anammox, was first identified in the Netherlands in 1990 and has since gained recognition for its high efficiency in removing nitrogen. anammox-based processes offer significant advantages over traditional nitrification/denitrification (N/DN) processes, including a theoretical reduction in aeration requirements by 60%, the complete elimination of the need for added organic carbon, and a 90% reduction in sludge generation [3]. These benefits highlight the potential for anammox technology to revolutionize nitrogen removal in wastewater treatment operations.

The anammox process has been specifically used in WWTPs with anaerobic sludge digestion, where 10–15% of the nitrogen load is provided by the return line from the centrifuge [4]. Sludge digestion liquid is a common type of wastewater characterized by a low C/N ratio. Sludge digestion liquid typically contains 500–2000 mg N-NH_4^+/L and approximately 500–1200 mg/L organic matter with limited biodegradability. With a pH range of 7.0–8.5 and temperatures typically between 30–37 °C, sludge liquid provides optimal growth conditions for anammox bacteria and ammonia-oxidizing bacteria (AOB) due to its high alkalinity and temperature [5]. Since anammox bacteria require nitrite to carry out their metabolism, it is necessary for AOB to develop alongside them to produce the required nitrite from ammonia. Depending on the system configuration, the production of nitrite from ammonia can occur in the same reactor where the anammox bacteria are located or in a preceding stage [6]. When both AOB and anammox are incorporated into the same reactor, it is referred to as a partial nitrification–anammox reactor, which operates with aeration periods when AOB are active, followed by anoxic periods when anammox bacteria are activated and consume the residual ammonia and newly formed nitrite. The AOB activity generates protons; thus, a high degree of alkalinity is desirable to prevent dramatic pH drops in the reactor that could affect the performance of the anammox, since their optimal pH is between 7–8 [7].

In contrast to their potential, anammox bacteria are sensitive to multiple environmental conditions such as the C/N ratio and the presence of heavy metals [8]. Among the most critical parameters is the concentration of nitrite, one of its substrates, which can, on the one hand, prove limiting if its concentration is lower than the saturation constant for anammox and on the other hand, exert inhibitory effects both in the short and long term [9], sometimes becoming irreversible if its concentration is too high. All of this contributes to the instability of the process, which, combined with the slow duplication rate of anammox (10–14 days), can lead to significant reductions in nitrogen removal, as well as extensive activity recovery times.

A literature review reveals a variety of studies on the inhibitory effect that nitrite may have on anammox bacteria; however, these studies differ in the methodology employed, as well as in the characteristics of the inoculum (intact granules, disaggregated granules, flocculent, or biofilm) and their origin (laboratory UASB, SBR, MBBR, etc.). Likewise, the studies do not agree on the inhibitory nitrite concentration. It has been reported that a concentration of 100 mg N-NO_2^-/L is sufficient to inhibit anammox activity in the short

term [10]. However, other experiments have shown that anammox activity increases up to 160 mg N-NO_2^-/L, with inhibition starting at 400 mg N-NO_2^-/L [7]. In another report, it is stated that inhibition begins at 103 mg N-NO_2^-/L, reaching IC_{50} at 185 mg N-NO_2^-/L [11]. Similarly, the published results regarding the saturation constant of anammox bacteria for nitrite show strong divergences.

Recent studies have explored various strategies to mitigate the effects of nitrite inhibition. For instance, the addition of intermediates such as hydrazine (N_2H_4) and hydroxylamine (NH_2OH) has been shown to alleviate inhibition caused by high substrate concentrations [11]; however, its prolonged use generates adverse effects [12]. On the other hand, the existence of an NO_2^-/NO_3^- transporter has been studied, which helps mitigate nitrite toxicity in anammox bacteria by adding external nitrate, exchanging external nitrate for nitrite inside the cell [13]. At a full scale, the most common approach remains controlling the nitrogen load applied to the reactor using anammox bacteria.

There are different methods to evaluate the presence of microbial toxicity in bacterial groups within WWTPs. In general, toxicity tests are carried out by comparing the substrate consumption rate or the metabolic product generation rate in the presence of the inhibitor with a control [14]. Specifically, regarding nitrogen removal, ISO 9509 outlines a method to determine the toxicity of a given contaminant on nitrification [15]. However, for both heterotrophic and autotrophic denitrification, there is no ISO standard for toxicity in liquid systems. Nevertheless, methods have been proposed in the literature to characterize denitrification. In Ref. [16], a methodology was developed to determine the toxicity of compounds found in wastewater on heterotrophic denitrification by monitoring gas production and its composition. Additionally, in Ref. [17], based on the work of Ref. [16], a method was developed to determine specific anammox activity and its inhibitory effects on autotrophic denitrification caused by foreign compounds or high substrate concentrations by tracking N_2 production.

In addition to the above, there are other parameters, such as pH and temperature, that show a significant and rapid influence on the performance of biological systems. Regarding the latter, there are reports indicating that anammox biomass increases its activity by around 30% for every 5 °C in the range of 25–35 °C [18]. Another study mentions that SAA increases by between 8.7% and 12.5% for each 1 °C increase in process temperature [19]. Recently, there has been growing interest in studying anammox activity at low temperatures (below 25 °C) and low ammonium levels to assess the potential use of anammox in the main treatment line of wastewater treatment plants. However, challenges in suppressing the emergence of nitrite-oxidizing bacteria under these conditions have limited this line of research [20]. Most temperature studies have been conducted with laboratory-scale biomass, with limited information available at full scale.

In the literature, there are different approaches to represent the kinetics of bacteria in relation to varying substrate concentrations. One of these approaches is the Andrews model [21], which is an empirical model that aims to interpret the behavior of microorganisms with respect to their limiting substrate. Like the Monod equation, which has a close formal relationship with the Michaelis–Menten expression for enzymes, the Andrews model is based on the mathematical expression proposed by Haldane, which links enzyme activity to substrate concentration, while accounting for the inhibitory effect of high substrate concentrations. Boon and Laudelot [22] were the first to successfully apply kinetic models originally proposed for enzymes to cases of microbial inhibition. Specifically, they demonstrated that *Nitrobacter winogradskyi* exhibits non-competitive inhibition kinetics in its rate of nitrite oxidation. Another noteworthy model is the Edwards model [23], which is distinguished by its use of the exponential constant *e* to capture the complex and nonlinear dynamics commonly observed in microbial systems. The fitting of the Andrews and Edwards models has been previously applied to anammox-based nitrogen removal systems by Ref. [19], yielding an R^2 greater than 0.92 for both cases.

In this work, the inhibitory effects of nitrite on specific anammox activity are studied in both the short and long term. Data generated from short-term experiments will be used as

input for fitting the kinetic models of Andrews and Edwards. Additionally, the short-term effect of temperature on specific anammox activity will also be investigated.

2. Materials and Methods

The anammox granular biomass was provided by the Mapocho-Trebal Biofactory of Aguas Andinas in Santiago de Chile. The biofactory treats wastewater through pre-, primary, and secondary treatment. Primary and secondary sludge are digested by anaerobic digestion. The effluent from the digester is centrifuged, and the liquid fraction is sent to an aerobic reactor where its COD is reduced, and subsequently, to a partial nitrification–anammox reactor to remove high levels of ammonia. The biomass comes from the latter. This reactor, operated under stable conditions, achieved an average nitrogen removal efficiency of 76.9% during the 30 days prior to biomass collection. Key operational parameters during this period included an average pH of 7.4, a temperature of 27 °C, and a volatile suspended solids (SSV) concentration of 3.37 g/L. Once collected, it was stored at 5 °C until use to ensure its viability and activity before the experiments. For the temperature study trials, granular anammox biomass from the La Farfana Biofactory of Aguas Andinas in Santiago, Chile, was used.

2.1. Specific Anammox Activity (SAA)

The activity assays were conducted based on a modified version of the method described by Ref. [17]. Vials holding 120 mL, with a liquid volume of 70 mL, were used for the assays. Each vial was inoculated with granular sludge that was previously washed and resuspended in phosphate buffer (0.14 g/L KH_2PO_4 and 0.75 g/L K_2HPO_4). The vial was sealed with a stopper, and both the liquid phase and headspace of the vial were gassed with argon for 10 min. To activate the sludge, the vial was incubated for 45 min in a shaker at 200 rpm and 30 °C. Then, a concentrated solution of N-NH_4^+ and N-NO_2^-, with a concentration defined according to the specific experiment, was injected. Ammonium chloride was used as the ammonium source, and sodium nitrite was used as the nitrite source. After substrate addition, the vial was incubated at 30 °C and 200 rpm, and the evolution of pressure in the vial headspace, an indirect measure of N_2 production resulting from the anammox activity of the granules, was recorded. The duration of the assay depended on the sludge activity. The rate of gaseous N_2 production was obtained, assuming that the gas confined in the headspace of the vial behaves as an ideal gas. Thus, for constant volume and temperature, each recorded pressure value corresponds to a certain number of moles of N_2 produced. SAA is calculated as described by Ref. [17], in which SAA is equivalent to the maximum rate of N_2 production (in mg/h) divided by the amount of biomass (gSSV) in the vial.

To evaluate the short-term effect of nitrite, batch assays were conducted with a constant initial ammonium concentration of 200 mg N/L and nitrite concentrations ranging from 5 to 250 mg N/L. Each assay was performed in duplicate.

2.2. Kinetic Model Fitting

The results obtained for SAA at different nitrite concentrations in the short-term experiment were fitted to two kinetic models: the Andrews model and the Edwards model.

At lower concentrations, substrates act as nutrients for microorganisms; however, at higher concentrations, they exhibit inhibitory effects. Elevated substrate levels hinder microbial growth and disrupt metabolic processes. The Andrews model, commonly applied to describe the kinetics of substrate inhibition in microbial systems, is derived from assumptions regarding enzyme–substrate complexes, specifically incorporating the formation of an inhibitory enzyme–substrate complex that reduces the overall reaction rate. The Edwards model is not based on mechanistic assumptions but rather uses the constant e to represent the complexity and non-linear relationships commonly observed in microbial processes. Although it is not based on any biochemical mechanism, this approach has proven to be flexible and successful in fitting experimental data [19,23].

Equation (1) shows the mathematical expression corresponding to the Andrews model, which is a non-competitive substrate inhibition equation.

$$\mathrm{SAA} = \mathrm{SAA_{max}} \cdot \frac{\mathrm{S}}{\mathrm{K_S} + \mathrm{S} \cdot \left(1 + \frac{\mathrm{S}}{\mathrm{K_I}}\right)} \quad (1)$$

where SAA_{max} corresponds to the theoretical maximum anammox specific activity (mgN eliminated/gSSV·h), K_S is the saturation constant (mg N-NO_2^-/L), K_I is the inhibition constant (mg N-NO_2^-/L), and S is the nitrite concentration (mg N-NO_2^-/L).

The values of the parameters SAA_{max}, K_S, and K_I were estimated using the GRG Nonlinear method provided by Solver (Microsoft Excel for Office 365 MCO, v2410), minimizing the error according to the least squares method.

Equation (2) shows the mathematical expression for the Edwards model.

$$\mathrm{SAA} = \mathrm{SAA_{max}} \cdot \left(e^{\left(\frac{-S}{K_I}\right)} - e^{\left(\frac{-S}{K_S}\right)}\right) \quad (2)$$

where SAA_{max} corresponds to the theoretical maximum anammox specific activity (mgN eliminated/gSSV·h), K_S is the saturation constant (mg N-NO_2^-/L), K_I is the inhibition constant (mg N-NO_2^-/L), and S is the nitrite concentration (mg N-NO_2^-/L).

The parameter values in the Edwards model were estimated using the same method used for the Andrews model.

2.3. Extended Nitrite Exposure Experiments

The extended exposure experiments were carried out in a sequential batch reactor (SBR) with a useful volume of 2 L. The reactor was constructed from glass and did not include baffles, ensuring smooth flow conditions. Complete mixing inside the reactor was achieved with a mechanical stirrer located near the bottom of the reactor, operating at a rotation speed of 200 rpm. The temperature was maintained at 30 ± 1 °C using a heat exchanger, and the pH was manually adjusted to remain between 7.4 and 8.0 by adding 2 M NaOH or 1 M HCl solutions as needed. Feeding and discharge operations were managed using peristaltic pumps, ensuring precise control of influent and effluent flows.

The reactor's operation was automated using a programmable logic controller (PLC) to manage cycles of 8 h, organized into four periods: feeding (10 min), mixing (420 min), settling (40 min), and discharge (10 min). The hydraulic retention time (HRT) was set at 32 h and kept constant throughout the experiments. The reactor was inoculated with anammox granular biomass from a partial nitritation–anammox reactor at the Mapocho-Trebal Biofactory of Aguas Andinas in Santiago de Chile. The inoculum had a volatile suspended solids concentration of 0.5 g/L at the beginning of the experiments. The reactor startup took 3 weeks to reach a steady state.

Once a steady state was reached in the reactor, the extended nitrite exposure experiments were initiated. These consisted of subjecting the sludge present in the reactor at the beginning of each cycle to three HRTs at a defined nitrite concentration. To ensure this condition, a sample was taken at the end of each cycle, and the feed concentration for the next cycle was adjusted, if necessary. At the beginning and end of each experiment (at the beginning and after three HRTs), the SAA of the sludge was measured, and significant changes in its values were evaluated. If there were no changes at the initial nitrite concentration studied, that experiment was concluded, and a new one was initiated at a higher concentration. The initial nitrite values studied were 15 mg N-NO_2^-/L and 30 mg N-NO_2^-/L. To replicate the typical concentration to which the granules were exposed in the reactor from which they originated, the ammonium concentration in the feed was modified as necessary to achieve an N-NH_4^+ concentration in the reactor vicinity of 200 mg N-NH_4^+/L.

Figure 1 presents a logical flowchart outlining the experimental procedures conducted.

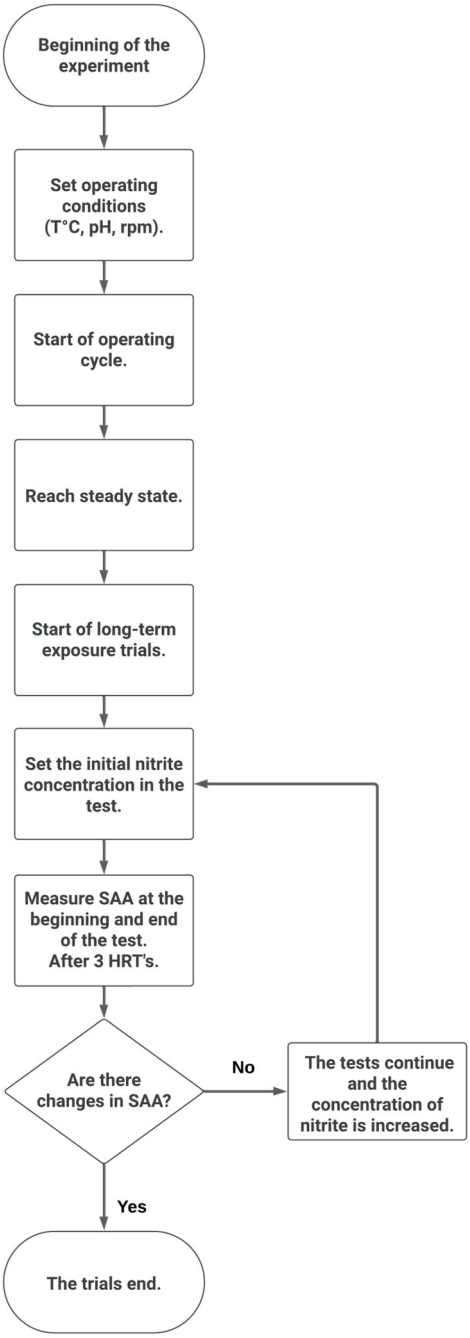

Figure 1. Logical flowchart of the experiment.

2.4. Analytical Methods

Ammonium was quantified spectrophotometrically using the phenol-hypochlorite method [24]. Calibration was performed using ammonium chloride standards prepared in deionized water at concentrations ranging from 0.2 to 1 mg $N\text{-}NH_4^+/L$. A calibration curve was constructed by measuring absorbance at 635 nm, yielding an R^2 value greater than 0.999.

Nitrite was quantified spectrophotometrically, according to standard method 4500-NO_2^- [25]. The calibration curve for nitrite was generated using sodium nitrite at concentrations of 0.04 to 0.72 mg $N\text{-}NO_2^-/L$ and measuring absorbance at 543 nm. The method showed linearity, with R^2 values above 0.999.

Nitrate was quantified spectrophotometrically by a modified version of standard method 4500-NO_3^- [25]. The calibration curve for nitrate was generated using sodium

nitrate at concentrations of 1.2 to 4 mg N-NO_3^-/L and measuring absorbance at 220 nm. At this wavelength, nitrite and nitrate both absorb. To correct for nitrite interference, a calibration curve for nitrite at 220 nm (concentrations from 3.04 to 12.16 mg N-NO_2^-/L) was prepared, allowing for subtraction of the nitrite absorbance from the total absorbance. Nitrate concentrations were then calculated from the corrected absorbance values.

Biomass concentration was measured as the amount of volatile suspended solids (gSSV/L), in accordance with standard methods [25]

3. Results and Discussion

3.1. Short-Term Nitrite Effect

The results obtained from the assays aimed at characterizing the response of SAA to short-term nitrite exposure are presented in Figure 2.

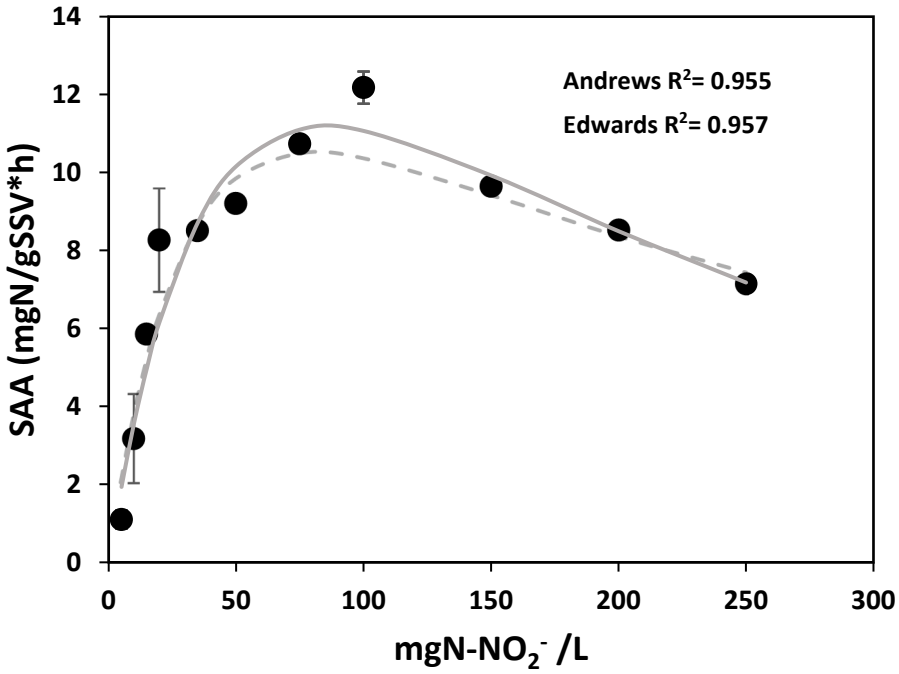

Figure 2. Effect of nitrite on SAA. The black circles represent the experimental results, the dashed line represents the prediction by the Andrews model of substrate inhibition, and the continuous line represents the prediction by the Edwards model of substrate inhibition.

The maximum specific anammox activity within the studied range was achieved at 100 mg N-NO_2^-/L, yielding a value of 12.2 mg N eliminated/(gSSV·h). Higher concentrations exhibited a detrimental effect on the SAA of the anammox biomass, with an IC_{60} observed at 250 mg N-NO_2^-/L. Similar behavior is reported by Ref. [22] (Table 1), in which activity tests were conducted with three types of biomasses: biofilm, granular, and flocculent from MMBR, UASB, and SBR reactors at laboratory scale, respectively. In Ref. [26], for biofilm biomass, the maximum SAA was obtained at 40 mg N-NO_2^-/L, while the IC_{50} was obtained at 85 mgN-NO_2^-/L, which are values significantly lower than those found in this study. For flocculent biomass, the maximum SAA was achieved at 81 mg N-NO_2^-/L and the IC_{50} at 98 mg N-NO_2^-/L, which, although approaching the maximum SAA, still significantly differs from the IC_{50} found in this study. In the case of the granular biomass from a UASB reactor, the maximum SAA was achieved at 83 mg N-NO_2^-/L and the IC_{50} at 240 mg N-NO_2^-/L, which is very similar to the values obtained in this study. This is an interesting fact, as it supports the idea that the biomass behavior is mainly influenced by its structure rather than the type of reactor and cultivation mode. Another case is presented by Ref. [11], where the SAA was evaluated for granular biomass and suspended biomass, resulting in the maximum activity obtained at 103 mg N-NO_2^-/L and the IC_{50}

corresponding to 185 mg N-NO_2^-/L for the granular biomass. Additionally, the study by Ref. [17] reports a maximum SAA at 140 mg N-NO_2^-/L and an IC_{50} at 350 mg N-NO_2^-/L. Differences may arise from the different origin of the granules, as well as their varying diameters, as previously reported [27]. Moreover, while the matrix of the granules provides mechanical stability, it also creates concentration gradients for substrates and metabolic products. Compounds such as nitrite and ammonium must diffuse from the external medium into the inner layers of the granule, where active bacteria reside. Therefore, the nitrite concentration observed for the bacteria is lower than that present in the bulk liquid, which translates into an apparent higher resistance to nitrite inhibition.

Table 1. IC_{50} values for nitrite inhibition and conditions for batch tests, according to the references.

IC_{50} [mgN-NO_2^-/L]	Temperature [°C]	Biomass Type	Source Reactor	Reference
250 [a]				
423.8 [b]	30	Granular	SBR	This study
321 [c]				
85	25	Biofilm	MBBR	[26]
98	25	Flocculent	SBR	[26]
240	25	Granular	UASB	[26]
185	30	Granular	EGSB	[11]
151	30	Suspended	Membrane reactor	[11]
350	30	-	-	[17]

[a] There is no experimental IC_{50}, so the IC_{60} is used. [b] IC_{50} obtained via the Andrews model. [c] IC_{50} obtained via the Edwards model.

With regards to fitting the experimental data to the kinetic model of non-competitive substrate inhibition proposed by Andrews, it was found that the best fit (R^2 = 0.955) was obtained for SAA_{max}, K_S, and K_I values of 25 mg N/(gSSV·h), 55.6 mg N-NO_2^-/L, and 116.7 mg N-NO_2^-/L, respectively. As shown in Figure 1, overall, the model interprets the biomass behavior in response to the nitrite concentration within the evaluated range, except for the value of 100 mg N-NO_2^-/L, for which the error is significant. According to the model, the maximum activity (dSAA/dS = 0) is achieved at 80.6 mg N-NO_2^-/L, and half of this activity is obtained at 15.3 mg N-NO_2^-/L. Furthermore, extrapolating the data suggests that the IC_{50} is reached at approximately 423.8 mg N-NO_2^-/L.

Regarding the fit of the experimental data to the Edwards inhibition model, the best fit (R^2 = 0.957) was obtained with an SAA_{max} of 17.2 mg N eliminated/(gSSV·h), a K_S of 36 mg N-NO_2^-/L, and a K_I of 287 mg N-NO_2^-/L. When calculating the maximum value of the Edwards model function, it is found to be 85 mg N-NO_2^-/L, while half of this activity is achieved at 17.5 mg N-NO_2^-/L. Similarly to the Andrews model, when extrapolating the data, it is found that the IC_{50} is reached at a nitrite concentration of 321 mg N-NO_2^-/L.

Both the Andrews and Edwards models presented a good fit to the experimental data. However, the values of the kinetic constants for both models differ significantly. The above is expected if we consider that the Andrews model is based on mechanistic enzyme–substrate assumptions, while the Edwards model is not. Although the evaluated system is not an enzyme–substrate but rather a bacteria–substrate, it is assumed that there is a limiting step in the biological process of anammox. In this sense, K_S and K_I describe that reaction. In contrast, K_S and K_I in the Edwards model do not have a direct biological interpretation. Regarding the comparison of the values obtained in this study with those available in Ref. [19], the anammox activity assays were conducted at 25 °C, obtaining a K_S and K_I for the Edwards model of 56.4 mg N-NO_2^-/L and 525 mg N-NO_2^-/L, respectively. Although both values are higher than those obtained in this study, the differences might be attributed to the temperature difference (30 °C in our assays), as it is known that kinetic constants decrease in value with rising temperatures. On the other hand, Ref. [28], applying the Andrews model, obtained a K_S of 29 mg N-NO_2^-/L and a K_I of 1779 mg N-NO_2^-/L

at 35 °C, where the Ks is about half and the Ki is more than 10 times what was obtained in this study. Closer values are reported in the publication by Ref. [29], where, using the Haldane (Andrews) model, a Ks of 15.36 mg N-NO_2^-/L and a Ki of 159.5 mg N-NO_2^-/L were obtained.

It is important to note that the calculated kinetic constants likely do not interpret the individual behavior of each anammox cell but rather incorporate the effect of external and internal diffusional constraints within the granules. The impact of the concentration gradients of the substrates and products involved in bacterial metabolism can be significant, as addressed by Ref. [30] in their review. The size of the granules, their composition in the EPS and proteins, as well as the distribution of different bacterial groups along the granule diameter are factors that influence the work of Ref. [31], and in this regard, the kinetic constants maximum SAA, K_S, and K_I determined in this study would be apparent kinetic constants.

The lack of direct measurements of granule size distribution and internal diffusion gradients limits the ability to fully interpret the observed kinetic constants as intrinsic microbial parameters. These values likely reflect a combined effect of microbial activity and physical–chemical constraints within the granules.

3.2. Long-Term Nitrite Effect

Figure 3 shows the evolution of nitrogen species during the long-term nitrite exposure experiment. For all cycles, the duration was the same, regardless of whether they were initially loaded with 15 mg N-NO_2^-/L or 30 mg N-NO_2^-/L. As can be seen, in general, the nitrite level at the end of the cycles was not less than 3 mgN/L (except for cycles 7 and 13), which could be interpreted as the consumption rate drastically decreasing below this value, consistent with the results of the short-term experiments presented earlier.

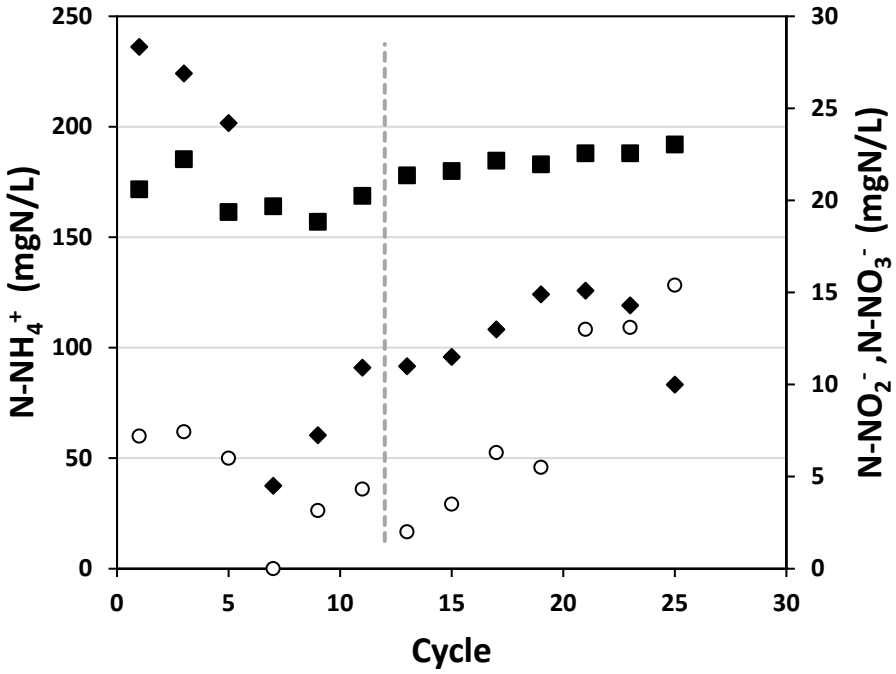

Figure 3. Ammonium (black square), nitrate (black diamond), and nitrite (white circle) in discharge during cycles performed in the long-term experiment in the SBR reactor. The dashed line (- -) represents the moment when the initial nitrite concentration was changed from 15 mgN-NO_2^-/L to 30 mgN-NO_2^-/L at the beginning of each cycle.

Table 2 presents the SAA values obtained during the execution of the extended exposure experiments. As can be observed, the biomass subjected to the regimen of 15 mg N-NO_2^-/L at the beginning of each cycle had no significant effect on SAA (cycle 1 and cycle

12). Therefore, it was decided to increase the nitrite concentration at the beginning of the cycles to 30 mg N-NO_2^-/L. After 12 more cycles of operation under this new condition, the activity assay was repeated, resulting in a decrease of approximately 50%. This can also be observed in Figure 3, where the residual nitrite concentration begins to increase as the cycles progress after week 12. The nitrogen load supplied to the system also decreased throughout this second experiment to avoid nitrite overload.

Table 2. SAA values of sludge samples taken from the SBR reactor during the extended nitrite exposure experiments.

Cycle	SAA (mgN/gSSV·h)
1	5.43
12	5.32
25	2.67 ± 0.3

The results obtained are consistent with those reported by Ref. [9], in which it was observed that nitrite concentrations higher than 15 mg N-NO_2^-/L should be avoided to maintain stable anammox system operation. Similarly, Ref. [32], using granular anammox biomass in a UASB reactor, determined that at 1.5 µg/L of free nitrous acid, the system begins to destabilize, which under the operational conditions used (pH and temperature) is equivalent to around 15 mg N-NO_2^-/L. On the other hand, it should be noted that in this experiment, the feed consisted only of sodium nitrite and ammonium chloride, without the addition of micronutrients or an inorganic carbon source. Regarding the latter, Ref. [33] showed that there could be a decrease of up to 40.5% in SAA between anammox bacteria fed with and without bicarbonate in the long term. It is possible that a percentage of the activity loss in the assay at 30 mg N-NO_2^-/L is a combined effect of carbon starvation and nitrite exposure.

From an operational standpoint, the results obtained in the short and long term indicate that under no circumstances should the nitrite concentration in the anammox denitrification reactor exceed 100 mgN-NO_2^-/L, as the SAA immediately decreases, which is a negative consequence, since nitrite will be consumed more slowly by the anammox bacteria until reaching non-inhibitory levels. In contrast, although long-term experiments show that levels between 30 and 100 mgN-NO_2^-/L constitutes an inhibitory range for prolonged exposure, the SAA is higher in the short term, so the system tends to naturally recover its balance, unless it is overloaded with new nitrite. Overall, the operational range that ensures stability is below 30 mgN-NO_2^-/L, even if part of the biocatalytic potential of the anammox is sacrificed.

3.3. Short-Term Effect of Temperature

The influence of temperature on SAA was evaluated. Figure 4 shows the results obtained, in which a clear increase in SAA value with temperature increment within the studied range can be observed. The temperature range selected for the study was chosen because it is close to the temperature at which the liquid originates from the anaerobic digester. Regarding the relationship between SAA and temperature, similar results have been reported previously in the literature [34]. Ref. [35] studied SAA variation in the temperature range of 10–45 °C, using both granular biomass and biofilm, finding that for both cases, the SAA at 25 °C is less than half the SAA at 35 °C, representing a more drastic effect than that obtained in this study (67.7%). One explanation could be that the biomass used by Ref. [35] came from a laboratory reactor operating at 30 °C, while the one in this study comes from a full-scale reactor, with an average temperature of 27 °C, which could impact the present community and its tolerance to lower temperatures. On the other hand, Ref. [36] measured SAA in the range of 10–55 °C, showing that approximately 50% of the SAA obtained at 35 °C is preserved at 25 °C. Similar results were obtained by Ref. [37], where a profile of SAA with respect to the temperature of its enriched culture showed that

55% of SAA at 35 °C is maintained at 25 °C. According to the review conducted by the authors of this study, the SAA variation in the range of 25–35 °C is the lowest reported.

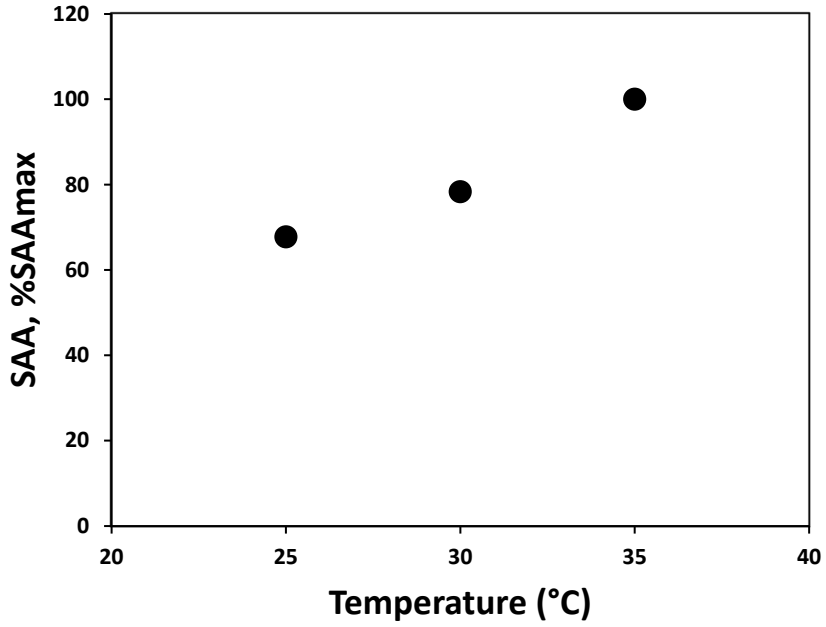

Figure 4. SAA at 25, 30, and 35 °C.

4. Conclusions

Under the studied conditions, nitrite concentrations equal to or exceeding 30 mgN-NO_2^-/L should be avoided to maintain the stable operation of anammox systems. Nevertheless, short-term experiments demonstrate that concentrations between 30–100 mgN-NO_2^-/L temporarily increase SAA, helping the system self-regulate to reach non-inhibitory long-term concentrations.

Both the Andrews and Edwards models accurately interpret the relationship between nitrite concentration and SAA, although the derived K_S and K_I values differ considerably compared to those of previous studies.

In the temperature range of 25–35 °C, SAA follows an increasing trend, supporting earlier reports. Temperature selection can be adjusted to enhance SAA in specific cases.

This study was limited by the controlled laboratory conditions under which the experiments were conducted, which may not fully replicate the dynamic environments of wastewater treatment plants. Despite these limitations, the study provides valuable insights into the operational boundaries for anammox-based systems. The results can guide the optimization of nitrite concentrations and temperature conditions in partial nitritation–anammox reactors to enhance process efficiency and minimize activity loss. Future research is essential to understand the long-term response to high nitrite concentrations without inorganic carbon limitation, which is more representative of real-scale conditions. Additionally, studying the recovery times from nitrite inhibition and their relationship with long-term concentrations and exposure times to high concentrations would be highly valuable for full-scale applications.

Author Contributions: B.I., R.C., Y.L. and C.C. conceived and planned the experiments. B.I., R.C., Y.L., C.G. and M.P. participated in the sample preparation, data analysis, and interpretation of the results. B.I. and R.C. wrote the manuscript draft. R.C. and Y.L. supervised the research work, contributed to the discussion of the data, and revised the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: The original contributions presented in this study are included in the article. Further inquiries can be directed to the corresponding author.

Conflicts of Interest: Authors Yves Lesty and Chloe Girard were employed by the company Aguas Andinas S.A. Author Mateo Pastur was employed by the company Cetaqua Chile. Author Celia Castro was employed by the company Cetaqua Barcelona. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

References

1. Smith, V.H.; Tilman, G.D.; Nekola, J.C. Eutrophication: Impacts of excess nutrient inputs on freshwater, marine, and terrestrial ecosystems. *Environ. Pollut.* **1999**, *100*, 179–196. [CrossRef] [PubMed]
2. Ward Mary, H.; Jones Rena, R.; Brender Jean, D.; De Kok Theo, M.; Weyer Peter, J.; Nolan Bernard, T.; Villanueva Cristina, M.; Van Breda Simone, G. Drinking water nitrate and human health: An updated review. *Int. J. Environ. Res. Public Health* **2018**, *15*, 1557. [CrossRef]
3. Lackner, S.; Gilbert, E.M.; Vlaeminck, S.E.; Joss, A.; Horn, H.; Van Loosdrecht, M.C. Full-scale partial nitritation/anammox experiences–an application survey. *Water Res.* **2014**, *55*, 292–303. [CrossRef] [PubMed]
4. Driessen, W.; Hendrickx, T. Two decades of experience with the granular sludge-based anammox® process treating municipal and industrial effluents. *Processes* **2021**, *9*, 1207. [CrossRef]
5. Ren, Z.Q.; Wang, H.; Zhang, L.G.; Du, X.N.; Huang, B.C.; Jin, R.C. A review of anammox-based nitrogen removal technology: From microbial diversity to engineering applications. *Bioresour. Technol.* **2022**, *363*, 127896. [CrossRef] [PubMed]
6. Bagchi, S.; Biswas, R.; Nandy, T. Autotrophic ammonia removal processes: Ecology to technology. *Crit. Rev. Environ. Sci. Technol.* **2012**, *42*, 1353–1418. [CrossRef]
7. Talan, A.; Tyagi, R.D.; Drogui, P. Critical review on insight into the impacts of different inhibitors and performance inhibition of anammox process with control strategies. *Environ. Technol. Innov.* **2021**, *23*, 101553. [CrossRef]
8. Gutwiński, P.; Cema, G.; Ziembińska-Buczyńska, A.; Wyszyńska, K.; Surmacz-Gorska, J. Long-term effect of heavy metals Cr(III), Zn(II), Cd(II), Cu(II), Ni(II), Pb(II) on the anammox process performance. *J. Water Process Eng.* **2021**, *39*, 101668. [CrossRef]
9. Fernández, I.; Dosta, J.; Fajardo, C.; Campos, J.L.; Mosquera-Corral, A.; Méndez, R. Short-and long-term effects of ammonium and nitrite on the Anammox process. *J. Environ. Manag.* **2012**, *95*, S170–S174. [CrossRef]
10. Strous, M.; Kuenen, J.G.; Jetten, M.S. Key physiology of anaerobic ammonium oxidation. *Appl. Environ. Microbiol.* **1999**, *65*, 3248–3250. [CrossRef] [PubMed]
11. Carvajal-Arroyo, J.M.; Sun, W.; Sierra-Alvarez, R.; Field, J.A. Inhibition of anaerobic ammonium oxidizing (anammox) enrichment cultures by substrates, metabolites and common wastewater constituents. *Chemosphere* **2013**, *91*, 22–27. [CrossRef] [PubMed]
12. Sari, T.; Akgul, D.; Mertoglu, B. Long-term response of anammox process to hydrazine under different exposure strategies. *J. Environ. Chem. Eng.* **2024**, *12*, 113600. [CrossRef]
13. Li, G.; Vilcherrez, D.; Carvajal-Arroyo, J.M.; Sierra-Alvarez, R.; Field, J.A. Exogenous nitrate attenuates nitrite toxicity to anaerobic ammonium oxidizing (anammox) bacteria. *Chemosphere* **2016**, *144*, 2360–2367. [CrossRef]
14. Strotmann, U.; Durand, M.J.; Thouand, G.; Eberlein, C.; Heipieper, H.J.; Gartiser, S.; Pagga, U. Microbiological toxicity tests using standardized ISO/OECD methods—Current state and outlook. *Appl. Microbiol. Biotechnol.* **2024**, *108*, 454. [CrossRef]
15. *ISO 9509*; Water Quality—Toxicity Test for Assessing the Inhibition of Nitrification of Activated Sludge Microorganisms. International Organization for Standardization: Geneva, Switzerland, 2006.
16. Buys, B.R.; Mosquera-Corral, A.; Sánchez, M.; Méndez, R. Development and application of a denitrification test based on gas production. *Water Sci. Technol.* **2000**, *41*, 113–120. [CrossRef]
17. Dapena-Mora, A.; Fernandez, I.; Campos, J.L.; Mosquera-Corral, A.; Mendez, R.; Jetten MS, M. Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. *Enzym. Microb. Technol.* **2007**, *40*, 859–865. [CrossRef]
18. Oshiki, M.; Shimokawa, M.; Fujii, N.; Satoh, H.; Okabe, S. Physiological characteristics of the anaerobic ammonium-oxidizing bacterium 'Candidatus Brocadia sinica'. *Microbiology* **2011**, *157*, 1706–1713. [CrossRef]
19. Marina, C.; Kunz, A.; Bortoli, M.; Scussiato, L.A.; Coldebella, A.; Vanotti, M.; Soares, H.M. Kinetic models for nitrogen inhibition in ANAMMOX and nitrification process on deammonification system at room temperature. *Bioresour. Technol.* **2016**, *202*, 33–41. [CrossRef]
20. Adams, M.; Issaka, E.; Chen, C. Anammox-based technologies: A review of recent advances, mechanism, and bottlenecks. *J. Environ. Sci.* **2024**, *148*, 151–173. [CrossRef]
21. Andrews, J.F. A mathematical model for the continuous culture of microorganisms utilizing inhibitory substrates. *Biotechnol. Bioeng.* **1968**, *10*, 707–723. [CrossRef]
22. Boon, B.; Laudelout, H. Kinetics of nitrite oxidation by Nitrobacter winogradskyi. *Biochem. J.* **1962**, *85*, 440. [CrossRef] [PubMed]
23. Edwards, V.H. The influence of high substrate concentrations on microbial kinetics. *Biotechnol. Bioeng.* **1970**, *12*, 679–712. [CrossRef] [PubMed]

24. Weatherburn, M.W. Phenol-hypochlorite reaction for determination of ammonia. *Anal. Chem.* **1967**, *39*, 971–974. [CrossRef]
25. American Public Health Association; American Water Works Association; Water Environment Federation; Lipps, W.C.; Braun-Howland, E.B.; Baxter, T.E. (Eds.) *Standard Methods for the Examination of Water and Wastewater*, 24th ed.; APHA Press: Washington, DC, USA, 2023.
26. Raudkivi, M.; Zekker, I.; Rikmann, E.; Vabamäe, P.; Kroon, K.; Tenno, T. Nitrite inhibition and limitation–the effect of nitrite spiking on anammox biofilm, suspended and granular biomass. *Water Sci. Technol.* **2017**, *75*, 313–321. [CrossRef] [PubMed]
27. Cho, S.; Takahashi, Y.; Fujii, N.; Yamada, Y.; Satoh, H.; Okabe, S. Nitrogen removal performance and microbial community analysis of an anaerobic up-flow granular bed anammox reactor. *Chemosphere* **2010**, *78*, 1129–1135. [CrossRef] [PubMed]
28. Zu, B.; Zhang, D.J.; Yan, Q. Effect of trace NO_2 and kinetic characteristics for anaerobic ammonium oxidation of granular sludge. *Huan Jing Ke Xue= Huanjing Kexue* **2008**, *29*, 683–687. [PubMed]
29. Baeten, J.E.; Batstone, D.J.; Schraa, O.J.; van Loosdrecht, M.C.; Volcke, E.I. Modelling anaerobic, aerobic and partial nitritation-anammox granular sludge reactors-A review. *Water Res.* **2019**, *149*, 322–341. [CrossRef]
30. Ni, B.-J.; Hu, B.-L.; Fang, F.; Xie, W.-M.; Kartal, B.; Liu, X.-W.; Sheng, G.-P.; Jetten, M.; Zheng, P.; Yu, H.-Q. Microbial and physicochemical characteristics of compact anaerobic ammonium-oxidizing granules in an upflow anaerobic sludge blanket reactor. *Appl. Environ. Microbiol.* **2010**, *76*, 2652–2656. [CrossRef] [PubMed]
31. Zhang, Y.; He, S.; Niu, Q.; Qi, W.; Li, Y.Y. Characterization of three types of inhibition and their recovery processes in an anammox UASB reactor. *Biochem. Eng. J.* **2016**, *109*, 212–221. [CrossRef]
32. Puyol, D.; Carvajal-Arroyo, J.M.; Sierra-Alvarez, R.; Field, J.A. Nitrite (not free nitrous acid) is the main inhibitor of the anammox process at common pH conditions. *Biotechnol. Lett.* **2014**, *36*, 547–551. [CrossRef] [PubMed]
33. Jin, R.-C.; Yu, J.-J.; Ma, C.; Yang, G.-F.; Zhang, J.; Chen, H.; Zhang, Q.-Q.; Ji, Y.-X. Transient and long-term effects of bicarbonate on the ANAMMOX process. *Appl. Microbiol. Biotechnol.* **2014**, *98*, 1377–1388. [CrossRef] [PubMed]
34. Lotti, T.; Kleerebezem, R.; Van Loosdrecht MC, M. Effect of temperature change on anammox activity. *Biotechnol. Bioeng.* **2015**, *112*, 98–103. [CrossRef] [PubMed]
35. Dosta, J.; Fernández, I.; Vázquez-Padín, J.R.; Mosquera-Corral, A.; Campos, J.L.; Mata-Alvarez, J.; Méndez, R. Short-and long-term effects of temperature on the Anammox process. *J. Hazard. Mater.* **2008**, *154*, 688–693. [CrossRef] [PubMed]
36. Sobotka, D.; Zhai, J.; Makinia, J. Generalized temperature dependence model for anammox process kinetics. *Sci. Total Environ.* **2021**, *775*, 145760. [CrossRef] [PubMed]
37. Hu, Z.; Lotti, T.; de Kreuk, M.; Kleerebezem, R.; van Loosdrecht, M.; Kruit, J.; Jetten, M.S.M.; Kartal, B. Nitrogen removal by a nitritation-anammox bioreactor at low temperature. *Appl. Environ. Microbiol.* **2013**, *79*, 2807–2812. [CrossRef] [PubMed]