Biomass aggregation influences NaN_3 short-term effects over anammox bacteria activity

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Abstract: The main bottleneck to maintain the long term stability of the partial nitritation-anammox processes, especially those operated at low temperatures and nitrogen concentrations is the undesirable development of nitrite oxidizing bacteria (NOB). When this occurs, the punctual addition of compounds with the capacity to specifically inhibit NOB without affecting the process efficiency might be of interest. Sodium azide (NaN_3) is an already known NOB inhibitor which at low concentrations does not significantly affect the ammonia oxidizing bacteria (AOB) activity. However, studies about its influence on anammox bacteria are unavailable. For this reason the objective of the present study was to evaluate the effect of NaN_3 on the anammox activity. Three different types of anammox biomass were used: granular biomass comprising AOB and anammox bacteria (G1), anammox enriched granules (G2) and previous anammox granules disaggregated (F1). No inhibitory effect of NaN_3 was measured on G1 sludge however the anammox activity decreased in the case of G2 and F1. Granular biomass activity was less affected (IC_{50} 90 mg/L, G2) than flocculent one (IC_{50} 5 mg/L, F1). Summing up not only the granular structure protects the anammox bacteria from the NaN_3 inhibitory effect but also the AOB act as a barrier decreasing the inhibition.

Keywords: Anammox; inhibition; granules; NOB; partial nitritation; sodium azide.

1. Introduction

The anammox process consists of the oxidation of ammonium to nitrogen gas, in anaerobic conditions, using nitrite as electron acceptor and producing small amounts of nitrate (Strous *et al.* 1998). It was discovered in 1990s and immediately identified as a promising process allowing for the establishment of the completely autotrophic nitrogen removal, with N_2 as main product. Required nitrite for this process is produced from the oxidation of half of the incoming ammonium to nitrite by ammonia oxidizing bacteria (AOB) in the so called partial nitritation (PN) process.

Nowadays, the technologies based on partial nitritation-anammox processes have been successfully implemented in more than 100 full scale plants, operated at mesophilic conditions and treating high nitrogen concentration effluents (Lackner *et al.* 2014). A relevant number of these plants face the problem of nitrate build up, revealing the presence of nitrite oxidizing bacteria (NOB), as the weak point of the partial nitritation-anammox system stability (Wang *et al.* 2015). From previous studies, performed in a stable single stage partial nitritation-anammox granular biomass system, the appearance of NOB has been associated to the development of flocs or small granules (Winkler *et al.* 2011; Morales *et al.* 2016). However this observation is not of general application and NOB wash-out strategies have not been found yet. For this reason to identify the operational conditions for the NOB suppression is crucial as it is also the main bottleneck to extend the application of the anammox based processes to mainstream conditions (nitrogen concentration < 70 mg N/L and temperature < 25 ºC) and to some industrial effluents.

Up to date, different strategies to induce the NOB depletion have been proposed in PN-anammox systems. One studied action in a one-stage partial nitritation-anammox system was the re-inoculation with anammox biomass to improve the nitrite depletion and avoid its use by

the NOB. However, this method is expensive, due to the large amounts of biomass needed in a full scale plant, and not permanent as nitrate concentrations appear again after some time (Wang *et al.* 2015). Another option relies on the control of the solids retention time (SRT) as anammox bacteria need higher SRT than the aerobic ones; however it is only feasible if the anammox bacteria are grown as biofilm and the NOB as suspended biomass (Han *et al.* 2016). Another strategy is based on the control of the dissolved oxygen (DO) concentration at low values profiting from the fact that at high temperature the oxygen affinity of AOB is lower than that of NOB. But for a long time operation at low temperatures this is not applicable as this behaviour is inverted (higher oxygen affinity for AOB than AOB) (Ma *et al.* 2016). This may be explained by the dominant NOB specie since *Nitrospira*-like NOB (K-strategists) abundance increase under DO limited conditions and lower temperature against *Nitrobacter*-like NOB (r-strategists) (Ma *et al.* 2016). Recently, new strategies arose such as the use free ammonia or free nitrous acid NOB inhibitory concentrations (more toxic to NOB than to AOB), real time control of parameters like pH, ammonia or nitrate concentrations (Ge *et al.* 2015; Wang *et al.* 2015). From these studies it is inferred that the stability problem due to NOB development is still far from being solved. Therefore, new strategies as the use of specific inhibitory compounds can be considered an alternative in some cases (Wang *et al.* 2015).

It is known that in general NOB are more sensitive than AOB to the presence of substances such as organic matter, sulphide, hydroxylamine, salts, chlorates, hydrazine or azide (Ge *et al.* 2015). However, substances like organic matter, salt or sulphides are also known to be inhibitors of the anammox bacteria (Dapena-Mora et al. 2007). Although some of them exert worse effects on the anammox bacteria than on the NOB, others affect preferentially the NOB. For example, the hydroxylamine, an intermediate in both nitrification and anammox process, is toxic only for NOB. Wang et al. (2015) proposed the use of this compound (20 mg/L) combined with the SRT control (down 40 days) to restore the stable operation of the partial nitritation-anammox and suppress the NOB activity. Another compound, the sodium azide (NaN_3), has been identified as a specific NOB inhibitor with no detrimental effect on AOB activity at low concentrations (IC_{50} values of 40 and 0.025 mg NaN_3/L for AOB and NOB, respectively) (López-Fiuza *et al.* 2002). However, the evaluation of its potential effect on the anammox biomass has not been evaluated yet either in suspended or aggregated biomass. Having in mind that anammox bacteria are operated in many cases in the form of granules together with AOB, this aggregation stage can be exploited as a beneficial parameter to resist the presence of the potential NOB inhibitors. It has been observed that the granule matrix acts as a mass transfer barrier that produces lower internal local concentrations of toxic compounds than those in the bulk liquid (Adav *et al.* 2008).

When the suppression of NOB activity is accomplished via inhibitors addition, certain amounts of nitrite might be accumulated in the system with the consequent production of N_2O gas (undesired due to its global warming effect). It is a byproduct of the nitrification process under aerobic conditions or of an incomplete denitrification carried out by nitrifiers or heterotrophic denitrifiers under anoxic conditions (Campos *et al.* 2016).

The aim of this study was to determine the NaN_3 inhibitory effects on the specific anammox activity of flocculent anammox biomass and granular biomass, performing the anammox and/or partial nitritation-anammox processes, respectively. The activity restoration capacity was also assesed for the anammox granules. The N_2O production in the tests was evaluated.

2. Material and Methods

2.1 Origin of biomass

Three types of biomass were used at concentrations approximately of 3 g VSS/L in the experiments. Granular biomass (G1) was collected from a pilot plant performing the partial nitritation-anammox in a single unit. The pilot plant was operated with the ELAN® process treating the reject water from a municipal WWTP containing 540-1045 mg NH_4^+-N/L at 30 °C (Morales *et al.* 2015). Granular anammox enriched biomass (G2) was taken from a laboratory reactor, inoculated with biomass from the ELAN® pilot plant, treating a synthetic medium with 60 mg NH_4^+-N/L and 60 mg NO_2^--N/L at 30 ºC. Finally, previous granular anammox enriched biomass was mechanically disaggregated to obtain flocculent biomass (F1). Previously performed Fluorescence *in situ* Hybridization (FISH) analysis revealed that the anammox dominant specie in all the samples was *Brocadia fulgida* (Morales *et al.* 2015).

2.2 Batch activity tests

The specific anammox activity (SAA) was determined in batch tests carried out according to Dapena-Mora et al. (2007). The biomass samples were washed with phosphate buffer. The headspace of the vials, hermetically closed, was flushed with Helium gas. The vials were incubated at 30 °C and 150 rpm. After substrates addition (70 mg N/L of ammonium and nitrite, respectively), and pressure equalization to the atmospheric one, the overpressure evolution during time was recorded using a differential pressure transducer (0-5 psi, linearity 0.5% of full scale) manufactured by Centerpoint Electronics.

To check the inhibitory effect of sodium azide on the SAA, experiments with eight different concentrations of this compound (in the range from 0 to 100 mg/L) were carried out. The desired amount of sodium azide solution was added previous to closing the vials and flushing them. SAA tests to evaluate the reversibility of the inhibitory effect or the NaN_3 were carried out only with G2. In this case each vial was washed with phosphate buffer by consecutive filling it to the top and draining the supernatant for 10 times. Then SAA tests were performed.

Heterotrophic denitrification activity tests were carried out to determine if some of the produced nitrogen in the vials of the SAA tests could be originated by the presence of denitrifying activity. A procedure similar to that used in the SAA tests was performed but only nitrite (70 mg N/L) was added as substrate.

2.3 Calculations

The SAA was estimated from the measurement, throughout the time, of the overpressure inside the headspace of the vials (Dapena-Mora *et al.* 2007). The measured overpressure corresponded to N_2 production (composition higher than 99%) when the anammox was the only activity inside the vial (Dapena-Mora *et al.* 2007). However, in this case gas samples at the end of the SAA test were analysed to determine the N_2 production when N_2O was simultaneously produced and determine the actual SAA in g N/(g VSS·d).

The inhibitory effect of sodium azide on the anammox activity was expressed as percentage of activity maintained and calculated according to (EC.1):

$$\%SAA = SAA/SAA_0*100 \qquad \text{EC.1}$$

Where SAA and SAA_0 are the specific anammox activity measured in the presence of NaN_3 concentrations and corresponding to the control test (maximum SAA), respectively.

The IC_{50} was determined as the concentration of the NaN_3 which led to an activity percentage (%SAA) of 50%.

2.4 Analytical methods

At the end of each activity test, the biomass concentration (g VSS/L) was determined according to Standard Methods (APHA-AWWA-WPCF 2005) and gas samples collected

from the headspace of the vials were analysed by gas chromatography (GC). The GC system, a Hewlett Packard 5890 Series II instrument, was equipped with a flame ionization detector (TCD) and 80/100 Porapak Q column (2x1/8'', Supelco). The mobile phase consisted of Helium gas with a flow rate of 16 mL/min and oven, detector and injector temperatures were 35, 110 and 110 ºC, respectively. The average diameter of the granules was determined using a stereomicroscope (Stemi 2000-C, Zeiss) for image acquisition and the software Image ProPlus® for image analysis.

3. Results and Discussion

3.1 Short term anammox inhibition by sodium azide

Results from the batch experiments performed with the three types of biomass showed that the inhibitory effect exerted by NaN_3 on the maximum specific anammox activity is highly dependent on the biomass aggregation state (Figure 1). The partial nitritation-anammox granular biomass (G1) showed no inhibitory effect to sodium azide concentrations from 0 to 100 mg/L and its SAA value remained around 0.330±0.051 g N/(g VSS·d) (Table 1). The granular structure (Figure 2.a) and the presence of AOB in the outer layer of the granules (Morales *et al.* 2015) presumably helped to mitigate the toxic effect of NaN_3 over the anammox bacteria, located in the core of the granule.

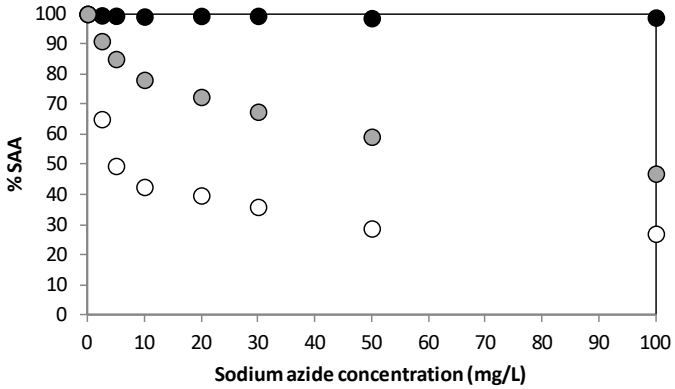

Figure 1. Effect of sodium azide at concentrations between 0 and 100 mg/L over the maximum specific anammox activity (SAA) of: partial nitritation-anammox granules (G1) (●), enriched anammox granules (G2) (●) and anammox flocs (F1) (○).

Table 1. Characteristics of sludge samples and sodium azide effects

Sample	Type of biomass	Diameter (mm)	SAA_0 (g N/g VSS·d)	NaN_3 inhibition	IC_{50} (mg /L)
G1	Granular PN-anammox	2.0	0.330±0.051	No	-
G2	Granular anammox	2.4	0.366±0.007	Yes	90
F1	Disaggregated anammox	-	0.366±0.007	Yes	5

However, when the enriched anammox biomass (G2 and F1) was evaluated the inhibitory effect of the NaN_3 was detected even at its lowest concentration. Mechanical disintegration did not affect the SAA measured in the absence of the inhibitor, which was of 0.366±0.007 g N/(g VSS·d) (Table 1). When both types of anammox biomass were exposed to increasing concentrations of NaN_3 a similar inhibition pattern was observed. The NaN_3 inhibitory effect increased faster at low inhibitor concentrations (up to 10 mg/L) showing a sharp decrease of anammox activity. The SAA diminution was less relevant for higher sodium azide

concentrations. These assays, with entire (Figure 2.b) and disintegrated (Figure 2.c) granules, showed the importance of the aggregation state of the biomass as the percentage of remaining activity is more than twice smaller for F1. The estimated value of IC_{50} with NaN_3 was around 90 mg/L for the anammox granular biomass (G2) and 5 mg/L for the disintegrated granules (F1). The granular structure originates presumably a gradient of the sodium azide (due to its diffusivity) throughout the granule that led to a much lower inhibition (Crank 1975). This effect can be also observed in unsteady state.

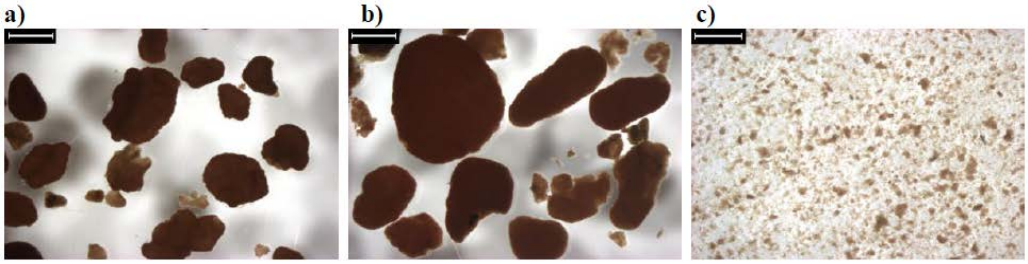

Figure 2. Images of **a)** partial nitritation-anammox granules (G1), **b)** enriched anammox granules (G2) and **c)** **a**nammox flocs (F1). The size bar represents 2 mm.

The partial nitritation-anammox granules (G1) presented a smaller average diameter than the anammox enriched granules (G2) with values of 2.0 mm and 2.4 mm, respectively (Figure 3). There were some bigger particles in G1 (up to 7.5 mm) but a wider size distribution was observed and huge amounts of small particles were present. More than 60% of the total number corresponded to particles with a size below 0.5 mm, while in the case of G2 about 50% were granules with sizes below 1.5 mm (Figure 3). Despite the mass transfer resistance is higher in the bigger granules, the present results shown higher inhibitory effect of NaN_3 in G2. Therefore, presumably not only the granular but also the presence of AOB located in the external layers of the granules could act as a physical barrier to avoid the direct contact of the anammox bacteria with this toxic compound. Moreover, the presence of extracellular polymeric substances (EPS) has been identified with the granular biomass resilience against toxic compounds (Chen *et al.* 2017).

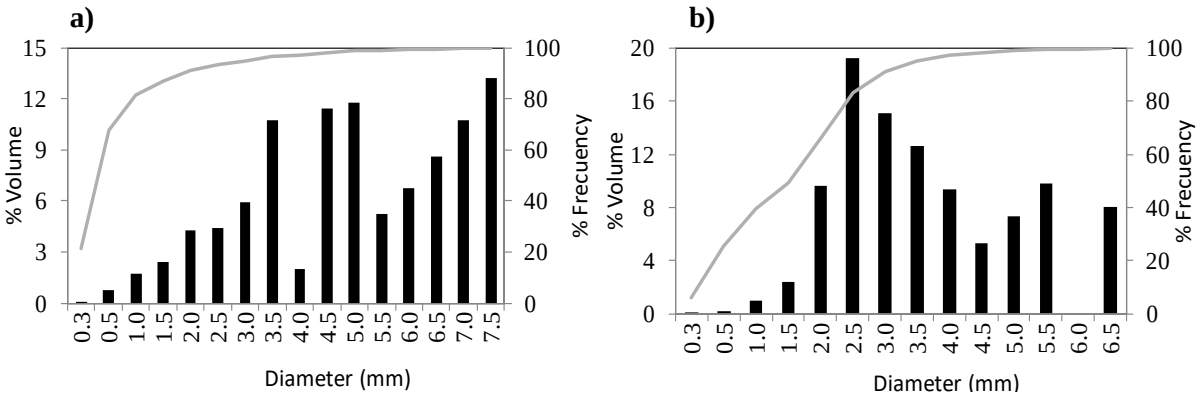

Figure 3. Size distributions of **a)** partial nitritation-anammox granules (G1) and **b)** enriched anammox granules (G2) in percentage of volume respect to the total volume. Grey line indicates the accumulative frequency of each class of particles.

López-Fiuza *et al.* (2002) has previously reported the IC_{50} value of 16.3 µg N_3/L for NOB, that is equivalent to 24.7 µg NaN_3/L, which is a value much lower than the one obtained for the anammox biomass in this study. Moreover, the sodium azide concentration reported to totally inhibit the NOB (in batch tests) is 1.5 mg/L according to Guisasola *et al.* (2005). This concentration caused, in the present study, the loss of around 5% of the specific anammox activity of anammox granules (G2) and 20% of flocculent anammox sludge (F1), respectively.

3.2 Production of N_2O

The composition of the gas phase in the SAA experiments was characterized. Although N_2 was the most abundant gas with a percentage between 98-99%, small amounts of CO_2 and N_2O were also detected. In all cases the higher the sodium azide concentration applied the higher the N_2O concentration measured (Figure 4), while the composition in terms of CO_2 was maintained practically constant (around 0.4% for G1 and G2 and 0.8% in the case of F1). Since the anammox bacteria metabolism does not produce N_2O (Kartal *et al.* 2007) further research about the reasons for these emissions is needed. Possible causes of the nitric oxide origin could be either the presence of heterotrophic denitrification, which used the products from the decayed biomass as organic carbon, or the occurrence of the nitrifying denitrification process (Ali *et al.* 2013). Moreover, N_2O might be also chemically produced by the reaction of sodium azide and nitrite in acid media to produce nitrogen and nitric oxide (Stedman 1959). However, a vial was incubated with the substrates (ammonia and nitrite) and phosphate buffer to test the gas production from the chemical reaction and no N_2O was detected.

Despite the unknown origin of this gas, again the aggregation state of the biomass has a strong influence. Comparing the results from the experiments with G2 and F1, the percentages of N_2O measured in the vials with the former (granules) was half of the amount produced with the latter (flocs). As the flocculent biomass (F1) was also more inhibited than the granular one (G2), the N_2O production might be related to the anammox bacteria activity inhibition, associated to their death, together with the production of another biological activity to be identified. Okabe *et al.* (2011) also found greater N_2O production in an anammox granules when its activity is inhibited (in that case due to the presence of HNO_2 due to the pH decrease). These authors concluded that the heterotrophic denitrification was the main responsible process for N_2O production due to the lack of enough organic matter to complete the process (Okabe *et al.* 2011). This might be also the cause in the present study since the only organic matter source present in the experiments comes from the decayed biomass.

In the case of the biomass G1 the SAA remained constant at all NaN_3 tested concentrations (Figure 1) but the N_2O percentage in the gas phase was similar to that from the flocculent biomass. The presence of the AOB that might carry out the nitrifying denitrification process might be responsible for this observation in G1. However in F1 the AOB population is not expected to be present in significant amounts.

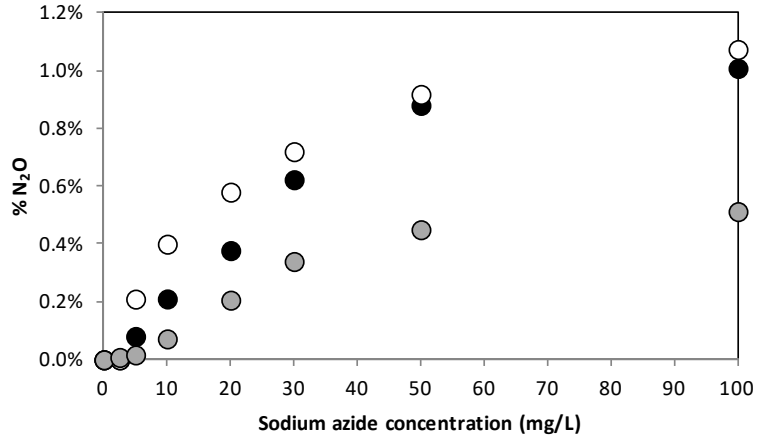

Figure 4. Effect of sodium azide at concentrations between 0 and 100 mg/L over the % of produced N_2O in the gas phase of: partial nitritation-anammox granules (G1) (●), enriched anammox granules (G2) (●) and anammox flocs (F1) (○).

In order to elucidate the possible heterotrophic denitrification role on the N_2O production, a denitrification test adding only nitrite as substrate was carried. The test was performed with the biomass G2 in the most unfavourable case (100 mg NaN_3/L). The denitrification activity measured was around 0.020±0.008 g N/(g VSS·d) and the gas composition was considerable different from that obtained in the SAA test (Table 2). The N_2O percentage obtained in the denitrification test was almost 6 times higher than the one measured in the anammox test, showing that presumably the heterotrophic denitrification activity may be the main process responsible for N_2O emissions. It should be pointed out that the gas phase is not greatly enriched in N_2 since it only accounts for 96%.

Based on the results obtained in this study it is not possible to conclude a clear explanation to identify the N_2O emissions source. But, it may be hypothesized that for G1 the main cause is the nitrifying denitrification and for G2 and F1 the heterotrophic denitrification. Presumably, the higher the inhibition of the anammox activity the higher the biomass decay and the more important the heterotrophic denitrification becomes on the gas production and therefore on the N_2O emissions. More biomass decay may take place in the F1 with the consequent higher heterotrophic denitrification as can be seen by the CO_2 production (almost double) and higher N_2O emissions (Table 2).

Table 2. Gas composition of the headspace in the SAA test and denitrification test with granular (G2) and flocculent (F1) anammox biomass at the presence of 100 mg sodium azide/L.

Biomass	Test	%N_2	%CO_2	%N_2O
F1	SAA	98.21±0.17	0.72±0.05	1.07±0.22
G2	SAA	99.08±0.03	0.40±0.02	0.51±0.01
G2	SAA*	99.64±0.01	0.19±0.01	0.17±0.01
G2	Denitrification	96.02±0.39	1.04±0.18	2.94±0.22

* Recovery SAA test, without NaN_3 after the exposition of the biomass to 100 mg/L.

3.3 Restoration of anammox activity

The possible reversibility of NaN_3 inhibitory effect over the anammox activity was evaluated in batch activity tests with anammox granules (G2) previously exposed to the toxic. Results indicated that the activity of the anammox biomass was almost completely restored after an exposure to sodium azide concentrations lower than 20 mg/L during 7 hours and after a washing step. However, in the case of the exposure to higher concentrations the activity was not completely recovered after biomass washing and about 30% of inhibition was detected in

the biomass exposed to 100 mg/L of NaN_3. The produced gas composition was also determined in these activity recovery experiments and the N_2O concentration was negligible in experiments performed with biomass previously exposed to concentrations up to 20 mg/L of NaN_3. In all cases it was lower than in the experiments with sodium azide. For example, the percentage of N_2O in the test with G2 and 100 mg/L was 0.5% and after washing the biomass the obtained percentage was of 0.2% (Table 2). However, after washing it was not possible to guarantee that sodium azide was fully removed from the biomass especially in the experiments with high concentrations and further research is required to evaluate the effects of the long term exposure.

In case these future long term inhibition experiments provide similar results to those obtained in the present study the sodium azide could be an option to be used to suppress the NOB activity not only in the one-stage partial nitritation-anammox process (not inhibition found) but also in the two-stage configuration using granular biomass in the anammox reactor. G2 at the concentration needed to achieve the complete inhibition of NOB (1.5 mg/L) (Guisasola *et al.* 2005) will lose around 5% of its SAA and this inhibition would be reversible meaning that to operate the reactor under this conditions is feasible.

4. Conclusions

To sum up, the biomass grown as granules together with the presence of AOB in the external layers can act as a physical barrier for the anammox bacteria to mitigate the toxic effects of chemical compounds as azides. The sodium azide did not affect the partial nitritation-anammox granular biomass. The enriched anammox biomass was inhibited by NaN_3 at all the concentrations tested, but the effect over anammox granules was considerably lower than over anammox flocculent biomass. Resulting in a IC_{50} of 90 mg/L for granular biomass and IC_{50} 5 mg/L for the flocculent one. For enriched anammox biomass (G2 and F1) N_2O emissions are related to the extension of SAA inhibition being lower when the inhibition is lower. In the case of partial nitritation-anammox biomass the nitrifying denitrification is the most probable source of N_2O and it increased at higher sodium azide concentrations.

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