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A N I N T E R N A T I O N A L J O U R N A L

The Influence of Stirring Speed, Temperature and Initial Nitrogen Concentration on Specific Anammox Activity

Tiago H. Martins[1]*, Theo S.O. Souza[2], Maria Bernadete Amâncio Varesche[1].

[1]*Department of Hydraulics and Sanitation, São Carlos School of Engineering, University of São Paulo, São Carlos, SP, Brazil.*

[2] *Department of Hydraulic and Environmental Engineering, Polytechnic School, University of São Paulo, São Paulo, SP, Brazil.*

ABSTRACT

This study evaluates the influence of initial nitrogen concentration, temperature and stirring speed on specific anammox activity (SAA). The biomass was tested in single batch reactors with different initial nitrogen concentrations (Assay 1) ranging from 60 to 140 mg N_{total}/L in equimolar ratio (NO_2^--N/NH_4^+-N) and in another test to 67.3 mg NH_4^+-N/L and 92.2 mg NO_2^--N/L (close to anammox stoichiometric ratio). The anammox biomass was also tested in single batch at different temperatures (from 20 to 37° C) to determine the short-term effects on SAA (Assay 2). In the third assay the stirring speed ranged from 50 to 150 rpm in a sequencing batch reactor (SBR) at 37 ºC. SAA was affected by the stoichiometric molar ratio but not by equimolar initial concentrations. The maximum specific anammox activities were 26.2 mg NH_4^+-N/g VSS.h in the single batch reactor at 37 ºC with NO_2^--N/NH_4^+-N stoichiometric ratio and 33.5 mg NH_4^+-N/g VSS.h in the SBR at 37 ºC and 50 rpm. The NO_2^--N/NH_4^+-N molar ratio affected specific anammox activity, and SAA showed to be more hindered by low increases of stirring speed than reported in the literature.

Key words: SBR, ammonium, nitrite, specific anammox activity, anammox

*Author for correspondence: dr.thmartins@gmail.com

INTRODUCTION

The pollution caused by nitrogen compounds, mainly characterized by an excess of ammonium used in fertilization practices, is a subject of great concern. The nitrogen released into the environment affects the biogeochemical cycles by stimulating photosynthetic organisms that fix carbon, increasing biomass concentration in water bodies and consequently causing eutrophication.

The conventional nitrification-denitrification processes play an important role in removing nitrogen from wastewater [1]. Besides the classical nitrification, ammonium (NH_4^+) can also be oxidized in anoxic environments in a process known as anammox (anaerobic ammonium oxidation), coupled to nitrite (NO_2^-) reduction. This process applies autotrophic nitrogen removal in wastewater under anaerobic conditions, and is advantageous in environments containing low levels of readily biodegradable organic matter [2,3]. Since it requires that approximately half of the NH_4^+-N is oxidized partially to NO_2^--N in a preceding nitrification step for producing an anammox-suitable influent, the process greatly reduces the oxygen demand [4]. Moreover, given that anammox bacteria do not require organic matter and the process presents low sludge production, from the viewpoint of environmental preservation, it is considered as a sustainable anaerobic wastewater treatment for nitrogen removal processes.

Strous et al. [5] estimated the anammox stoichiometry based on mass balance on anammox enriched cultures, as shown in Eq. 1. The anammox physiology was studied by Strous et al. [6] in batch reactors. The specific anammox activity (SAA) obtained was 1.1 g NH_4^+-N/g protein. day. Concentrations of up to 1 g N/L in ammonium or nitrate forms did not inhibit anammox activity. However, values above 100 mg NO_2^--N/L (for several days) inhibited the process. Moreover, the addition of 1.4 mg N/L hydrazine and 0.7 mg N/L hydroxylamine (intermediate compounds of the anammox process) reestablished metabolism.

$$1\ NH_4^+ + 1{,}31\ NO_2^- + 0{,}045\ HCO_3^- \longrightarrow$$
$$\longrightarrow 1{,}045\ N_2 + 0{,}22\ NO_3^- + 0{,}045\ CH_2O_{0,5} + 1{,}87\ H_2O + 0{,}09\ OH^- \quad (1)$$

Egli et al. [7] enriched a culture with 88% of anammox bacteria (determined using quantifying FISH method) in a rotating biological contactor treating ammonium-rich leachate. The culture was tolerant to up to 182 mg N-NO_2^-/L, which is a higher value than that observed for *Brocadia anammoxidans*. However, the catalytic anammox activity was 20 times smaller than B. *anammoxidans*.

The first studies on anammox showed enhanced activities at higher temperatures, in the range of 37 to 40 °C [8,9]. Dalsgaard and Thandrup [10] reported anammox activity in marine sediments ranging from 6.5 to 35.7 ºC. In long-term tests using a SBR, Dosta et al. [11] and Hu et al. [12] showed SAA between 12 and 30 ºC.

Aside from nitrite concentration and temperature, many other factors affect the anammox process, such as ammonium concentration, organic matter, salinity, heavy metals, phosphate and sulfide [13,14]. Moreover, visible light and stirring speed are also known to influence anammox activity [4].

Among them, operational conditions such as initial nitrogen concentration, temperature and stirring speed are particularly important, and must be studied systematically. In this context, the present work aimed to assess the specific anammox activity varying these three parameters. Its experimental determination was conducted by measuring the specific nitrogen compounds consumption under these different conditions to obtain information about how they influence the anammox process and to provide data that could be used to improve systems using this biotechnology.

MATERIAL AND METHODS

Biomass from a 5-litre sequencing batch reactor (SBR) enriched for anammox was used as inoculum source [15], and contained 0.6 g VSS/L. The SBR was fed with synthetic medium according to Graaf et al. [8]. Assays 1 and 2 were carried out as single batch tests using the same synthetic medium in 250 mL flasks filled with 90mL of medium and 10ml of anammox biomass. The influence of different parameters on anammox activity was evaluated as described in Table 1. The initial nitrogen concentrations applied to the SBR enrichment were the same used in Assay 2 and also used by Dosta et al. [11] in SAA temperature tests.

The short-term experiments with different concentrations of nitrogen compounds and temperature (Assays 1 and 2) were performed in triplicate. Assays 1 and 2 were carried out at 37 ºC and the temperature in Assay 2 was tested between 20 to 37 ºC (Table 1).

Table 1. Parameters evaluated in specific anammox activity assays

Nitrogen concentration (Assay 1) (mg NO_2^--N/L / mg NH_4^+-N/L)	Temperature (Assay 2) (70mg NO_2^--N/ L and 70mg NH_4^+-N/L)	Stirring speed (Assay 3) (Specific Power)
31.6 / 32.8	37 °C	50 rpm (0.008 kW/m^3)
73 / 67	35 °C	80 rpm (0.032 kW/m^3)
92.2 / 67.3	30 °C	100 rpm (0.064 kW/m^3)
	25 °C	150 rpm (0.216 kW/m^3)
	20 °C	

The influence of stirring speed on anammox activity was evaluated using time profiles obtained for the SBR operated by Martins et al. [15]. The SBR was operated between 383 and 433 days under different stirring speeds. The SBR in standard conditions was operated continuously at 50 rpm (0.008 kW/m^3). During the assays to measure the influence of stirring speed, the stirring speed of the reactor was only changed during one batch cycle. The standard stirring speed was then reestablished to 50 rpm after the test; therefore, only the short-term effects of changing the stirring speed were tested. One week after the original conditions were reestablished, the reactor was subjected to the next test.

The specific power consumption in a stirred vessel depends on the various geometrical parameters of the impeller (height (h) and diameter (d)), reactor (diameter (D) and height (H)), the rotating speed (N) and the fluid properties (density (ρ) and viscosity (μ)). The calculation of the energy consumed in the stirring process depending on the rotating speed (N) was performed according to Arrojo et al. [16]. The reactor was made in borosilicate glass (diameter of 20.3 cm and height of 17 cm). A six-bladed turbine carried out the stirring at 50 rpm (each blade with length of 6.8 cm and height of 2 cm).

SAA was estimated from the slope of the curve describing the NH_4^+-N consumption as a function of time and related to the biomass concentration in the flasks, as shown in Eq. (2). Values of k_{NO2-} and k_N (for nitrite consumption and nitrogen production) were calculated similarly.

Analysis of ammonium, nitrate and nitrite were performed using flow injection analysis (FIA), and biomass concentration was expressed as g VSS/L in accordance to APHA [17].

Effects of temperature on bacterial anammox activity were modeled using the integrated form of the Arrhenius equation [18]. The activation energy (E_a) was estimated from the slope of an Arrhenius plot of ln (k) as a function of T^{-1} (Eq. 3).

The initial nitrogen concentrations used to calculate E_a in the Assay 2 were in according to Dosta et al. [11].

$$C_n/C_x = -k(t - t_0) + C_{no}/C_x \quad (2)$$

Where: C_n/C_x (mg N/g VSS) = nitrogen concentration (C_N) per unit of biomass (C_x) as a function of time (t);k (mg N/g VSS.h) = zero-order kinetics constant (SAA, for NH_4^+-N consumption); C_{no}/C_x (mg N/g VSS) = initial nitrogen concentration (C_{no}) per unit of biomass (C_x).

$$\ln(k) = \ln(A) - \frac{Ea}{R}\frac{1}{T} \quad (3)$$

Where: A is the Arrhenius constant; k is the reaction rate (mg N/g VSS.h); R is the gas constant (8.31 kJ/mol); and T is the absolute temperature (K).

RESULTS AND DISCUSSION

SINGLE BATCH ASSAYS: NITROGEN CONCENTRATION (ASSAY 1) AND TEMPERATURE INFLUENCE (ASSAY 2)

Single batch assays were performed to verify the behavior of biomass regarding SAA under different temperatures and nitrogen concentrations. In Tests 1 and 2, in the Assay 1 series (Table 2), ammonium was not completely consumed (Figure 1 (a, b)) while in Test 3 (when nitrite concentration was increased to reach the stoichiometric ratio: 1.36 NO_2^--N / 1 NH_4^+-N) both ammonium and nitrite were 100% removed (Figure 1 (c)). This phenomenon was probably due to the fact that the biomass was not subject to nitrite limiting condition, and thus the two nitrogen compounds could be completely consumed, reaching values close to zero simultaneously.

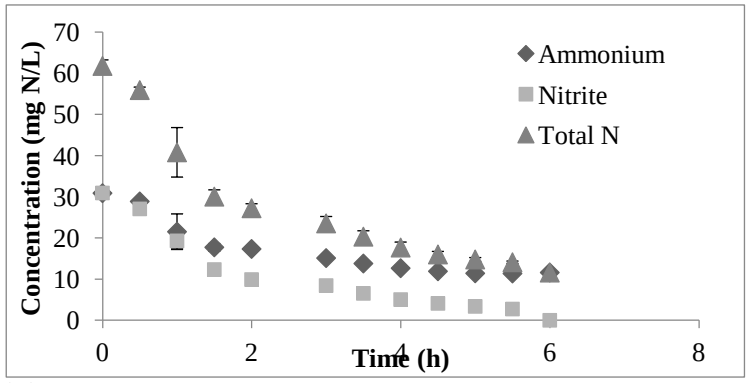
As shown in Table 2, the values of SAA and k_{NO2-} from Tests 1 and 2 (equimolar initial concentrations) were similar. Analysis of variance (95% confidence) revealed that the difference between Test 1 and 2 on Assay 1 was not statistically significant, but both were statistically different from Test 3. However, in Test 3, the SAA and k_{NO2-} were 23 and 30% higher than those obtained in Tests 1 and 2, respectively. This indicated that the initial concentration of the compounds in Tests 1 and 2 did not influence the SAA, but the initial molar ratio (nitrite/ammonium) had a significant influence on SAA.

Lopez *et al.* [19] performed the start-up and enrichment of the anammox process based on the influent molar ratio of nitrite/ammonium, gradually increasing it from 0.76 to 1.32, and exponentially increasing the nitrogen load applied from 0.01 to 1.60 kg N/d. m^3. The ammonium removal efficiencies were 53, 79 and 99.9% for the ratio of 0.76, 1 and 1.32, respectively, corroborating the influence of the nitrite/ammonium molar ratio on the performance of the process. However, it was not possible to compare the SAA values as a response of nitrogen molar ratios, because the authors did not present the values in specific terms.

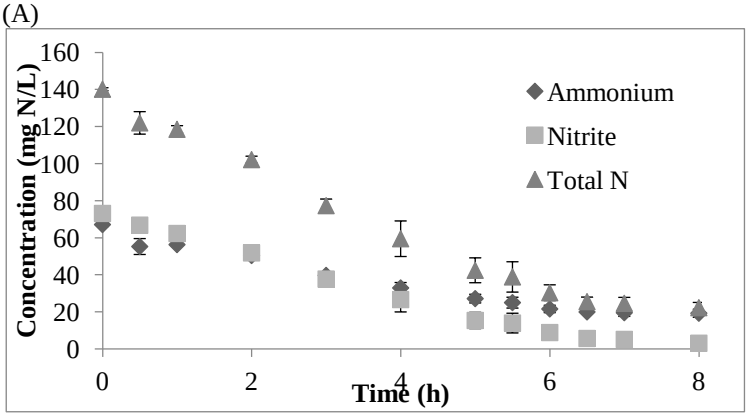
The evaluation of temperature influence was also performed in single batch tests (Table 3). The initial nitrogen concentration of each test was approximately equal to Test 2 of Assay 1.

Table 2. Initial nitrogen concentration influence in Assay 1.

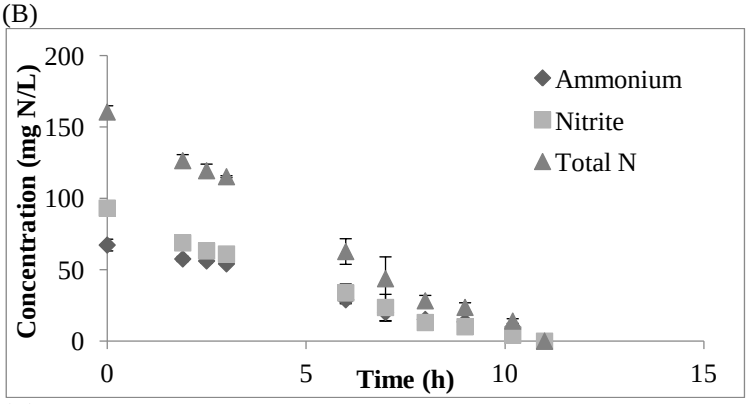
Test	Concentration (mg NO_2^--N/L / mg NH_4^+-N /L)	Molar Ratio (Ammonium consumed : Nitrite consumed : Nitrate produced)	SAA (mg NH_4^+-N/ g VSS.h)	Complete Ammonium consumption
1	31.6 / 32.8	1 : 1.24 : 0.24	20.1±1.2	No
2	73 / 67	1 : 1.43 : 0.34	19.5±1.9	No
3	92.2 / 67.3	1 : 1.38 : 0.21	26.2±2.1	Yes



(A)



(B)



(C)

Figure 1. Temporal variation of nitrogen compounds in batch tests with (A) 32.8 mg NH_4^+-N/L and 31.6 mg NO_2^--N/L, (B) 67 mg N-NH_4^+-N/L and 73 mg NO_2^--N/L and (C) 67.3 mg NH_4^+-N/L and 92.2 mg NO_2^--N/L. Error bars represent the standard deviation.

At 35 °C, nitrite was almost completely removed in 6 hours. The final concentration of ammonium, nitrite and nitrate were respectively 9.9 mg NH_4^+-N/L, 2.4 mg NO_2^--N/L and 17.4 mg NO_3^--N/L (10 hours). The test at 30° C was also carried out for 10 hours, with final concentrations of 10 mg NH_4^+-N/L, 5 mg NO_2^--N/L and 13.5 mg NO_3^--N/L. In the tests at 25 and 20º C, a much greater inhibition of anammox

activity by low temperature was observed, mainly at 20 °C. These experiments ended at 15 hours with 88 and 55% nitrite removal and 68 and 45% ammonium removal, for 25 and 20 ^{o}C, respectively.

The specific anammox activity increased gradually with temperature ranging between 20 and 35 °C, with values increasing from 1.20 to 6.6 mg NH_4^+-N/g VSS.h (Figure 2). However, there was an abrupt SAA increase at 37 °C (Test 2 of Assay 1) reaching values of 19.5 mg NH_4^+-N/g VSS.h. These values are similar to those obtained by Strous et al. [6] and Jetten et al. [9] for optimal temperatures ranging between 37 ºC and 40 °C. Table 3 shows the molar ratio on each Test. The nitrite consumption rate by anammox equation was higher than expected by the anammox molar ratio, indicating the presence of denitrifying activity in the biomass.

Egli et al. [7] performed SAA assays at the following temperatures: 11, 20, 25, 30, 37 and 45 °C. Maximum SAA (as N_2 production rate) was observed at 37 °C. No anammox activity was observed at 45 °C, and after the temperature was reduced to 37 °C the activity could not be reestablished. At 11°C, SAA was approximately 24% of the activity observed at 37 °C. In contrast, in the Assay 2 the biomass at 20 ºC presented only 9.2 % of the activity at 37 °C, under equimolar initial concentration.

Table 3. Temperature influence on Assay 2.

Temperature (ºC)	Molar Ratio (Ammonium consumed : Nitrite consumed : Nitrate produced)	SAA (mg NH_4^+-N/ g VSS.h)	Complete Ammonium consumption
20	1 : 1.47 : 0.39	1.8±0.3	No
25	1 : 1.43 : 0.26	2.4±0.3	No
30	1 : 1.46 : 0.26	4.06±0.2	No
35	1 : 1.35 : 0.45	6.6±0.7	No
37	1 : 1.43 : 0.34	19.5±1.9	No

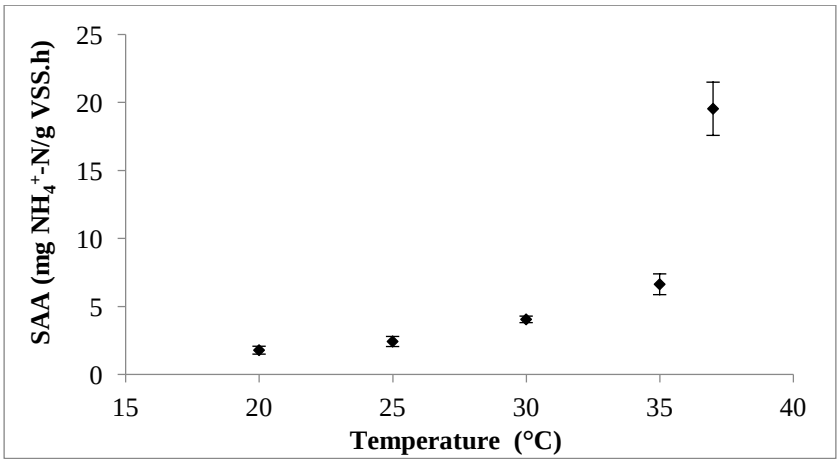


Figure 2. Specific Anammox Activity under different temperatures. Error bars represent the standard deviation.

Strous et al. [20] studied the anammox process in a wastewater treatment plant sludge digester. Preliminary experiments indicated that the biomass was not inhibited by the characteristics of the effluent. 37 °C and pH 8 were the optimum values for the anammox activity. In these physiological tests, specific anammox activity (SAA) was 0.58 mg N-NH_4^+/g VSS.h, five times lower than the SAA of the biomass in the reactor. The biomass in the batch assays presented 78.2% of activity in the SBR.

An exponential increase of the SAA was observed for temperatures up to 37 °C. Activation energy (E_a) of 104 kJ mol^{-1} was calculated for anammox biomass

according to the Arrhenius model (Eq. 2). The activation energies of the anammox process in other reactors treating wastewater were 63 and 70 kJ/mol, respectively [6,11]. The E_a of 61 and 51 kJ/mol in marine sediments were lower than the wastewater treatment reactors [10,18].

Sequencing batch (Assay 3): stirring speed influence

The same behavior pattern of nitrogen compound concentrations was observed in all assays regarding stirring speed influence (Assay 3), with concentration increasing up to 250 mg N_{total}/L at 2.5 hours of the batch cycle, coinciding with the filling period of the reactor (Figure 3). In all assays the influent concentration was averagely 219 mg NH_4^+-N/L and 289 mg NO_2^--N/L.

In the standard operating conditions (50 rpm/0.008 kW/m^3) (Figure 3 (a)), it could be observed that nitrite (limiting substrate) was consumed up to the 10th hour. When the SBR was set to 80 rpm (0.032 kW/m^3), the concentration of nitrogen compounds reached a maximum of 120.1 mg NH_4^+-N/L and 115.5 mg NO_2^--N/L in the second hour (Figure 3b), slightly higher than 50 rpm (96.8 mg NH_4^+-N/L and 108.3 mg NO_2^--N/L). At 100 rpm (0.064 kW/m^3), maximum ammonium (104.5 mg NH_4^+-N/L) and nitrite values (144.1 mg NO_2^--N/L) occurred in the second hour of the batch cycle. At the time profile of the test at 150 rpm (0.216 kW/m^3) (Figure 3d) maximum values of ammonium (113.8 NH_4^+-N/L) and nitrite (154.6 mg NO_2^--N/L) were observed three hours after the beginning of the test.

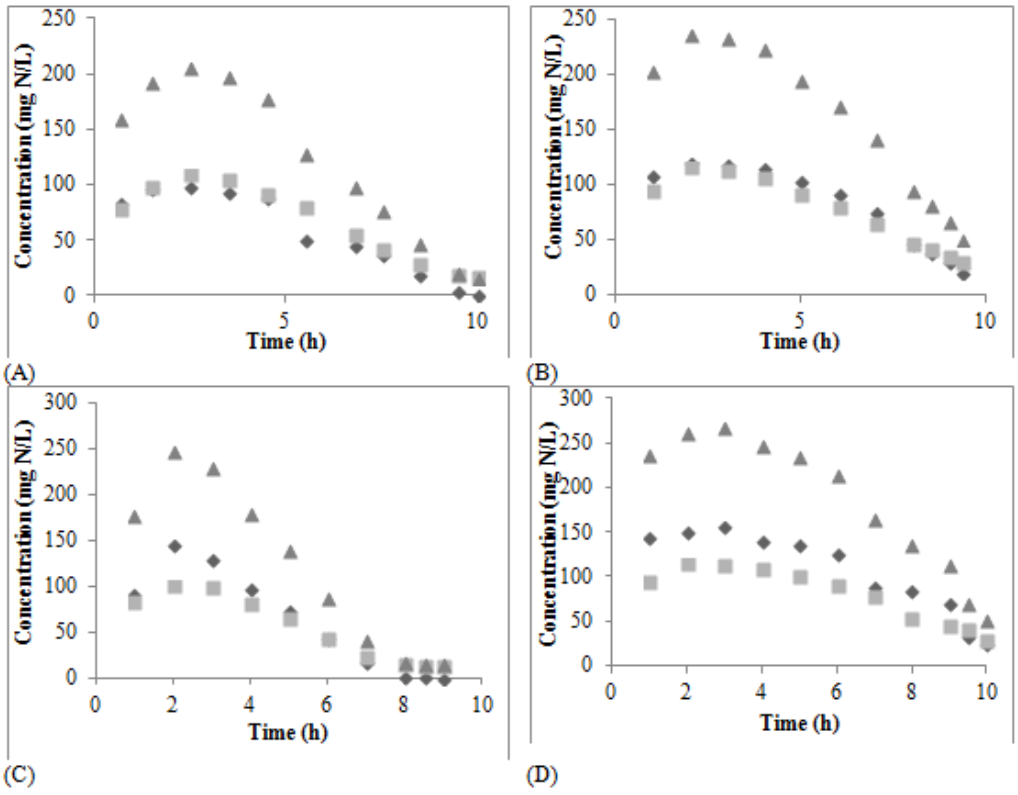


Figure 3. Temporal variation of nitrogen compounds in a SBR subjected to (A) 50 rpm, (B) 80 rpm, (C) 100 rpm and (D) 150 rpm. Nitrite nitrogen (Squares), Ammonium nitrogen (Diamonds) and Total nitrogen (Triangles)

As shown in Tables 4 and 5, at 50rpm, values of k_{NO2-} = 34.8 mg NO_2^--N/g VSS.h and specific anammox activity SAA = k_{NH4+} = 33.5 mg NH_4^+-N/g VSS.h were obtained. The SAA at 80 rpm was 28.8 mg N-NH_4^+/g VSS.h, lower than that obtained for 50 rpm. The SAA at 100 rpm test was 29.2 mg NH_4^+-N/g VSS.h, and

the k_{NO2-} was 43.3 mg NO_2^--N/g VSS.h. In this case, the highest k_N of all the assays tested was reached, resulting in 72.5 mg N/g VSS.h (Figure 3). The SAA at 150rpm was 24.1 mg NH_4^+-N/g VSS h (Table 4). This test had the highest nitrate production over consumption of ammonium (0.57 mol NO_3^--N/ mol NH_4^+-N).

Table 4. Stirring speed influence on Assay 3.

Stirring speed (rpm)	Molar Ratio (Ammonium consumed : Nitrite consumed : Nitrate produced)	SAA (mg NH_4^+-N/ g VSS.h)	Complete Ammonium consumption
50	1:1.32:0.19	33.5	No
80	1:1.33:0.27	28.8	No
100	1 : 1.4 : 0.25	29.2	No
150	1 : 1.44 : 0.57	24.1	No

There was a tendency to the decrease of SAA values with increasing agitation of the SBR (Table 5). The values ranged from 33.6 to 24.1 mg NH_4^+-N/g VSS.h, when stirring speed was increased from 50 (0.008 kW/m^3) to 150 rpm (0.216 kW/m^3), respectively. However, an increase in the kinetic parameter k_{NO2-} (43.3 mg NO_2^--N/g VSS.h) was observed at 100 rpm. In the other tests, the values remained almost constant, ranging from 34.8 to 36.9 mg NO_2^--N/g VSS.h for 50 and 150 rpm, respectively.

Table 5. Kinetic parameters under different stirring speeds in SBR.

Stirring speed (rpm)	$k_{nitrite}$ (mg NO_2^--N/g VSS.h)	$k_{ammonium}$ (mg NH_4^+-N/g VSS.h)	$k_{total\ N}$ (mg N/g VSS.h)
50	34.8	33.5	67.1
80	35.5	28.8	64.2
100	43.3	29.2	72.5
150	36.9	24.1	61.0

The increase in k_{NO2-} at 100 rpm was probably due to the following possibilities: (i) endogenous denitrification of biomass lysed by shearing at this stirring speed, probably by micro-organisms related to *Pseudomonas* sp. and *Comamonas* sp. present in the SBR [15]; and/or (ii) the Ar/CO_2 atmosphere in the headspace of the reactor may not have been sufficient to maintain the anaerobic agitation conditions at this speed, enabling the occurrence of nitrification by nitrite oxidizing bacteria (NOB) and subsequent reduction of nitrate, because at 100 rpm there was no more nitrate accumulation than the one produced by the anaerobic oxidation of ammonium.

At 150 rpm, the biomass presented a different behavior. Nitratation probably occurred but there was no nitrate reduction, since in this condition there was more nitrate accumulation than expected, regarding the anammox process. It can be noted that although the values of k_{NO2-} were similar at 50 and 80 rpm, at 80rpm the k_{NO2-} was proportionally higher than k_{NH4+} (SAA), indicating the favored use of nitrite compared to ammonium at higher stirring speeds (Table 5).

The molar ratio of nitrate formation should be of approximately 0.26 [5]; however, a value of 0.57 was obtained at a stirring speed of 150 rpm (Table 4). In addition, there were higher nitrite conversions at the two higher stirring speeds, since the nitrite / ammonium molar ratios were 1.4 and 1.44 at 100 and 150 rpm, respectively.

Arrojo et al. [16] tested stirring speeds between 60 rpm (0.003 kW/m^3) and 180 rpm (0.09 kW/m^3) in a SBR and observed no significant changes in SAA, but there was a significant decrease in this parameter at 250 rpm (0.23 kW/m^3). The authors reported

a sharp decrease of the SAA only when the specific power was higher than 0.09 kW/m^3. Nevertheless, in the present study a decrease of the SAA was observed for lower ranges of specific power (14% when comparing 0.008 to 0.032 kW/m^3) (Table 4).

It was also observed that 154.6 mg NO_2^--N/L in the third hour of the batch at 150 rpm did not cause biomass inhibition. This value was higher than that reported by Strous et al. [6] but lower than that one reported by Egli et al. [7]. The anammox biomass used by Bettazzi et al. [21] showed 25% inhibition for 60 mg NO_2^--N/L in short-term tests, but repeated additions of nitrite higher than 30 mg NO_2^--N/L caused activity losses. It was also observed that 154.6 mg NO_2^--N/L in the third hour of the batch at 150 rpm did not cause biomass inhibition. This value was higher than that reported by Strous et al. [6] but lower than that one reported by Egli et al. [7]. Considering the studied conditions, the best stirring speed (and specific power applied) in the SBR was 50 rpm (0.008 kW/m^3), because increasing the applied power did not significantly increase the SAA and, for optimization purposes, 50 rpm was sufficient for good results.

In this study, two values of the maximum specific anammox (MSAA) were determined: 1) 26.2 mg NH_4^+-N/g VSS.h in Test 3 of Assay 1 (in single batch), and 2) 33.5 mg NH_4^+-N/g VSS.h (in the SBR test with stirring speed of 50 rpm). The MSAA was lower for single batch tests when compared to the SBR, probably due to improved agitation, enhanced mass transfer in the SBR and differences on the reactors configuration. Strous et al. [20] also investigated the reason for the lower activities in experiments with single batch reactors, but did not reach any precise conclusions about this phenomenon.

The SAA values found in this study were 43% of the value obtained by Jetten et al. [9]. However, the SAA of 33.5 mg NH_4^+-N/g VSS.h obtained in assay 3 (at 50 rpm) was higher or close to most of the relevant data. Table 6 presents a data compilation from different SAA studies in the literature.

Table 6. Specific anammox activity values found in literature.

Specific anammox activity (mg NH_4^+- N/g VSS.h)	Reactor Type	Temperature (°C)	Reference
4.25[b]	Batch	35	[22]
37.1[a, b]	Batch	37	[7]
47.91	Gas-lift	30	[23]
7.5	SBR 50rpm	35	[24]
27.08	SBR 70 rpm	35	[24]
3.54	SBR	35	[25]
3.64	SBR 50 rpm	35	[26]
18.33[b]	Batch	35	[27]
16.67[b]	SBR 60-180rpm	30	[16]
10.41[b]	SBR 250 rpm	30	[16]
66.67	Fixed-bed	37	[28]
33.5	SBR 50 rpm	37	This work
26.2	Batch	37	This work

[a] The amount of biomass in the original article was given as protein, and it was converted to VSS using a factor of 0.6 g protein = 1g VSS [5,29]

[b] The nitrogen was given as N-N_2

CONCLUSIONS

The specific anammox activity increased abruptly at 37° C. The NO_2^--N/NH_4^+-N molar ratio affected specific anammox activity, and SAA decreased with increasing stirring speed. For the single batch assays, 92.2 mg NO_2^--N/L and 67.3mg NH_4^+ N /L at 37 ºC was the best condition among the tested. The present study showed a decrease of the SAA for low increments of specific power. An increase in the value of the kinetic parameter k_{NO2-} at 100 rpm was observed, and for the other stirring speeds the values remained practically constant. Maximum specific anammox activity (MSAA) occurred at 50 rpm and 37 °C in the SBR and was 33.5 mg NH_4^+-N/g VSS.h with initial concentrations close to the stoichiometric NO_2^--N/NH_4^+-N molar ratio (1.32).

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