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Mainstream anammox reactor performance treating municipal wastewater and batch study of temperature, pH and organic matter concentration cross-effects

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Abstract

The anammox process is an energy efficient promising alternative to biologically remove the nitrogen. Thus, a 5-L anammox granular reactor was inoculated with sludge coming from a sidestream partial nitritation and anammox reactor (> 200 mg N/L and 30 °C) and it was directly subjected to 15 ± 1°C treating mimicked municipal wastewater (50 mg N/L). Results indicated that an acclimation period (commonly used) to progressive reach the mainstream conditions is not needed, shortening the start-up periods. The long-term anammox process stability was proved to treat synthetic wastewater with decreasing alkalinities and nitritified primary settled municipal wastewater. The low pH values (6.2 ± 0.1) of the municipal wastewater fed did not affect the process stability. Residual organic matter concentrations augmented the nitrogen removal efficiency from 80 % (with the synthetic medium) to 92 % achieving effluent concentrations below 10 mg TN/L. Finally, the effect of pH (6 - 8), temperature (15 - 30 ° C) and organic matter concentration (0 - 75 mg TOC/L) over the specific anammox activity (SA_{AMX}) was evaluated at short-term. pH and temperature and their interactions exerted significant influence on the SA_{AMX} value while the TOC concentrations itself did not significantly change the SA_{AMX}.

Keywords: alkalinity; autotrophic nitrogen removal; inorganic carbon; mainstream; low temperature; specific anammox activity.

1. Introduction

The contribution of the partial nitritation and anammox (PN/AMX) processes, to the achievement of energy autarky in wastewater treatment plants (WWTPs), when implemented in the mainstream is undeniable. However, up to now, mainstream anammox based processes have never been implemented at full-scale (Qiu et al., 2020). The main identified limiting factors that challenge its application are the slow growth rate of the anammox bacteria (exacerbated at low temperature, < 20 °C), and the low biomass yield associated to the low total nitrogen (TN) concentrations in the mainstream (< 100 mg TN/L) (Hoekstra et al., 2018; Qiu et al., 2020). To tackle these issues, the biomass retention maximization inside the reactor is fundamental.

The operation of anammox systems at temperatures below their optimal range (< 30 °C) has a detrimental effect on the anammox population activity. Lotti et al. (2015) found that the anammox energy activation cannot be considered constant for the temperature range from 15 to 30 °C, as it is generally accepted for ammonium oxidizing bacteria (AOB) in the activated sludge models (Henze et al., 2006). For this reason, AOB and anammox activities are unbalanced, limiting one-stage PN/AMX stability and the specific treatment capacity of the system. To implement the PN/AMX processes in separate units represents a beneficial solution to optimize both independently. In such circumstances, in the anoxic reactor, anammox bacteria competition is absent or minimized with nitrite oxidizing bacteria (NOB), since dissolved oxygen (DO) would not be available; and with heterotrophic denitrifying bacteria, as negligible organic matter concentrations will be present. These competitions are also widely reported as challenging aspects of the mainstream anammox implementation (Qiu et al., 2020).

The feasibility of performing the anammox process at low/moderate temperature was assessed in the long-term using different reactor configurations. Most available studies, up to now, have been performed by subjecting the inoculum to long acclimation periods (several months) until low temperature and low TN concentrations were reached leading to stable performance but long start-up periods (De Cocker et al., 2018; Reino et al., 2018; Sánchez Guillén et al., 2016). Moreover, limited research works were performed using municipal wastewater (Laureni et al., 2015; Lotti et al., 2014b; Ma et al., 2013; Reino et al., 2018) and the achieved TN concentrations in the effluent were up 40 mg TN/L, higher than 10 mg TN/L the established discharge limit in the European Union, among others, for sensitive areas.

Furthermore, in most research works, anammox reactors are usually fed with an excess of alkalinity while, in practice, the inorganic carbon (IC) concentration in municipal wastewater is low (Burton et al., 2014; Seuntjens et al., 2018). As chemolithoautotrophic bacteria, anammox bacteria utilize IC during the anabolism being vulnerable to the IC limited conditions, decreasing the biomass activity (Strous et al., 1998). Moreover, IC has an important role in maintaining the pH value adequate for the biological reactions. With this in mind, Kimura et al. (2011) defined as optimal for the anammox process a maximal influent IC to ammonium ratio of 5.83 mg NH_4^+-N/mg IC to maintain the anammox activity. Contrary, Liao et al. (2008) found that the nitrogen removal rate (NRR) decreased when the influent ratio decreased from 0.32 to 0.28 mg NH_4^+-N/mg IC. Moreover, Jin et al. (2014) reported that an IC shortage increases the inhibition caused by excess substrates. Anammox bacteria are sensitive to environmental conditions such as pH, temperature, DO, organic matter or substrate concentrations (Jin et al., 2012). The reported threshold values were mainly determined at high temperatures (≥ 30 °C) and might vary at mainstream conditions. Daverey et al. (2015) evaluated the simultaneous effect of temperature and pH and observed that the optimal pH increases at low temperatures. Since the nitritation process consumes alkalinity, the low pH of the stream entering the mainstream anammox reactor might be an issue. Moreover, the presence of residual organic matter concentration could favour the denitrification process development. Scarce information and unclear conclusions are available on the interaction of combined inhibitory conditions (Daverey et al., 2015; Tomaszewski et al., 2017).

Hence, the main objective of this study is to evaluate the long-term performance and stability of a granular anammox reactor operated at mainstream conditions, 15 °C and 50 mg TN/L, and inoculated with biomass which was not previously acclimated to low nitrogen neither lower temperature. First, the effect of alkalinity supply over the long-term reactor performance was assessed using synthetic feeding as well as the effect of treating partially nitritified municipal wastewater, evaluating the influence of the residual organic matter concentration. Additionally, the influence of individual and combined effects of temperature, organic matter concentration and pH on the specific anammox activities (SA_{AMX}) was tested in batch experiments.

2. Materials and Methods

2.1. Reactor description and operating conditions

A 5-L sequencing batch reactor (SBR) with a volume exchange ratio fixed at 25 % was used to perform the anammox process (Figure 1). The temperature was controlled at 15 ± 1 °C. The complete mixture was provided by a mechanical stirrer with a rotating speed of 60 - 80 rpm. A slow flow of Argon gas (95 % Ar and 5 % CO_2) was bubbled in the liquid media to ensure anoxic conditions until day 200. From this day onwards, the anoxic conditions were maintained by the nitrogen produced during the anammox process itself. The SBR was inoculated with granular PN/AMX sludge from a 1.2-m^3 ELAN® pilot plant (Morales et al., 2015), which treated, at 30 °C, the supernatant from an anaerobic sludge digester in a municipal WWTP.

The SBR operation lasted 485 days divided into 8 different Stages (Table 1). It was fed with synthetic media, between days 0 and 392 (S-I to S-VI), and with municipal wastewater from day 393 onwards (S-VII to S-VIII). In Stage I, nitrate was supplied to the feeding, and its concentration was gradually decreased in Stages II and III. Then, during Stages IV to VI, the alkalinity concentration fed was also step-wise decreased to have values for this parameter close to mainstream conditions.

Finally, during Stage VII and VIII, a nitritified municipal wastewater was treated in the SBR and in Stage VIII the cycle duration was shortened in order to treat higher load.

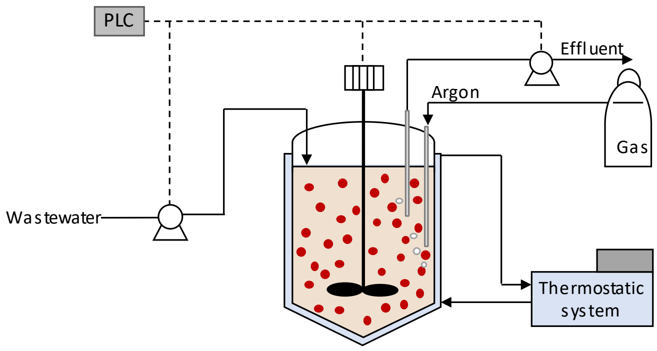

Figure 1. Scheme of the experimental set-up for establishing the anammox process.

The synthetic feeding composition was adapted from the one described by Dapena-Mora et al. (2004), containing 25 mg NH_4^+-N/L (as NH_4Cl) and 25 mg NO_2^--N/L (as $NaNO_2$). Nitrate was supplied, 0 to 25 mg NO_3^--N/L (as KNO_3), to prevent biomass methanization under anaerobic conditions. The feeding media was supplemented with, in mg/L: 96 – 1,250 of $KHCO_3$ (to provide variable alkalinities), 147 of KH_2PO_4, 300 of $CaCl_2 \cdot 2\ H_2O$, 200 of $MgSO_4 \cdot 7\ H_2O$, 11 of $FeSO_4 \cdot 7\ H_2O$, 8 of EDTA-Na·2 H_2O and 0.2 mL/L of trace solution (Vishniac and Santer, 1957).

From day 393 onwards, the anammox reactor was fed with primary settled municipal wastewater (Table 1), which was partially treated in a nitritation reactor based on the *in situ* free nitrous acid (FNA) accumulation strategy (Pedrouso et al., 2017). During this period, up to 85 % of the ammonium fed to this nitritation unit was oxidized to nitrite. Therefore, this effluent was mixed with raw primary settled municipal wastewater (containing only ammonium as nitrogen) to obtain a stream with a nitrite to ammonium ratio of approximately 1.2 g NO_2^--N/g NH_4^+-N (Table S1 in Supporting Material).

Table 1. Summary of the operating conditions and feeding composition throughout the operational stages.

Feeding	Stages	Days	Nitrate mg NO_3^--N/L	Nitrite mg NO_2^--N/L	TOC mg TOC/L	Alkalinity mg IC/L	NH_4^+-N/IC ratio g N/g IC
Synthetic media	I	0 – 118	25	25	-	130	0.19
	II	119 - 154	10	25	-	130	0.19
	III	155 - 197	0	25	-	130	0.19
	IV	198 - 248	0	25	-	65	0.38
	V	249 - 338	0	25	-	30	0.83
	VI	339 - 392	0	25	-	10	2.50
Municipal wastewater	VII	393 -432	1	24	19 ± 4	5	3.67
	VIII*	433 - 485	1	14	14 ± 4	2	7.50

IC: inorganic carbon; N: nitrogen; TOC: total organic carbon.

*The cycle length was shortened from 6 to 4 hours.

The SBR cycle lasted 6 hours (S-I to S-VII) comprising: 300 min of mixed feeding and reaction, 30 min of mixing, 15 min of settling and 15 min of effluent withdrawal (Figure S1.A in Supporting Material according to Dapena-Mora et al. (2004)). The SBR operated 24 hours/day and the different cycle phases were controlled by a programmable logic controller (PLC, Siemens, S7-224 CPU). Finally, in Stage VIII, the cycle length was shortened to 4 hours (Figure S1.B in Supporting Material), shortening the hydraulic retention time (HRT) from 24 to 16 hours.

2.2. Microbial activity batch tests

Maximum specific anammox activity (SA_{AMX}) was determined according to Dapena-Mora et al. (2007). Monthly activity tests were performed at 30 °C (as reference temperature) and 15 °C. Tests at 20 and 25 °C were performed as well to assess the effect of the temperature changes over the SA_{AMX}. When municipal wastewater was fed to the reactor, the specific activity of heterotrophic denitrifying

(SA_{HDN}) bacteria was measured at 15 and 30 °C, adding nitrate or nitrite (50 mg N/L) and acetate (100 mg COD/L) as substrates. All the activity tests were performed in triplicate.

2.3. Response surface methodology

The effect of temperature, pH and organic matter content over SA_{AMX} was assessed by a three-level-three-factor Box-Behnken design (BBD) and the response surface methodology (RSM) using SBR biomass taken in days 410 - 420. Temperature (x_1) was evaluated in the range of the optimal value for the test (30 °C) and the SBR operational temperature (15 °C), organic matter concentration (x_2) between 0 and 75 mg TOC/L as they are typical values found in mainstream effluents and pH (x_3) in the range of 6 to 8.

A total of 15 experiments (in triplicate), including the three replicates in the central point, were conducted (Table S.2 in Supporting Material). All the experiments were repeated to assess the reproducibility of the results. Organic matter (as sodium acetate) was added with the substrates and initial pH value was adjusted to the target value by adding NaOH or HCl in the washing step. Since the liquid media was phosphate buffer, the pH value did not significantly ($p > 0.8$) change during the test execution (data not shown). Biomass concentration in the vials was similar with average values of 2.6 ± 0.1 g VSS/L.

The relationships between the response (SA_{AMX}) and the independent variables tested were analyzed by linear regression and fitted to a second-order polynomial model using Equation 1.

$$Y = b_{0j} + \sum_{i=1}^{3} b_{ij}x_i + \sum_{i=1}^{3}\sum_{k=1}^{3} b_{ikj}x_i x_k \qquad \text{Eq. 1}$$

where Y represents the predicted response (SA_{AMX}), b_{0j}, b_{ij}, and b_{ikj} are the regression coefficients calculated from the experimental results by the least-squares method, and x_i and x_k ($k \geq i$) are the independent variables in coded values, with variation ranges from -1 to 1 (Table S2).

The statistical analysis was performed using the analysis of variance (ANOVA), including the F-test value, which established the global model significance, the lack of fit, the determination coefficients (R^2) and the adjusted R^2 ($R^2_{adjusted}$). The significant factor affecting each dependent variable was selected according to the Student t-test establishing a 95 % confidence level. The statistical software IBM SPSS 24 was used to generate the regression analysis and analysis of factor contribution. Excel tool was used to plot the response surface and contour plots.

2.4. Analytical methods

Influent and effluent samples were periodically taken and were filtered using 0.45 µm pore-size filters before analysis. Ammonium (Bower and Holm-Hansen, 1980), nitrite and nitrate (American Public Health Association et al., 2017) concentrations were spectrophotometrically determined. Dissolved total organic and inorganic carbon concentrations (TOC and IC, respectively) were measured with a Shimadzu analyzer (TOC-L-CSN). Total nitrogen (TN) concentration was determined in the same Shimadzu analyzer with a TNM-L Unit. pH was measured using an electrode connected to Crison 506 measurer. Total suspended solids (TSS) and volatile suspended solids (VSS) concentrations, as well as sludge volume index at 30 min (SVI30), were determined according to Standard Methods (American Public Health Association et al., 2017). The average diameter of the granules and size distribution were measured using a stereomicroscope (Stemi 2000-C, Zeiss) incorporating a digital camera (Coolsnap, Roper Scientific Photometrics) for image acquisition. The obtained images were processed using the Image ProPlus® software.

2.5. Calculations

Statistical differences between the results obtained in the different operational stages were tested by one-factor ANOBA using the statistical software IBM SPSS 24. Frist, variance homogeneity was confirmed by Levene's test and normal distribution by the Shaphiro's test. Then, if the ANOVA confirmed the difference between mean values, a post hoc analysis (Tukey's HSD) was applied to determine between which values the difference was significant, considering a level of significance of 0.05. If data variance homogeneity and/or normal distribution was not met, the non-parametric Kruskal-Wallis analysis was applied and afterwards the Wilcoxon post hoc one.

3. Results and discussion

3.1 Anammox process establishment and maintenance

3.1.1 Reactor start-up

Stable anammox process was quickly established (Figure 2.A) when the inoculum, coming from one-stage PN/AMX system (enriched in anammox bacteria but also AOB (Morales et al., 2015)), was directly exposed to mainstream conditions. During the first days, nitrate was consumed and biomass concentration decreased. A possible explanation is the lysis of the aerobic bacteria (like AOB or heterotrophs) happening as no oxygen was available in the SBR. Then, the organic matter coming from the biomass death was used to denitrify the fed nitrate. Once no organic matter was available, heterotrophic denitrifying activity decayed and significant nitrate consumption was no longer observed from day 10 onwards. The biomass concentration decreased from 1.6 g VSS/L (day 0) to 1.2 g VSS/L (day 32). Then, it remained constant until Stage VI, indicating that the solids washout (5 - 10 mg VSS/L in the effluent) was compensated by the anammox growth (Figure 2.C).

As equimolar ammonium and nitrite concentration was fed, even nitrite was fully depleted, effluent ammonium concentration was approximately 5 mg NH_4^+-N/L and TN concentration was 11 ± 2 mg N/L leading to NRE from 74 to 79 % (Figure 2.A). During this Stage (except for the first 10 days), the obtained average values of nitrite to ammonium consumed ratio was 1.26 ± 0.12 g NO_2^--N/g NH_4^+-N (Figure 3) fitting well with the anammox stoichiometry (Lotti et al., 2014a; Strous et al., 1998). The nitrate produced to ammonium consumed ratio was 0.34 ± 0.09 g NO_3^--N/g NH_4^+-N (Figure 3), higher than the one expected suggesting the presence of NOB, which might profit from small DO concentrations entering to the non-hermetically closed reactor.

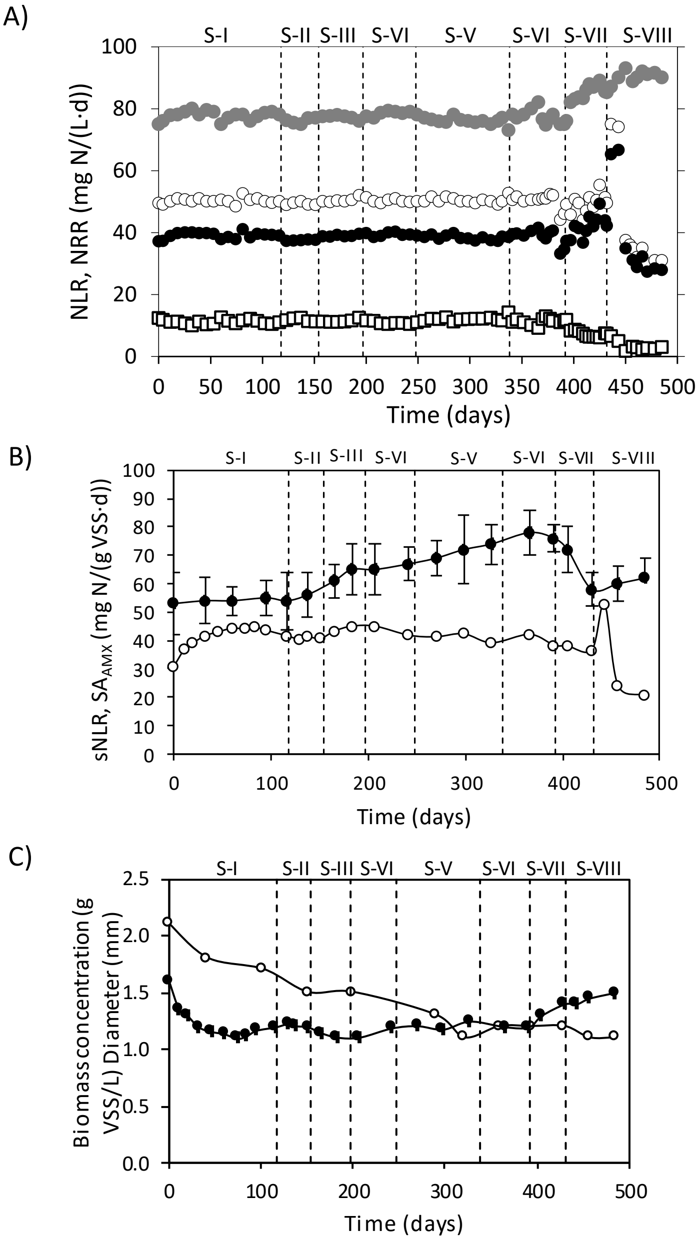

Figure 2. Evolution throughout the operational time of A) applied nitrogen loading rate (NLR, ○) and achieved nitrogen removal rate (NRR, ●), in mg TN/(L·d), Total nitrogen (TN, □) effluent concentration, in mg TN/L, and nitrogen removal efficiency (NRE, ●) in percentage; B) specific NLR (sNLR, ○) to the reactor and the maximum specific anammox activity (SA_{AMX}, ●) obtained in batch tests at 15 °C. and C) Biomass concentration inside the reactor (●), in g VSS/L, and the average diameter of the granules (○), in mm.

Then, during Stages II and III, the nitrate supply in the feeding was first reduced and then stopped (Table 1) since the produced nitrate by the anammox process was enough to maintain the anoxic environment. No significant differences regarding the NRE were detected ($p > 0.3$) when Stage III is compared with previous stages (Figure 2.A). The SA_{AMX} was also maintained from Stage I to Stage III with values of 53 ± 11 and 65 ± 9 mg N/(g VSS·d) on day 0 and day 183, respectively ($p > 0.10$; Figure 2.B). Moreover, the applied sNLR was always below the SA_{AMX} (Figure 2.B) proving that the NRR was limited by the applied NLR in agreement with the negligible nitrite concentration in the effluent during the whole operational period (< 0.01 mg NO_2^--N/L).

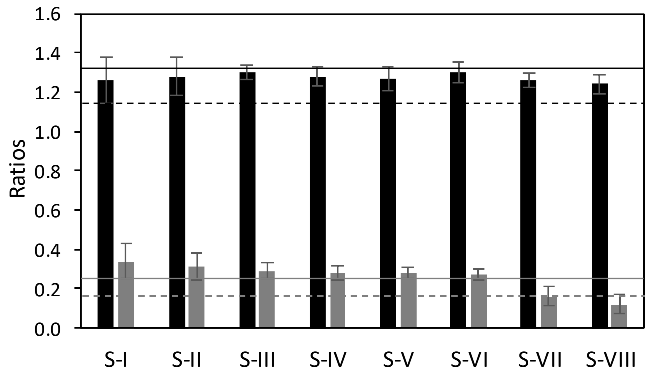

Figure 3. Evolution of the nitrite to ammonium consumed ratio (■) and nitrate produced to ammonium consumed ratio (■) in the different operational stages. Horizontal lines represent the stoichiometric values according to: according to Strous et al. (1998) solid lines and Lotti et al. (2014a) plotted as dashed lines.

3.1.2 Anammox process performance at decreasing alkalinity concentrations

Lack of alkalinity was reported in conventional activated sludge systems (Seuntjens et al., 2018) and it can be almost fully depleted in the mainstream nitritation process (Pedrouso et al., 2017). Different research works studied the short-term N/IC optimal ratio finding that the anammox activity decreased when the ratio is higher than 6 g NH_4^+-N/g IC and it must be maintained at least lower than 12 g NH_4^+-N/g IC (Kimura et al., 2011). Strous et al. (1998) and Lotti et al. (2014a) reported the anammox process stoichiometric NH_4^+-N/IC consumption ratios of 17.7 and 16.4 g NH_4^+-N/g IC, respectively. Nevertheless, Kimura et al. (2011) reported that the ammonium to IC consumption ratio depends on the influent ratio that should be lower than the stoichiometric one.

In the present study, no significant effect was observed over the NRR ($p = 0.38$) (Figure 2.A) due to the different IC concentrations (Table 1) in the feeding with ratios ranging between 0.19 and 2.50 g NH_4^+-N/g IC. Moreover, the effluent pH value remained at 7.3 ± 0.3. Liao et al. (2008) studied at 30 °C the effect of different IC concentrations in an anammox system fed with 80 mg NH_4^+-N/L. These authors reported an NRR increase when the influent NH_4^+-N/IC ratio decreased from 0.56 to 0.32 mg NH_4^+-N/mg IC but it decreased when the ratio further dropped to 0.28 mg NH_4^+-N/mg IC. In the present study during Stages IV-VI, the NRE was kept at 82 ± 3 % (Figure 2.A), limited by the equimolar ammonium and nitrite concentrations in the feeding and the effluent TN concentration stand at 11 ± 3 mg TN/L (Figure 2.A, $p=0.81$). The process stoichiometry fits well with the anammox one with average ratios of 1.28 ± 0.05 g NO_2^--N/g NH_4^+-N and 0.28 ± 0.03 g NO_3^--N/g NH_4^+-N (Figure 3).

Compared with the inoculum, the SA_{AMX} augmented ($p= 3\ 10^{-7}$) up to 78 ± 8 mg N/(g VSS·d) at the end of Stage VI (Figure 2.A). Whether this SA_{AMX} improvement was due to the alkalinity depletion in the feeding or due to the increase of the anammox enrichment level is uncertain. However, it is worth pointing out that, contrary to other studies, the SA_{AMX} did not decrease despite the long-term operation of the reactor at low temperature (15 °C) and low nitrogen concentrations (Hoekstra et al., 2018; Qiu et al., 2020). A comparison between the sNLR and the SA_{AMX} values revealed that, at the end of the Stage VI, the system was able to treat almost double of the applied sNLR (Figure 2.B). Microbial populations analysis will help to understand if the anammox enrichment degree increase and whether or not the commonly reported shifts on the predominant species happened when conditions change from sidestream to mainstream (Yang et al., 2018).

3.1.3 *Dependence of the anammox activity with temperature*

The SA_{AMX} is known to be highly affected by temperature (Lotti et al., 2015; Morales et al., 2016), experiencing a decrease when the temperature of operation diminishes (Figure 4.A). The SA_{AMX} measured at 15 °C significantly increased ($p=3\ 10^{-7}$) from the start-up to Stage VI whereas the SA_{AMX} values at 30 °C decreased from 270 ± 11 to 200 ± 10 mg N/(g VSS·d) ($p=2\ 10^{-15}$).

In the present study, the highest SA_{AMX} was always obtained at 30 °C, whilst Hu et al. (2013) reported that the optimal temperature of the anammox biomass after the long-term operation (300 days) at 12 °C changed from 35 °C to 25 °C. Adaptation of the anammox biomass to operate at low temperatures was also found by other authors (Dosta et al., 2008; Lotti et al., 2015). Some authors recommend to slowly adapt the sludge to low temperatures (De Cocker et al., 2018; Hoekstra et al., 2018; Reino et al., 2018). However, the decrease of the SA_{AMX} was similar to that observed in studies where the biomass was step-wise acclimated or directly exposed to mainstream conditions (Morales et al., 2016). Thus, progressive anammox adaptation might not be required shortening the start-up periods.

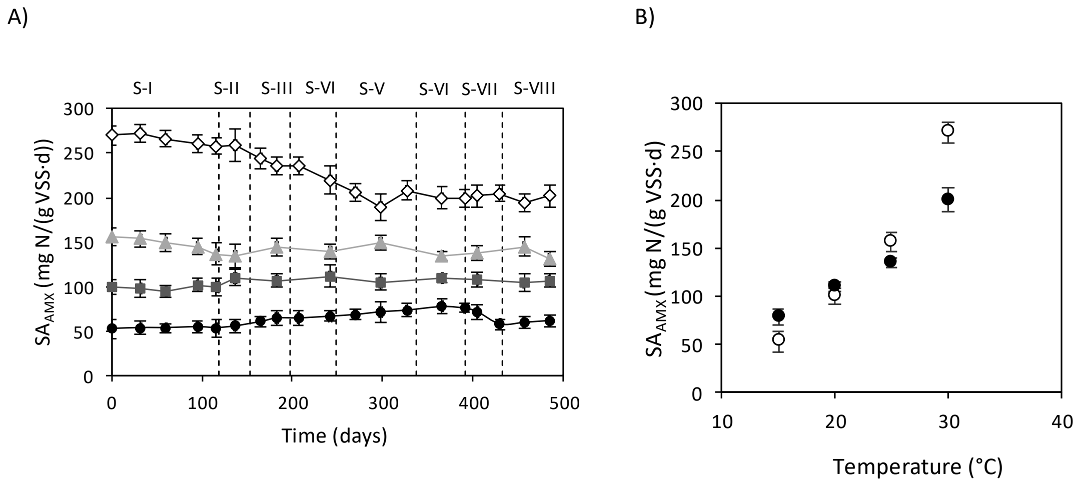

Figure 4. Evolution of the maximum specific anammox activities (SA_{AMX}): A) throughout the reactor operation measured at different temperatures: 15 °C (●), 20 °C (■), 25 °C (▲) and 30 °C (◊); B) Maximum specific anammox activity (SA_{AMX}) temperature dependency from samples collected on day 0 (○) and day 370 (●).

To better understand the effect of the temperature, Figure 4.B shows the SA_{AMX} values of the inoculum (considered acclimated to 30 °C) obtained at different temperatures and those determined for a SBR biomass sample collected on day 370. The SA_{AMX} values for biomass on day 370 are higher at 15 and 20 °C, and their diminishing tendency is smoother than in the case of the inoculum. Indeed, the inoculum SA_{AMX} at 30 °C is more than 5 times higher than at 15 °C, whereas this ratio decreased to 2.6 for sample on day 370 when the SA_{AMX} measured at 15 °C amounted to the 39 % of that one measured at 30 °C (Figure 4.B). Similarly, De Cocker et al. (2018) reported that the anammox activity measured at 15 °C was the 22.4 % of the value obtained at 30 °C operating an anammox SBR at temperatures decreasing from 30 to 10 °C for 257 days.

3.2 Anammox process performance treating nitritified municipal wastewater

Finally, in Stages VII-VIII the anammox reactor was fed with municipal wastewater (Table 1 and Table S1 in Supporting Material). During Stage VII, the NRE slightly increased to average values of 88 ± 5 % due to the presence of residual organic matter in the feeding (< 20 mg TOC/L, Table 1), mainly from the fraction of the fed stream which was not treated in the nitritation reactor (< 30 % in volume). The ratio of nitrate produced to ammonium consumed decreased to 0.12 - 0.16 g NO_3^--N/g NH_4^+-N (Figure 3) as nitrate was partially denitrified. The low TOC concentration limited the growth of heterotrophic denitrifying bacteria but contributed to increasing the NRE with effluent TN concentration below 6 mg TN/L (Figure 2.B). Thanks to this beneficial role polishing the reactor effluent, the nitrogen EU discharge limits (10 mg TN/L for sensitive areas) were accomplished. The anammox process was the main pathway of TN removal, while the denitrification process contribution to the TN removed accounts for approximately 5 - 7 %. Indeed, the SA_{HDN} values were under the detection limit even when the tests were repeated at 30 °C (using both nitrate and nitrite as substrates).

On day 433, the cycle length was shortened (Figure S1 in Supporting Material) to increase the NLR from 58 ± 9 to 75 ± 1 mg TN/(L·d) but, after 12 days, it decreased again to 33 ± 3 mg TN/(L·d) (Figure 2.A) due to the decrease in the municipal wastewater load (Table S1 in Supporting Material) with TN concentration below 25 mg TN/L (Figure 2.B). In this Stage VIII, the NRE was 91 ± 2 %. At this point, it was not possible to further decrease the HRT (16 h) due to the limited availability of nitritified wastewater.

The pH in the influent, and therefore in the SBR, decreased to average values of 6.2 ± 0.1, lower than 7.0 - 7.5 recommended to maintain the anammox process stability (Tomaszewski et al., 2017). 10 days after switching the feeding to municipal wastewater, the SA_{AMX}, at 15 °C, was 72 ± 8 mg N/(g VSS·d) and it decreased to 58 ± 6 mg N/(g VSS·d) after 40 days in Stage VII (Figure 4.A). Laureni et al. (2015) also observed a decrease in the anammox activity to 40 mg N/(g TSS·d) when the reactor was fed with municipal wastewater containing up to 20 mg TN/L and 47 mg sCOD/L and operated at 12 and 29 °C. Higher SA_{AMX} (60 mg N/(g VSS·d) at 11 °C) was reported by Reino et al. (2018) after it decreased more than 50 % once the synthetic feeding was replaced by municipal wastewater. The activity decrease might be attributed to the development of heterotrophic bacteria. However, in the present study, the anammox bacteria inhibition by the low operational pH should also be considered.

During Stages VII and VII, when the SBR was fed with municipal wastewater, the VSS concentration in the reactor progressively increased (Figure 2.C) and a flocculent biomass fraction developed corresponding to 10 - 15 % of VSS concentration. The development of flocculent biomass was attributed to the development of fast-growing heterotrophic bacteria. However, the limited organic matter concentration fed to the system (< 20 mg TOC/L) prevents that the heterotrophic denitrifying bacteria overgrowth the anammox bacteria. Thus, the controlled development of heterotrophic bacteria did not compromise the long-term system stability but promote effluent quality. The ratio of VSS/TSS remained at 0.78 ± 0.06 during the whole reactor operation ($p = 0.3$). Thus, no inorganic solids accumulation was observed using municipal wastewater as feeding like previously reported by

Reino et al. (2018). Despite the effluent VSS concentration from 10 mg VSS/L to 20 mg VSS/L, associated to the presence of residual organic matter in the feeding, the SVI remained at average values of 60 ± 5 mL/ g TSS and biomass was successfully retained.

In the present study, the SBR biomass accumulation capacity was limited with 1.4 ± 13 g VSS/L whereas higher biomass concentrations were reached in up-flow anaerobic sludge bed anammox reactors (e.g., 16.8 ± 0.5 g VSS/L (Reino et al., 2018) or 6.7 g VSS/L (Lotti et al., 2014b)). These differences were associated more to the NLR than to the reactor type. In the present study, the SBR was fed under substrate limiting conditions hindering the biomass growth. Consequently, the NRR achieved of 40 ± 10 mg TN/(L·d) was much lower than that reported by Reino et al. (2018) and Lotti et al. (2014b) of 1,200 ± 500 mg TN/(L·d) and 510 mg TN/(L·d), respectively, but better effluent quality was achieved in the present study. The low sNLR applied compared with the SA_{AMX} is also related to the deterioration of the granules integrity (Sánchez Guillén et al., 2016). The measured average granule size decreased (from 2.1 mm in the inoculum to 1.1 mm at the end of the operation) and the number of small granules increased (Figure 2.C and Figure S2)

Limited information is available about the long-term performance of anammox reactors treating municipal wastewater and fulfilling the nitrogen discharge limits. As an example, Jin et al. (2019) using a two-stage system managed to produce an effluent containing 5 - 12 mg TN/L, but the anammox reactor temperature was 29 - 30 °C, far from the expected values at the mainstream.

Results from the present study demonstrated that it was possible to perform the nitritation process, based on the *in situ* FNA production strategy (Pedrouso et al., 2017), followed by the anammox one to remove nitrogen from primary settled municipal wastewater at 15 °C, even at the low pH values (6.2) and alkalinity (between 3.67 and 7.50 g NH_4^+-N/g IC) of operation. High-quality effluent in terms of TN, TOC and TSS was achieved fulfilling the discharge standards in the EU for sensitive areas.

3.3 Effect of pH, COD and temperature over the specific maximum anammox activity

The SA_{AMX} drop when municipal wastewater was fed (Figure 2.B) might be mainly attributed either to the low pH value (6.2 ± 0.1) or to the presence of organic matter. The experiments defined according to BBD (Table S2) revealed that SA_{AMX} was severely affected by the low pH, being barely detected in the experiments carried out at pH 6 even at high temperature (30 °C) (Figure 5). Results agreed with those found by Tomaszewski et al. (2017).

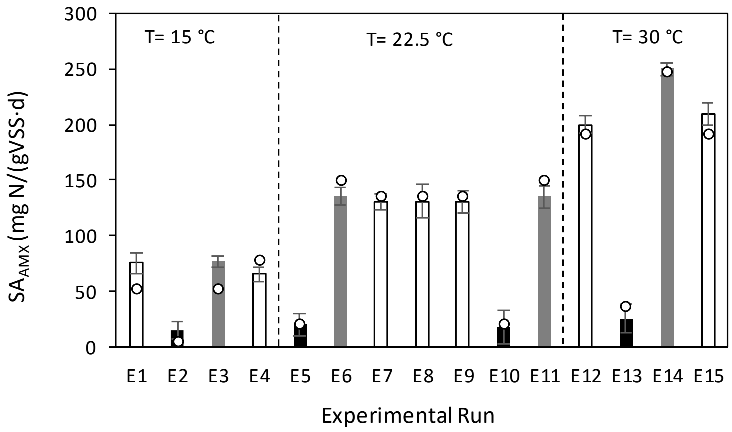

Figure 5. Maximum specific anammox activity (SA_{AMX}) results obtained from the Box-Behnken design experiments. Colours indicate different pH values: 6.0 (■), 7.0 (□) and 8.0 (■). Circles (○) are the predicted values by the model. Refer to Table S2 to see the used total organic carbon (TOC) in each experimental run.

By applying the multiple regression analysis to the experimental data, the SA_{AMX} value was modelled (Equation 2) as a function of temperature, TOC concentration and pH values (in the tested interval).

$$SA_{AMX} = 134.4 + 56.7\ T + 60.8\ pH + 41.0\ T \cdot pH - 50.2\ pH^2 \qquad \text{Eq. 2}$$

Those terms that were not significant at a 95 % of confidence were excluded from the model one by one starting from the lowest significant one (backward method) (Table S3 in Supporting Material). The BBD results were subjected to Student's test (t) (Table S4). Low values of t and P indicate a high significance of the corresponding model term. The TOC concentration and its interactions were not significant ($p < 0.05$). The same occurred in the case of the quadratic interaction for temperature while pH interaction with temperature and its square term were found to be significant indicating that SA_{AMX} was very sensitive to these factors. The ANOVA analysis from the model showed an R^2 of 0.975, R^2_{adj} of 0.966 and a high Fisher's F value (equal to 99), suggesting a high degree of correlation between the experimental and predicted values. The points cluster close to the diagonal line (45°) indicating a good fit of the model (Figure S3 in Supporting Material).

Daverey et al. (2015) studied the interaction of pH (5.38 - 9.62) and temperature (21.9 - 43.1 °C) over the SA_{AMX} and found that the pH value was the most influential factor. Temperature was not significant in their model but its effect cannot be neglected. Indeed, when they validated the obtained model at low temperature, the SA_{AMX} at 15 °C and pH value of 6.5 was zero whereas at 25 °C was 9 mg N/(g VSS·d). (Tomaszewski et al. (2017)) stated that despite a statistical correlation between temperature and pH over the SA_{AMX} was not found; the optimal pH range was narrower at low temperatures. Contrary to those findings, the interaction between temperature and pH in the model obtained in the present study was significant (Table S4).

The response surface (Figure 6) obtained for the significative dependent variables (pH and temperature) helps to visualize its relationships and effect over the SA_{AMX}. Maximum SA_{AMX} was measured at 30 °C and pH 8. pH value positive correlate with SA_{AMX} being its effect shielded by the great effect caused by the low temperatures. Curve sections further revealed that the interaction between temperature and pH is also significant.

Model was validated at pH 6.2 (the one in the SBR), 6.5 and 7.8 (the standard buffer) at different temperatures. The predicted and the experimental values are compared in Table S5. The experimental values obtained were close to the predicted ones, confirming the validity of the model except for the low pH values for which the error was 26 % and 62 % at 30 °C and 15 °C, respectively. This fact might be explained by the low SA_{AMX} observed that challenge to measure the SA_{AMX} with precision.

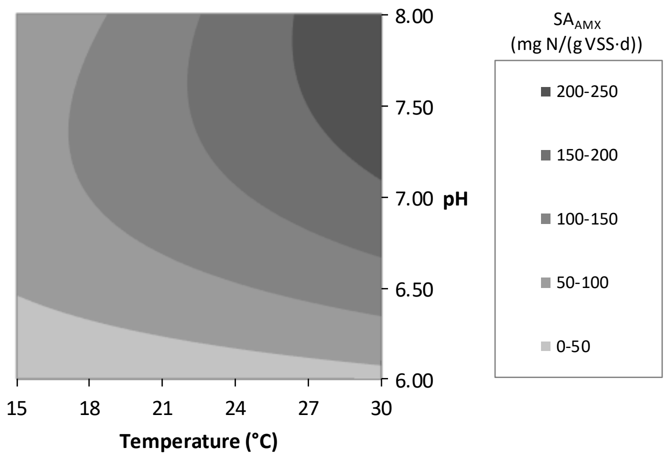

Figure 6. Surface response plot showing the interactive effects of temperature and pH on the specific anammox activity (SA_{AMX}).

4 Conclusions

Results demonstrate that acclimation periods to mainstream conditions (low temperature and nitrogen concentration) might be no needed for the establishment of the anammox process shortening the start-up periods. Stable anammox process at 15 °C to treat both synthetic wastewater and nitritified primary settled wastewater reaching a TN effluent concentration lower than 10 mg TN/L. The presence of low organic matter concentrations contributed to polishing the effluent increasing the NRE (up to 92 %). Although the temperature of operation affects the SA_{AMX}, once the biomass is adapted to low temperatures, its effect becomes less relevant.

Anammox granular biomass was successfully retained in the system and the anammox biomass growth compensated biomass washout. SA_{AMX} increase during the SBR operation fed with synthetic media and it decreased when SBR treated municipal wastewater probably due to the heterotrophic denitrifying bacteria development. The two-stage system limits the organic matter that enters to the anammox reactor, and therefore, heterotrophic bacteria did not overgrowth anammox ones.

Long-term stable anammox process was kept despite the low pH value of 6.2 of municipal wastewater. Short-term tests indicated that anammox activity is highly influenced by pH and its effect is also affected by temperature.

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