Claros Bedoya, JA.; Jiménez Douglas, E.; Aguado García, D.; Ferrer, J.; Seco Torrecillas, A.; Serralta Sevilla, J. (2013). Effect of pH and HNO2 concentration on the activity of ammonia-oxidizing bacteria in a partial nitritation reactor. Water Science and Technology. 67(11):2587-2594. doi:10.2166/wst.2013.132.

# Effect of pH and $HNO_2$ concentration on the activity of ammonia oxidizing bacteria (AOB) in a partial nitritation reactor

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**Abstract**
Ammonia oxidizing bacteria (AOB) are very sensitive to environmental conditions and WWTP operational parameters. One of the most important factors that affect their activity is the pH. Its effect is associated to: $NH_3/NH_4^+$ and $HNO_2/NO_2^-$ chemical equilibriums and biological reactions rate. The aim of this study was to quantify and model the effect of pH and free nitrous acid concentration on the activity of the AOB present in a lab-scale partial nitritation reactor. For this purpose, two sets of batch experiments were carried out using biomass from this reactor. FISH analysis disclosed that *Nitrosomona eutropha* and *Nitrosomona europaea* species were dominant in the partial nitritation reactor (>94%). The experimental results showed that free nitrous acid inhibits the AOB activity. This inhibition was properly modeled by the non-competitive inhibition function and the half inhibition constant value was determined as 1.32 mg $HNO_2$-N $L^{-1}$. The optimal pH for these AOB was found to be in the range 7.4-7.8. The pH inhibitory effect was stronger at high pH values than at low pH values. Therefore, an asymmetric inhibition function was proposed to represent the pH effect on these bacteria. A combination of two sigmoidal functions was able to reproduce the experimental results obtained.

**Key words** AOB, free nitrous acid, nitrite, partial nitritation, pH

## INTRODUCTION

Nitrification and denitrification processes are used in wastewater treatment plants (WWTP) in order to meet increasingly stringent discharge standards. Nitrification is the sequence of two stages: ammonia oxidation and nitrite oxidation. In the first stage, three groups of ammonia oxidizing organisms can be discriminated: ammonia oxidizing bacteria (AOB), heterotrophic ammonia oxidizers and ammonia oxidizing archaea. However, there are no clear evidences that ammonia oxidizing archaea or heterotrophic ammonia oxidizers play a significant role in conventional activated sludge processes (Kampschreur *et al.*, 2009). During the second stage, nitrite oxidation is carried out by Nitrite Oxidizing Bacteria (NOB). *Nitrospira* spp. and *Nitrobacter* spp. are the NOB species most frequently detected in WWTPs. On the other hand, during the denitrification process, several organisms, bacteria as well as archaea, can reduce nitrate ($NO_3^-$), nitrite ($NO_2^-$), nitric oxide (NO), and nitrous oxide ($N_2O$) using organic and inorganic substrates through different metabolic routes (Thorndycroft *et al.*, 2007).

Biological nitrogen removal by means of ammonia oxidation to nitrite (nitritation) and its further reduction to nitrogen gas ($N_2$) have become a feasible alternative to treat wastewater streams with high ammonia concentration, like supernatants from anaerobically digested sludge, piggery wastewater and landfill leachate (Mulder *et al.*, 2001; Hwang *et al.*, 2005; Ganigué *et al.*, 2007). Technologies such as SHARON process (Single reactor High activity Ammonia Removal over Nitrite) (Hellinga *et al.*, 1999) and SHARON-ANAMMOX process (combination of partial nitritation with anaerobic ammonium oxidation) allow removing high ammonia concentrations via the nitrite route (Van Dongen *et al.*, 2001). The SHARON-ANAMMOX concept gets much

attention as organic matter is not needed for denitrification, reducing operational costs. Since ANAMMOX process converts ammonium to nitrogen gas using nitrite as the electron acceptor, partial nitritation is required to obtain an effluent with a composition of 50% ammonium and 50 % nitrite (Van Hulle *et al.*, 2007).

Several AOB species are involved in the nitritation process. *Nitrosomonas eutropha* and *Nitrosomonas europaea* have been identified as the most abundant species in the nitritation process from high ammonia concentration streams. This abundance may be consequence of their tolerance to both high salinity (Claros *et al.*, 2010) and high nitrite concentrations (Beaumont *et al.* 2002). However, the bacterial acclimation could play an important role (Ganigué *et al*., 2007).

pH is an important parameter that affects the AOB activity. Its effect is associated to: $NH_3/NH_4^+$ and $HNO_2/NO_2^-$ chemical equilibriums and biological reactions rate. The pH value affects directly the enzymatic activity by means of the inhibition of active sites of the enzymes for the union of $H^+$ and $OH^-$ (García and Fernández-Polanco, 1996). High free ammonia (FA) concentrations at high pH, as well as high free nitrous acid (FNA) concentrations and lack of substrate availability at low pH values have been reported as the main causes of the AOB activity decrease. However, the isolated effect of the pH value on the AOB activity is still unclear. This study provides further experimental information about the effect of pH and FNA concentration on the AOB activity in a partial nitritation process. These effects were determined by means of respirometric batch experiments. A mathematical expression to represent the effect of pH and FNA concentration on AOB was proposed and the inhibition constants were determined.

## MATERIALS AND METHODS

### Partial nitritation reactor

Partial nitritation was achieved in a laboratory-scale continuous stirred tank reactor of 7 L-capacity. The system was operated under aerobic conditions to achieve an ammonium/nitrite ratio close to 50% (ANAMMOX suited influent). Oxidation reduction potential (ORP), pH, temperature, dissolved oxygen (DO) and conductivity were monitored. The pH (SP10B, Consort), temperature (ST10N, Consort), ORP (SP50X, Consort), conductivity (SK10B, Consort) and DO (CellOx 325, WTW) probes were connected to a multi-channel analyser (Consort C832) and an oxymeter (Oxi340 WTW), respectively. These devices were in turn connected via RS232 to a PC with Visual Basic 6.0 software for data monitoring and storage (every 30 seconds). A thermostated water bath was used to maintain the temperature at 30ºC. An on-off controller was used to keep the DO concentration at 2 mg $L^{-1}$.

The reactor was fed with synthetic wastewater which composition emulates the typical one of the supernatant from the anaerobic sludge digestion. The synthetic influent stream contained per liter: 4.7 g $(NH_4)_2SO_4$; 4 g $KHCO_3$; 0.05 g $MgSO_4.7H_2O$; 0.03 g $K_2HPO_4$; 0.003 g $CaCl_2$ and trace compounds (0.015 g $FeCl_3 \cdot 6H_2O$; 0.150 g $H_3BO_3$; 0.03 g $CuSO_4 \cdot 5H_2O$; 0.637 g KI; 0.12 g $MnCl_2 \cdot 4H_2O$; 0.06 g $Na_2MoO_4 \cdot 2H_2O$; 0.12 g $ZnSO_4 \cdot 7H_2O$; 0.15 g $CoCl_2 \cdot 6H_2O$). The molar alkalinity/ammonium ratio was kept close to 1, typical ratio in supernatant liquors from anaerobically digested sludges. The hydraulic retention time (HRT) was established at 4.6 days. The sludge retention time (SRT) varied within the range 6-8 days. The difference between HRT and SRT is due to the evaporation rate along the experimental period. Since the evaporation rate varied

due to variations in laboratory ambient temperature, the SRT was not constant. The mixed liquor volatile suspended solids concentration in the reactor was in the range 100-130 mg/L.

**Off-line batch reactor**

Several batch experiments were carried out in a 0.3 L water jacketed reactor using biomass from the partial nitritation reactor. The batch reactor was equipped with DO and pH sensors connected via RS232 to a PC. An on-off controller was installed in the experimental set-up to determine Oxygen Uptake Rates (OUR) along the experiments. The pH was controlled by means of a fuzzy-logic controller adding sodium carbonate ($Na_2CO_3$). The salt volume added was recorded to take into account the dilution effect.

**Respirometric off-line batch experiments**

The batch experiments were designed to identify the effect of FNA concentration and pH value on the AOB activity. The general methodology of these experiments can be summarized as follows: Initially, a sample from the nitritation reactor was withdrawn and washed to remove the ammonium and nitrite present in the sample. The initial ammonium and nitrite concentrations were established at the desired values and the batch reactor was filled. The initial pH value was adjusted adding chlorhydric acid (HCl) or sodium carbonate ($Na_2CO_3$). The bacterial activity was measured by Oxygen Uptake Rates (OUR) throughout all experiments. OUR measurements were performed between 4 and 2 mg $O_2$ $L^{-1}$. The pH value was maintained at the desired level for each experiment by means of the aforementioned fuzzy-logic controller. Temperature was kept at 30ºC in all the experiments.

Two sets of batch experiments were carried out: the first set consisted in 3 experiments aimed at identifying the effect of FNA concentration at pH values of 6.25, 6.50 and 7.00. Along these experiments the OUR values were recorded at different total nitrite concentrations ($TNO_2$) ranging from 75 to 5000 mg $NO_2$-N $L^{-1}$. Each $TNO_2$ level was achieved by adding the required amount of $NaNO_2$ and it was kept for around one hour. Preliminary experiments showed that this time length was adequate to observe the inhibition caused by FNA. FNA concentration was calculated through acid-base equilibrium equation at 30ºC. In order to avoid substrate limitation, the same ammonium concentration (1000 mg $NH_4$-N $L^{-1}$) was added in each experiment. According to previous experimental results (Claros *et al.*, 2010) this ammonium concentration assures that the maximum AOB activity is reached.

The second set of experiments (9 experiments) was carried out in order to evaluate the pH effect in the range 6 to 9. These experiments were carried out with different ammonium and nitrite concentrations. Table 1 shows the initial ammonium and nitrite concentrations used in each experiment. In each experiment pH was varied from 6 to 9 with a step size of 0.5 pH units. Each pH level was maintained around 1 hour. The OUR values were continuously recorded along the whole experiment.

Table 1. Ammonium and nitrite concentrations evaluated in the second set of batch experiments

| **Experiment** | **1** | **2** | **3** | **4** | **5** | **6** | **7** | **8** | **9** |
|---|---|---|---|---|---|---|---|---|---|
| **TAN ($NH_4$-N mg $L^{-1}$)** | 550 | 550 | 550 | 1100 | 1100 | 1100 | 1100 | 1100 | 2000 |
| **$TNO_2$ ($NO_2$-N mg $L^{-1}$)** | 700 | 1000 | 2000 | 1000 | 4000 | 4000 | 500 | 200 | 2000 |

**Analytical methods and Fluorescent *in situ* hybridization**

Ammonium ($NH_4$-N), nitrite ($NO_2$-N), nitrate ($NO_3$-N), total suspended solids (TSS), volatile suspended solids (VSS), total nitrogen and alkalinity were determined off-line according to standard methods (APHA/AWWA/WEF 2005).

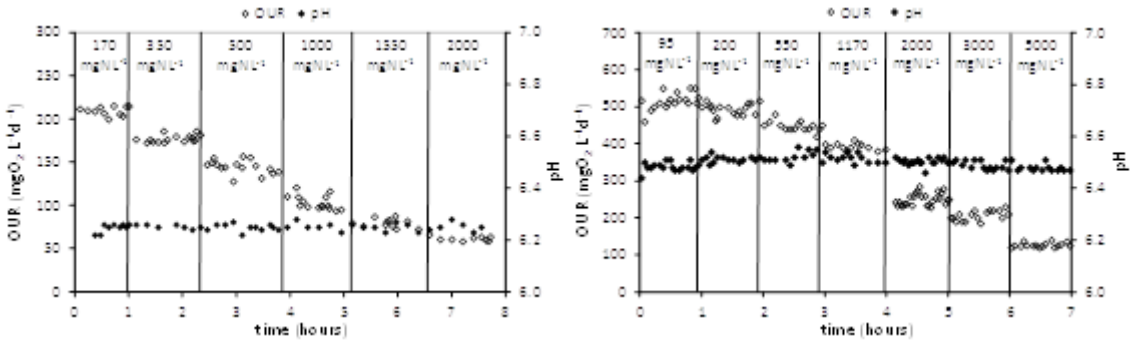
Fluorescent *in situ* hybridization (FISH) was performed on fixed activated sludge as specified by Manz *et al.* (1992). The oligonucleotide probe sequences used in this study were: NSO190 for the detection of *Beta-proteobacterial ammonia-oxidizing bacteria*, Nsv443 for *Nitrosospira spp*, Nmo218 for *Nitrosomonas oligotropha-lineage*, Nse1472 for *Nitrosomonas europea, N. halophila, N. eutropha and Kraftisried-Isolat*, Nmv for *Nitrosococcus mobilis ("Nitrosomonas") lineage* and EUBmix probe for the domain *Eubacteria*, which consist of a mixture of EUB338, EUB338II and EUB338III. Hybridized cells were enumerated using an automated bacteria quantification software (Borrás, 2008). Further information can be found elsewhere (Claros *et al.*, 2010).

## RESULTS AND DISCUSSION

**Effect of FNA concentration on the AOB activity**

Figures 1a and 1b show the OUR and pH evolution at the different nitrite concentration levels evaluated in the experiments carried out at pH 6.25 and 6.5, respectively (experiment at pH 7 not shown). As can be seen in these figures, the highest bacterial activity was measured at the beginning of the experiments. As nitrite was progressively added the AOB activity progressively decreased.

Average values of OUR were determined for each nitrite concentration level evaluated. In order to compare the effects of different $TNO_2$ concentrations at different pH values, the bacterial activity was normalized and expressed as percentage of the maximum OUR recorded in each experiment. Figure 1c shows the bacterial activity (expressed as OUR percentage) versus the $TNO_2$ concentration. Bacterial activity was also plotted versus the FNA concentration (Figure 1d).



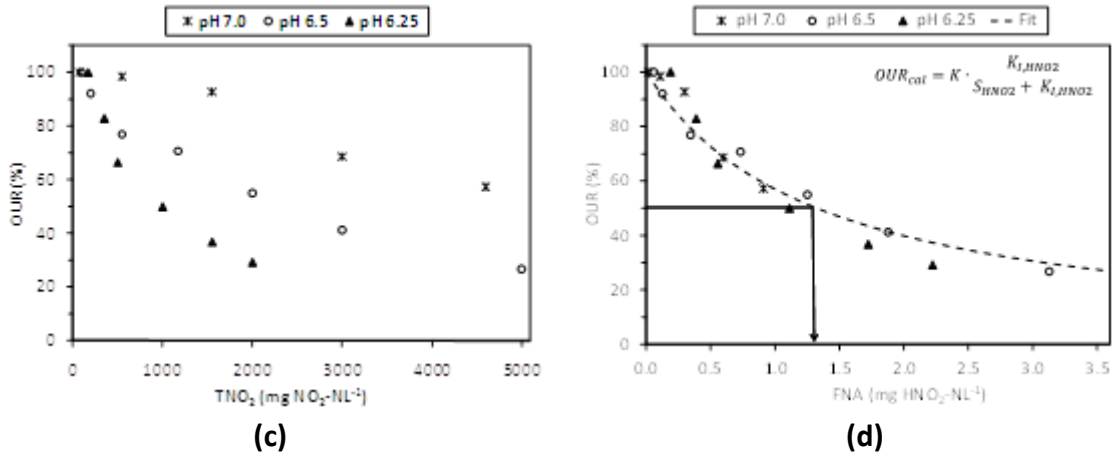



**Figure 1**. OUR evolution (mg $O_2$ $L^{-1}d^{-1}$) and total nitrite concentrations (mg $NO_2$-N $L^{-1}$) in the experiments carried out at pH 6.25 (a) and 6.50 (b). AOB activity expressed as OUR percentage versus $TNO_2$ (c) and FNA (d) concentrations (mg N $L^{-1}$).

The results show that the inhibition of the AOB activity is consequence of the FNA concentration. As can be seen in Figure 1d, all the experiments followed the same trend in the bacterial activity decrease as FNA concentration was increased, irrespective of the pH value of the experiment. The experimental results could be accurately reproduced using the non-competitive inhibition function ($K_I/(K_I + I)$ where I is the FNA concentration and $K_I$ represents the FNA concentration at which 50% of inhibition occurs).

The FNA inhibition constant was determined by minimising the root mean square error between the experimental data and model predictions as 1.32 mg $HNO_2$-N $L^{-1}$. This value is close to the values determined by Van Hulle *et al.* 2007 ($K_{IHNO2}$=2.04 mg $HNO_2$-N $L^{-1}$), Wett and Rauch 2003 ($K_{IHNO2}$=2.8 mg $HNO_2$-N $L^{-1}$) and Hellinga *et al.* 1999 ($K_{IHNO2}$=0.21 mg $HNO_2$-N $L^{-1}$) for side-streams with high ammonium concentrations. However, the value obtained in this study is considerably higher than the value obtained by Jiménez *et al.* 2011 ($K_{IHNO2}$=0.013 mg $HNO_2$-N $L^{-1}$) in an activated sludge pilot plant treating domestic wastewater. This difference is due to the different AOB species present in both systems. According to FISH results *Nitrosomona eutropha* and *Nitrosomona europaea* species were dominant in the partial nitritation reactor (over 94%), while *Nitrosomona oligotropha* predominates in activated sludge systems treating urban wastewater. Beaumont *et al.* (2002) indicated that *Nitrosomona europaea* spp. possesses a gene that encodes a functional copper-type NirK, which confers tolerance to high nitrite concentrations. This result is coherent with the high inhibition constant value obtained in this study.

**Effect of pH on the AOB activity**

Figures 2a and 2b show the OUR evolution along the pH levels evaluated in experiments 2 and 8, respectively. As can be seen in these figures, as the pH was increased, the OUR also increased until the maximum activity was reached. Further pH increments led to a clear OUR reduction. The main difference between these experiments was the pH value at which the maximum activity was reached. In experiment 2, the OUR significantly increased when the pH was raised from 7.0 to 7.5, but it was nearly the same between pH 7.5 and 8.0. However, in experiment 8, the OUR was almost constant between pH 7.0 and 7.5, but it clearly decreased when the pH was

raised from 7.5 to 8.0. The time-evolution profiles of pH and OUR along the rest of the experiments (data not shown) presented the same pattern, exhibiting experiments 1 to 4 the maximum activity at pH values in the range 7.5 to 8.0 while experiments 5 to 9 in the pH range 7.0 to 7.5. It is hypothesized that the different optimum pH is due to the different pH value in the partial nitritation reactor when the experiments were carried out. The batch experiments were performed along the 8 months of experimental period using biomass from the partial nitritation reactor. The same operational conditions (influent flow-rate and composition, dissolved oxygen concentration, temperature) were maintained along the whole period and the effluent ammonium to nitrite ratio was always close to 1 (ranging from 0.95 to 1.15). The main difference was the pH value in the partial nitritation reactor which could be due to the different evaporation rate along the operational period related to variations in laboratory ambient temperature. Figure 2c shows the pH evolution in the partial nitritation reactor along the experimental period jointly with the dates when the batch experiments were carried out. As can be seen in Figure 2c experiments 1 to 4 were carried out when the average pH in the partial nitritation reactor was around 6.6, while experiments 5 to 9 were carried out when the average pH in the partial nitritation reactor was around 6.2. These results suggest an adaptation of AOB to the pH value in the reactor: the pH value at which maximum AOB activity is reached depends on the prevailing environmental conditions in the reactor. The lower pH in the reactor, the lower the optimum pH.

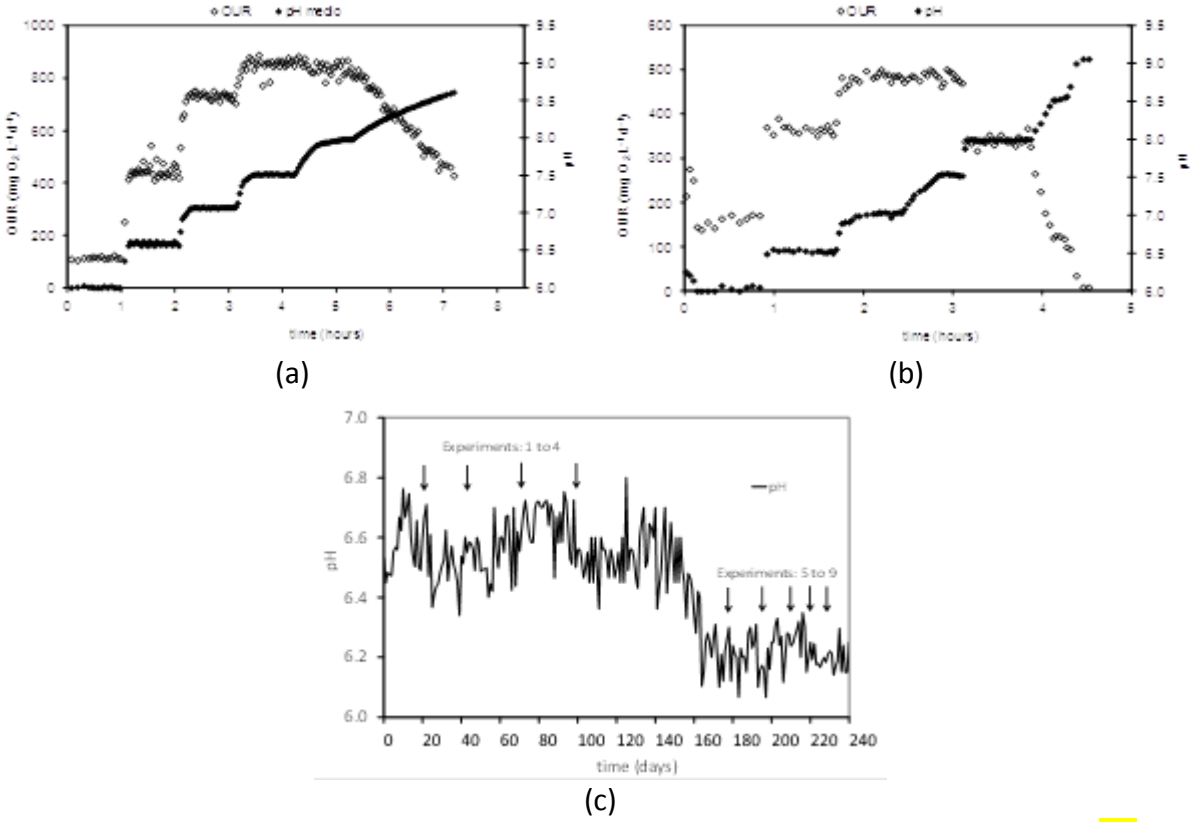



**Figure 2**. OUR evolution at different pH evaluated along: (a) experiment 2 and (b) experiment 8. (c) pH evolution in the partial nitritation reactor along the experimental period. Arrows indicate the dates when the experiments were made.

Figure 3 shows the normalized AOB activity versus pH in the nine experiments. As can be observed in this figure, high pH values have a significant inhibitory effect on the AOB activity (bacterial activity is reduced from over 90% at pH 8 to below 10% at pH

9). The AOB activity reduction at pH values above the optimum pH is due to the high pH values reached, since it cannot be attributed neither to low substrate concentrations (free ammonia concentration increased as the pH increased along the experiment) nor to high FNA concentrations (FNA concentration decreased due to the pH increment). FA inhibition is not considered since previous experimental results (Claros *et al.*, 2010) showed that salinity and not FA concentrations was responsible for the reduction in the AOB activity. The AOB activity reduction observed at high pH values is not due to salinity as it is the same along each experiment. Since the reduction in the AOB activity is only due to the high pH values, all the experiments of each group present the same activity decrease at pH values above the optimum (see Figure 3).

On the other hand, the AOB activity reduction at pH values lower than the optimum pH is due to both the low pH values and the FNA concentrations, since a significant reduction in AOB activity was observed in experiment 8 carried out at very low FNA concentrations (<0.35 mg $HNO_2$-N $L^{-1}$). The rest of the experiments were carried out at higher FNA concentrations and higher activity reductions were observed. The higher FNA concentration, the sharper activity decrease was observed.

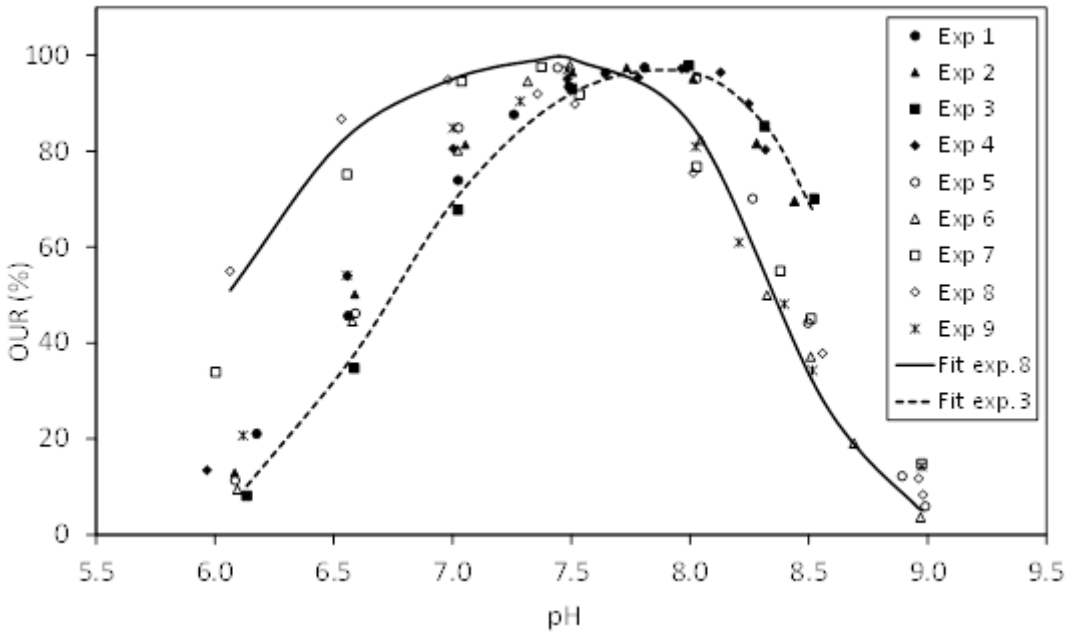



**Figure 3**. OUR versus pH in all the experiments.

Equation 1 is proposed to model the asymmetric form that the pH inhibition presents on both sides of the optimum pH value jointly with the FNA inhibition. pH inhibition is considered in this equation through a combination of two sigmoidal functions normalized respect to the value obtained at optimum pH (see Equation 2). This normalization is required since the product of the two sigmoidal functions is lower than 1 even at optimum pH.

$$\frac{OUR}{OUR_{max}} = \frac{K_{IHNO2}}{K_{IHNO2}+S_{HNO2}} \cdot I_{pH} \qquad \textbf{Equation 1}$$

$$I_{pH} = \frac{f(pH)}{f(pH_{opt})} = \frac{\frac{1}{1+\exp(A_H(K_H-pH))} \cdot \frac{1}{1+\exp(A_{IH}(pH-K_{IH}))}}{\frac{1}{1+\exp(A_H(K_H-pH_{opt}))} \cdot \frac{1}{1+\exp(A_{IH}(pH_{opt}-K_{IH}))}} \qquad \textbf{Equation 2}$$

$K_H$, $K_{IH}$ = inhibition constants for low and high pH, respectively (mol $H^+$ $L^{-1}$); $A_H$, $A_{IH}$ = fit parameters; $K_{IHNO2}$ = inhibition constant for $HNO_2$ (mg $HNO_2$-N $L^{-1}$), pHopt= optimum pH.

The proposed equation has been used to reproduce all the experiments. Simulations were carried out using the previously determined value for FNA inhibition constant ($K_{IHNO2}$=1.32 mg $HNO_2$-N $L^{-1}$). Model parameters related to pH inhibition ($K_H$, $K_{IH}$, $A_H$, $A_{IH}$) were obtained by minimising the root mean square error between the experimental data and model predictions. Since the optimum pH observed in experiments 1 to 4 was higher than the optimum pH observed in experiments 5 to 9, different parameter values were obtained for both groups of experiments. Accurate model predictions for all the experiments could not be achieved using the same pH inhibition parameters. Table 2 shows the pH inhibition parameters determined for both groups of experiments.

Table 2. pH inhibition constants and optimum pH obtained for both groups of experiments.

| Experiments | pH reactor | $K_H$ | $A_H$ | $K_{IH}$ | $A_{IH}$ | Optimum pH value |
|---|---|---|---|---|---|---|
| **1, 2, 3, 4** | 6.60 | 6.40 | 1 | 8.55 | 3 | 7.75 |
| **5, 6, 7, 8, 9** | 6.20 | 5.70 | 1 | 8.20 | 3 | 7.40 |

The values of the pH inhibition parameters obtained in this study indicate that AOB activity is half-inhibited at pH values around 6 (6.4 for the first group of experiments and 5.7 for the second group of experiments) and 8.4 (8.55 for the first group of experiments and 8.2 for the second group of experiments). The inhibition observed in this study at high pH values is similar to that observed by Van Hulle *et al.* (2007) (the AOB activity was half-inhibited at pH 8.2, and no AOB activity was detected at pH values over 8.6). The significant differences between the values obtained in this work and those obtained by Jimenez *et al.*, (2011) ($A_H$ = 2.64, $K_H$=6.05, $A_{IH}$ = 2.74 and $K_{IH}$ = 9.98) are due to the different AOB species prevailing in the studied systems. Jimenez *et al.* (2011) determined the effect of pH on the activity of the AOB present in a biological nitrogen removal pilot plant (*Nitrosomonas oligotropha spp.* was the dominant AOB specie in this plant). Since the pH value in activated sludge reactors is usually between 7 and 8, the activity of the AOB species present in these systems is half-inhibited at pH values higher than the AOB species present in SHARON reactors where the pH is around 6.5.

Model predictions for experiments 3 and 8 are shown in Figure 3. As can be seen in this figure, the equation proposed accurately reproduced the decrease observed in the AOB activity at both sides of the optimum pH in these experiments. Similar accurate predictions were obtained for the rest of the experiments (simulations are not included in Figure 3 for clarity reasons). The model predictions at pH values above the optimum are more or less the same for all the experiments of each group since the term ($K_{IHNO2}/(K_{IHNO2} + S_{HNO2})$) is close to 1 due to the low FNA concentrations. However, at pH values below the optimum, experimental data as well as model predictions are different for every experiment of each group since FNA concentrations were different and high enough to inhibit AOB activity.

## CONCLUSIONS

In this work, the effect of FNA concentration and pH on the activity of the AOB present in a partial nitritation reactor has been quantified through respirometric batch experiments. For this purpose, the activity of biomass from a partial nitritation reactor was measured at different TAN and $TNO_2$ concentrations, as well as at different pH values. The main conclusions that can be drawn from this study are:

- According to FISH analysis, *Nitrosomonas europaea* and *Nitrosomonas eutropha* were the only AOB species in the partial nitritation reactor (94 ± 1% of total bacteria).
- FNA inhibits the activity of *Nitrosomonas europaea* and *Nitrosomonas eutropha*. This inhibition can be properly described by the non-competitive inhibition function. The value of the half inhibition constant for these bacteria was determined as $K_{IHNO2}$=1.32 mg $HNO_2$-N $L^{-1}$.
- The optimal pH for these AOB species was found to be in the range 7.4 to 7.8. High pH values show a stronger inhibitory effect than low pH values. Therefore, an asymmetric inhibition function is required to represent the pH effect on these bacteria. A combination of two sigmoidal functions was able to reproduce the experimental results obtained. Different pH inhibition constants were obtained according to the pH prevailing in the partial nitritation reactor. These results suggest an adaptation of the bacterial population to the environmental conditions.

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