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Experimental sulphide inhibition calibration method in nitrification processes: a case-study.

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Abstract

Sulphide is one of the inhibitors in the nitrification process in WWTP in regions with sulphate rich soils. As little information is currently available on sulphide nitrification inhibition, the aim of this study was to develop a method based on a modification of the Successive Additions Method to calibrate the effect of sulphide on the activity of ammonia-oxidising bacteria (AOB) and nitrite-oxidising bacteria (NOB). The developed method was then applied to activated sludge samples from two WWTPs with different influent sulphide concentrations. In both cases, sulphide had a greater inhibitory effect on NOB than AOB activity. The sulphide inhibition was found to be lower in the activated sludge fed with sulphide-rich wastewater. The AOB and NOB activity measured at different sulphide concentrations could be accurately modelled with the Hill inhibition equation.

Keywords: Sulphide inhibition; nitrification inhibition; ammonia-oxidising bacteria calibration; nitrite-oxidising bacteria calibration; calibration methodology.

1. INTRODUCTION

The accumulation of nutrients such as nitrogen is one of the main reasons for eutrophication and toxicity in aquatic systems, the main sources being agricultural run-off and urban wastewaters. Removing these components in wastewater treatment plants (WWTPs) is therefore essential to maintain high quality receiving waters. Nitrogen removal in WWTPs is typically carried out by nitrification and de-nitrification processes.

The oxidation of ammonium to nitrate during nitrification is carried out by two different autotrophic bacteria which use inorganic carbon as their carbon source: ammonium-oxidising bacteria (AOB), which oxidise ammonia to nitrite (Eq. (1)), and nitrite-oxidising bacteria (NOB), which oxidise nitrite to nitrate (Eq. (2)). Three enzymes are involved in these processes: ammonium monooxygenase (AMO), which catalyses the oxidation of ammonium to hydroxylamine, hydroxylamine oxidoreductase (HAO), which catalyses the oxidation of hydroxylamine to nitrite, and nitrite oxidoreductase (NOR), which catalyses the oxidation of nitrite to nitrate (Arp et al., 2002; Bejarano-Ortiz et al., 2015).

Nitrification is a highly sensitive process in which the nitrification rate is affected by several parameters such as: pH, dissolved oxygen concentration (DO), alkalinity (ALK), temperature (T), Sludge Retention Time (SRT) and inhibitory or toxic substances (Tang and Chen, 2015). The cellular synthesis reactions, in which the cellular composition is represented as $C_5H_7O_2N$, are included below (Eqs. (1)(2)):

Ammonium oxidising bacteria (AOB)

$$NH_4^+ + 2.45\,O_2 + 6.71HCO_3^- \rightarrow 0.11\,C_5H_7O_2N + 2.50\,NO_2^- + 1.03\,H_20 + 6.51\,H_2CO_3 \quad (1)$$

Nitrite oxidising bacteria (NOB)

$$NO_2^- + 0.001\,NH_4^+ + 0.01\,H_2CO_3 + 0.003\,HCO_3^- + 0.339\,O_2 \rightarrow 0.006\,C_5H_7O_2N + 0.003\,H_20 + 1.34\,NO_3^- \quad (2)$$

Nitrification has been reported to be a crucial issue in WWTP design due to the slow growth rate of nitrifying bacteria (Urgun-Demirtas et al., 2008), which means that WWTPs with nitrification are less resilient to the possible shock loads of inhibitory substances (Choi et al., 2010) and have a reduced capacity to comply with discharge limits.

Subbarao et al. (2009) listed more than 30 substances that can inhibit nitrification, including those formed by reduced sulphur compounds capable of de-activating the AMO enzyme and competing for the enzyme's active site, sulphide being the most common of these.

Sulphide is present in both industrial and urban wastewaters (in regions with sulphate rich soils) because of sulphate reduction in the sanitation system. Sulphate is biologically reduced to sulphide in anaerobic conditions by sulphate-reducing bacteria (SRB) and has a negative impact on WWTPs.

Several studies (Sánchez-Ramírez et al., 2015; Sears et al., 2004) have shown that sulphide inhibits the activity of AOB and NOB in different ways, although the inhibition

constants proposed by different authors vary significantly (Delgado Vela et al., 2018). For example, Sears et al., 2004 reported that 0.5 mg $S \cdot L^{-1}$ as total soluble sulphide within nitrifying cultures can completely inhibit the oxidation of ammonia. Several authors also emphasize that the inhibition is reversible, and that nitrification is recovered after eliminating the sulphide present. Other authors (Erguder et al., 2008; Sánchez-Ramírez et al., 2015) found that the presence of sulphide in a batch reactor generated an accumulation of nitrite and highlighted the sensitivity of NOB to this compound.

Respirometry-based assays, such as specific oxygen uptake rate (SOUR), are the most commonly used approach to determine the level of nitrification inhibition (Kapoor et al., 2016). However, measuring AOB and NOB inhibition requires isolating AOB and NOB activity by means of, for instance, a selective inhibitor for each group. Although an NOB inhibitor seems to be the most direct way of measuring the activity of AOB, it is necessary to verify that: (i) it completely blocks NOB activity, while (ii) it does not inhibit AOB, and (iii) the inhibition should be instantaneous. In the absence of an appropriate inhibitor, other methods should be considered to measure separately the activity of both groups. In the present study, a modified version of the method developed by Moussa et al., (2003), based on successive additions of nitrite and ammonium, was used to measure AOB activity when NOB were most active.

The aim of this work was thus to develop an experimental method for evaluating the level of sulphide inhibition in AOB and NOB activity during the nitrification process comparing two activated sludges with different sulphide exposition.

2. MATERIALS AND METHODS

Respirometric techniques have been applied to evaluate and quantify the effect of sulphide on NOB and AOB activity individually. This technique is based on measuring the evolution of dissolved oxygen concentration to determine the Oxygen Uptake Rate (OUR), which represents biomass activity and depends on the degradation of an external substrate and internal biomass reservoirs. When all the external substrate has been consumed, the OUR is related to the bacteria's activity using their internal reservoirs (endogenous OUR, OUR_{end}) so the consumption associated to the ammonium released by the biomass decay is included in it. The difference between the OUR in the presence of a substrate (exogenous OUR, OUR_S) and the OUR_{end} is the activity of the bacteria associated with the substrate consumed (Eq. (3)).

$$OUR_X = OUR_S - OUR_{end} \quad (3)$$

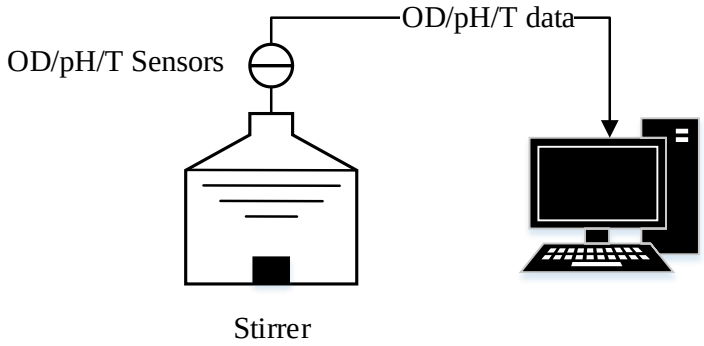
To quantify the biomass activity at different substrate concentrations the sludge must be in endogenous conditions to guarantee that any increase in OUR is due to NOB or AOB activity after adding an external substrate. To achieve endogenous conditions, biomass has to be exposed to aerobic conditions until the external substrate is completely depleted.

A modification of the Successive Additions Method (Moussa et al., 2003) (see Section 2.3) was applied to determine the influence of sulphide inhibition on AOB and NOB activity.

2.1. Experimental design

Figure 1 shows the experimental device used for the calibration process. It consisted of a 0.5 L effective volume Erlenmeyer flask, a magnetic stirrer, a pH/T Cell pH sensor (WTW©, Weilheim, Germany), a dissolved oxygen sensor Cell OX325 (WTW©,

125 Weilheim, Germany), a Multi 350i (WTW©, Weilheim, Germany) and a computer to
126 register and analyse the data obtained from these sensors.



127

128 *Figure 1. Experimental set-up*

129 **2.2. Analytical methods and reagents.**

130 The sludge was characterized and monitored during the endogenous phase, analysing the
131 following parameters: Total Suspended Solids (TSS), Volatile Suspended Solids (VSS),
132 Total Chemical Oxygen Demand (COD_T), sulphate (SO_4-S), sulphur (S^2-S) and nutrients
133 (ammonium (NH_4-N), nitrate (NO_3-N), nitrite (NO_2-N) and orthophosphate (PO_4-P)). The
134 analytical measurements were carried out based on the Standard Methods (APHA, 2005):
135 Solids (2540-D and 2540-E), COD (5220-B), sulphide (4500-S2--D), sulphate (4500-
136 SO4-2-F), ammonium (4500-NH3-G), nitrate (4500-NO3), nitrite (4500-NO2-B) and
137 orthophosphate (4500-P-F). Sodium nitrite (98%- PANREAC), ammonium chloride
138 (100%- VWR), sodium sulphide (>=98% SIGMA ALDRICH) and sodium dihydrogen
139 phosphate (99%-PANREAC) were purchased from laboratory suppliers and used as
140 received.

141

142 **2.3. Experimental procedure**

143 The experimental procedure was based on measuring the maximum AOB and NOB
144 activity in the absence and in the presence of different concentrations of sulphide, and

obtaining the degree of inhibition by the level of activities measured. For that purpose, the oxygen consumption was evaluated as indicator. The OD range was between 7 and 4 mg $O \cdot L^{-1}$. The substrate concentration (NH_4^+ or NO_2^-) required for AOB and NOB to reach their maximum activity was previously determined. Biomass activity was then measured by adding the substrate concentration and different sulphide concentrations, as described in the following sections.

2.4. Measuring maximum nitrifying bacteria activity

2.4.1. Maximum NOB activity

The method used to determine maximum NOB activity was in 5 steps:

1. A volume of 500 mL of activated sludge (AS) in the endogenous phase was placed in a stirred Erlenmeyer flask to determine the OUR_{end}.
2. The pH value was recorded to control possible pH inhibition. If necessary, acid or base was added to keep pH constant 7.5 ± 0.2.
3. The DO time evolution was recorded during around 3 minutes to obtain the OUR_{end}.
4. After that, a known amount of substrate (NO_2-N) was added recording again the DO time evolution (OUR_{NO2}).

The method was repeated for the different concentrations of nitrite shown below to find the concentration that caused the maximum NOB activity (NO_{2max}). A new aliquot of sludge was used in every test. The oxygen consumption associated with nitrite oxidation (OUR_{NOB}) was obtained from Eq. (4) and can be described by a Monod expression, Eq. (5), which represents the variation of bacterial activity expressed as Oxygen Uptake Rate as a function of the substrate concentration in the medium. Furthermore, Eq. (6) shows the components which define the specific maximum oxygen consumption.

$$OUR_{NOB} = OUR_{NO2} - OUR_{end} \quad (4)$$

$$OUR_{NOB} = M_{NOB} \frac{NO_2}{K_{sNO2} + NO_2} I_{H2S} \quad (5)$$

$$M_{NOB} = \mu_{max} \cdot X \cdot \frac{(1 - 1.14 Y_{NOB})}{Y_{NOB}} \quad (6)$$

Where M_{NOB} ($mg \cdot L^{-1} \cdot d^{-1}$), K_{sNO2} ($mg \cdot L^{-1}$) NO_2 ($mgNO_2\text{-}N \cdot L^{-1}$) and I_{H2S} are the specific maximum oxygen consumption due to the presence of a substrate, in this case nitrite, the nitrite half-saturation constant, the concentration of nitrite in the medium and the inhibition function used to represent the sulphide inhibition, respectively. It is important to mention that, in the determination of NOB and AOB maximum activity sulphide is not present, so the numerical value of the switch function is one. M_{NOB} depends of the maximum specific rate (μ_{max} (d^{-1})), the concentration of NOB (X ($mg \cdot L^{-1}$)) and the yield coefficient of NOB (Y_{NOB}). As all the experiments carried out in a short time, the amount of bacteria could be considered as a constant.

2.4.2. Determining AOB maximum activity

The fact that AOB transforms ammonium into nitrite, which is the NOB substrate, makes it difficult to measure AOB activity in isolation. De-activating NOB by adding an inhibitor or during maximum NOB activity are the usual alternatives. However, the inhibitor must de-activate one type of bacteria without affecting the others, which is rather difficult. Claros et al. (2010) recommended the Successive Addition Method (Moussa et al. 2003), which is based on the consecutive injection of $NaNO_2$ (to measure the OUR related to NOB activity) and NH_4Cl (OUR related to the activity of both bacteria) to determine the activity of the ammonia and nitrite oxidisers separately. The method used is as follows:

Steps 1, 2 and 3 as previously indicated.

4. Adding the amount of NO_{2max} calculated in the previous section and recording the concentration of dissolved oxygen to obtain OUR_{NO2max}.
5. When OUR_{NO2max} was determined (around 3 minutes) a known amount of substrate (NH_4-N) was added and the increase in the oxygen consumption rate is recorded (to determine OUR_{NH4}).

This process was repeated with different concentrations of ammonium using a new aliquot of sludge in each assay to determine the ammonium concentration required for AOB to reach their maximum activity (NH_{4max}). The oxygen consumption associated with the oxidation of AOB activity (OUR_{AOB}) is obtained from Eq. (7) and also can be described by a Monod expression Eq. (8).

$$OUR_{AOB} = OUR_{NH4} - OUR_{NO2max} \quad (7)$$

$$OUR_{AOB} = M_{AOB} \frac{\mathrm{NH_4}}{K_{sNH4} + \mathrm{NH_4}} I_{H2S} \quad (8)$$

Where M_{AOB} ($mg \cdot L^{-1} \cdot d^{-1}$), K_{sNH4} ($mg \cdot L^{-1}$) and NH_4 ($mgNH_4\text{-}N \cdot L^{-1}$) are the specific maximum oxygen consumption due to the presence of a substrate, in this case ammonia, the ammonia half-saturation constant and the concentration of ammonia in the medium, respectively.

2.5. Determining NOB and AOB sulphide inhibition

For this, the maximum activity of both groups of bacteria was evaluated at different sulphide concentrations. Adding sulphide to sludge has a double effect on oxygen consumption: the consumption associated with bacterial activity is reduced while that due to sulphide oxidation rises. The latter must be previously determined in order to quantify the inhibitory effect of sulphide on bacterial activity.

2.5.1. Determining oxygen consumption related to sulphide oxidation

The method used to determinate the oxygen consumed by sulphide oxidation in the activated sludge samples consisted of the following steps:

Steps 1, 2 and 3 as previously indicated.

4. When OUR_{end} was determined (around 3 minutes) a known amount of sulphide (S^{2-}) was added recording the OD time evolution to determine the $OUR_{maxS}{}^{-2}$.

This process was repeated with different concentrations of sulphide and new aliquots of sludge. The oxygen consumption associated with the oxidation of sulphide (OUR_{S2-}) obtained from Eq. (9) can also be described by a Monod expression (10).

$$OUR_{S^{-2}} = OUR_{maxS^{-2}} - OUR_{end} \quad (9)$$

$$OUR_{S^{-2}} = M_{S2-} \frac{S^{2-}}{K_{SS}2- + S^{2-}} (10)$$

Where M_{S2-} ($mg \cdot L^{-1} \cdot d^{-1}$), K_{sS2-} ($mg \cdot L^{-1}$) and S^{2-} ($mg\ S^{2-}\text{-}S \cdot L^{-1}$) are the specific maximum oxygen consumption due to the presence of sulphide, the sulphide half-saturation constant and the concentration of sulphide in the medium, respectively.

2.5.2. Determining NOB activity in the presence of sulphide.

The method used to determine the oxygen consumption by NOB in the presence of sulphide in the different samples consisted of the following steps:

Steps 1, 2 and 3 as previously indicated.

4. When OUR_{end} was determined (around 3 minutes), the nitrite concentration at maximum NOB activity is reached (NO_{2max}) and a certain amount of sulphide (S^{-2}) were added simultaneously recording the OD time evolution to determine the $OUR_{maxNO2 + S-2}$.

239 This process was repeated with different sulphide concentrations and new aliquots of
240 sludge. The oxygen consumption associated with sulphide oxidation and NOB activity in
241 the presence of sulphide was obtained from Eq. (11).

242 $$OUR_{NO_2+S^{-2}} = OUR_{maxNO_2+S^{-2}} - OUR_{end} \qquad (11)$$

243 The percentage of sulphide inhibition of NOB activity was calculated by comparing the
244 maximum NOB activity (OUR_{NOB}) and the NOB activity in the presence of sulphide
245 (OUR_{S-2}) measured at the established sulphide concentration (Eq. (12)).

246 $$\%\ InhibitionNOB = \frac{(OUR_{S^{-2}+OUR_{NOB}}) - OUR_{NO_2+S^{-2}}}{OUR_{NOB}} \times 100 \qquad (12)$$

247

248 **2.5.3. Determining AOB activity in the presence of sulphide**

249 The method used to determine AOB oxygen consumption in the presence of sulphide
250 consisted of the following steps:

251 Steps 1, 2 and 3 as previously indicated.

252 4. When OUR_{end} was determined (around 3 minutes), the nitrite concentration at
253 maximum NOB activity is reached (NO_{2max}) was added recording the OD time
254 evolution to determine the OUR_{NOB}.

255 5. When OUR_{NOB} was determined (around 3 minutes), the ammonium concentration
256 for maximum AOB activation (NH_{4max}) and the established amount of sulphide
257 (S^{2-}) were added simultaneously recording the OD time evolution to determine
258 the $OUR_{maxNO2 + (S-2 + NH4)}$.

259 This process was repeated for different concentrations of sulphide and new aliquots of
260 sludge. The oxygen consumption associated with sulphide oxidation and AOB activity in
261 the presence of sulphide was obtained from Eq. (13).

262 $$OUR_{(S^{-2}+NH_4)} = OUR_{maxNO_2+(S^{-2}+NH_4)} - OUR_{NO_2 max} \quad (13)$$

263 The percentage of sulphide inhibition on AOB activity was calculated by Eq. (14).

264 $$\%\, Inh\, AOB = \frac{(OUR_{S^{-2}+OUR_{AOB}-OUR_{NOB}\left(\frac{\%\, Inh\, NOB}{100}\right))-OUR_{(S^{-2}+NH_4)}}}{OUR_{AOB}} \times 100 \quad (14)$$

265

266 **2.6. Operating conditions and activated sludge characterisation**

267 The samples of activated sludge were taken from two full-scale WWTPs in eastern Spain
268 with conventional activated sludge processes including biological nitrogen removal. Both
269 samples were taken from the aerobic reactor. Both WWTPs treat urban wastewater with
270 high concentrations of sulphate, which is typical in wastewaters of this area. Table 1
271 shows the main sludge parameters as characterised at the beginning of the experiment.
272 The most remarkable parameters are the sulphide and sulphate concentrations. As above-
273 mentioned, both sludges were exposed at high sulphate concentrations but only in one
274 case (sulphide exposed sludge) sulphide was detected in the wastewater entering to the
275 biological reactor. The presence of sulphide could determine the sludge behaviour in the
276 inhibition process in terms of the sulphide adaptability of the sludge. For further
277 information of the WWTPs the characterization of the primary settler effluent is provided
278 in the supplementary material (Table S1).

279 *Table 1. Sludge characterisation (s.d.: standard deviation)*

Parameter	**Average ± s.d.**	
	Sulphide exposed sludge	**Sulphate exposed sludge**
TSS (mg· L^{-1})	4851 ± 63	3725 ± 99
VSS (%)	85.6 ± 1.7	82.8 ± 1.3

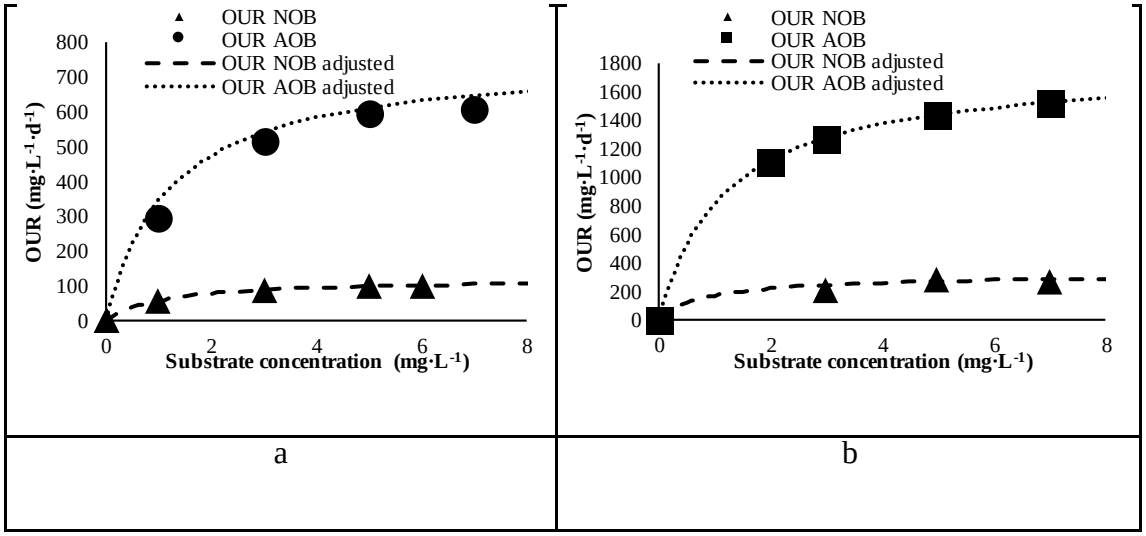
COD_T (mg COD· L^{-1})	6325 ± 336	4450 ± 54
NH_4-N (mg N· L^{-1})	0.7 ± 0.1	<0.03
PO_4-P (mg P· L^{-1})	<0.02	2.11 ± 0.02
NO_3-N(mg N· L^{-1})	38.6± 4.9	16.18 ± 0.36
NO_2-N (mg N/ L)	0.54 ± 0.01	0.08 ± 0.01
S^{2-}-S (mg S/L)*	10.0 ± 1.2	<0.02
SO_4-S (mg S· L^{-1})	230± 6.2	99.2± 7.1
SRT (d)	11	12
HRT (h)	20	15

*Concentration in wastewater entering to the biological reactor.

3. RESULTS AND DISCUSSION

3.1. Maximum bacterial activity

Maximum NOB and AOB activity of both activated sludge samples was determined following the method described in Sections 2.4.1 and 2.4.2, respectively. The sulphide-exposed sludge's response is shown in Figure 2a for additions of: 1.5; 3; 5 and 6 mg N-NO_2·$L^{-1}$and 1.5; 3; 5 and 7.5 mg N-NH_4·L^{-1}. As can be seen in this figure, the oxygen consumption associated with AOB activity is much higher than that for NOB. However, the maximum growth rate was achieved at 5 mg·L^{-1} in both substrates, equivalent to 99.19 mg O_2·L^{-1}·d^{-1} for NOB and 611.21mg O_2·L^{-1}·d^{-1} for AOB.



290 *Figure 2. AOB and NOB oxygen uptake rate for a) sulphide-exposed; b) sulphate-exposed sludge*

291 Figure 2b show the results of the sulphate-exposed sludge for additions of: 3; 5; and 7 mg
292 $N\text{-}NO_2 \cdot L^{-1}$ and 2; 3; 5 and 7 mg $NH_4\text{-}N \cdot L^{-1}$. This sludge also had a higher oxygen
293 consumption associated with AOB (8 times higher than oxygen consumption related to
294 NOB). When comparing both sludges, the oxygen consumption in the sulphate exposed
295 sludge is higher for both groups of bacteria. Based on these results, it was assumed that
296 the maximum activity was reached at a substrate concentration of 5 ppm for both groups
297 of bacteria, which corresponds to an oxygen consumption of 270.81$mg \cdot L^{-1} \cdot d^{-1}$ for NOB
298 and 1439.51 $mg \cdot L^{-1} \cdot d^{-1}$ for AOB.

299 The Monod expression was adjusted to the experimental results obtained. The parameter
300 values obtained are shown in Table 2. Half saturation constant values obtained were
301 similar in all the experiments. The main difference was the maximum oxygen
302 consumption for AOB and NOB (M). The influent composition, especially the presence
303 of sulphide, is directly related with the difference between both sludges. Furthermore, the
304 differences between AOB and NOB oxygen consumption are in the same way as obtained
305 by Delgado Vela et al., (2018) who affirm that NOB are more sensitive. However, further
306 microbiological research is needed to evaluate these differences attending to the different
307 species involved and their relative abundance.

Table 2. Calibration of AOB and NOB for each sludge

		Specific maximum oxygen consumption ($M_{Bacteria}$ ($mg \cdot L^{-1} \cdot d^{-1}$))	Half saturation constant (K_s($mg \cdot L^{-1}$))
Sulphide exposed sludge	**AOB**	758.12	1.20
	NOB	120.02	1.10
Sulphate exposed sludge	**AOB**	1,785.50	1.05
	NOB	319.70	1.00

3.2. Sulphide inhibition

The oxygen consumption related to sulphide oxidation was also evaluated by the method described in Section 2.5.1. For the sulphide-exposed sludge, the concentrations evaluated were between 1.25 and 15 mg $S^{2-} \cdot L^{-1}$, as can be seen in Figure 3a. For the sulphate-exposed sludge, the concentrations evaluated varied from 1.25 and 7.5 mg $S^{2-} \cdot L^{-1}$. The maximum oxygen consumption seems to be reached at this level.

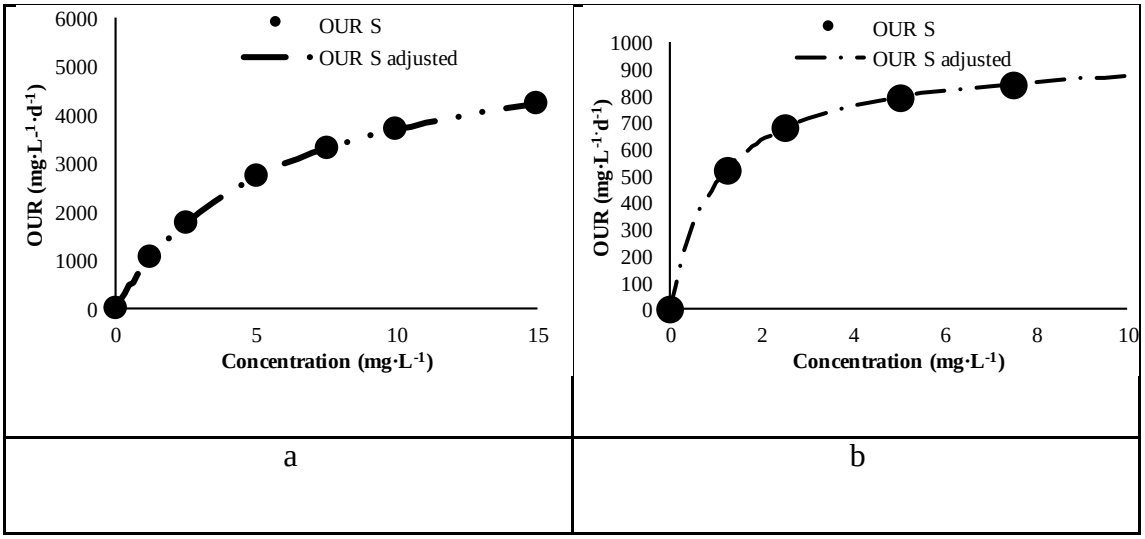


Figure 3. Oxygen consumption by sulphide oxidation in a) sulphide-exposed; b) sulphate-exposed sludge

The competition between chemical and biological oxidation of sulphide for dissolved oxygen is reported in literature (Bejarano Ortiz et al., 2013). As it can be seen in Figure 3, the oxygen consumption associated with sulphide addition was higher in the sulphide-exposed sludge. Although it is not possible to know the percentage of biological and chemical oxidation of the total consumption, the difference between both sludges could be attributed to the first process. The presence of sulphide in the wastewater at the entrance of the biological reactor enhances the growth of this type of bacteria and its adaptability. So, the difference between the oxygen consumptions could be associated to the presence of a bigger bacteria population capable to use sulphide to grow.

Table 3 shows the experimental OUR values obtained in both sludge types applying the method described in Sections 2.5.2 and 2.5.3. Eqs. 4, 6, 8, 10 and 12 were applied to calculate the OUR values. Graphs related to the tests carried out to analyse the sulphate exposed sludge with a sulphide concentration of 7.5 mg $S^{2-} \cdot L^{-1}$ are included as Supplementary Material.

Table 3. Experimental oxygen uptake rates (OUR) obtained in the different experiments carried out.

	Sulphide Concentration ($mg \cdot L^{-1}$)	**1.25**	**2.5**	**5**	**7.5**	**10**
Sulphide exposed sludge ($mg\ O \cdot L^{-1} \cdot d^{-1}$)	OUR NOB	-	103.68±10	250.56±25	103.68±9	207.36±20
	OUR AOB	-	613.44±46	613.44±46	613.44±46	613.44±46
	OUR S^{2-}	-	1,261.44±112	2,488.32±174	3,300.48±241	3,957.12±109
	OUR (S^{2-} +NO_{2max})	-	1,339.2±90	2,617.92±192	3,326.4±217	4,008.96±74

	OUR ($NH_{4max}+S^{2-}$)	-	1,581.12±78	3,196.8±102	3525.12±184	4,112.64±267
Sulphate exposed sludge (mg O·L^{-1}· d^{-1})	OUR NOB	267.84±10	276.48±10	267.80±19	267.84±15	-
	OUR AOB	1,434.24±51	1,434.24±51	1,434.24±51	1,434.24±51	-
	OUR S^{2-}	449.28±25	682.56±15	794.88±8	838.08±15	-
	OUR (S^{2-} +NO_{2max})	596.16±27	777.6±16	829.44±42	846.72±31	-
	OUR ($NH_{4max}+S^{2-}$)	1,546.56±13	1,520.64±15	1,123.2±64	1,123.2±14	-

Table 4 shows the NOB and AOB inhibition degree obtained for the different sulphide concentrations evaluated according to Eq. (11) and Eq. (13).

Table 4. Sulphide inhibition on AOB and NOB.

	Sulphide Concentration (mg S^{2-} · L^{-1})	**1.25**	**2.5**	**5**	**7.5**	**10**
Sulphide exposed sludge	% NOB inhibition	-	25	48	75	75
	% AOB inhibition	-	44	53	51	50
Sulphate exposed sludge	% NOB inhibition	4	66	88	97	-
	% AOB inhibition	15	29	60	62	-

It can be seen that in the case of the sulphide-exposed sludge the degree of inhibition of NOB activity does not vary for sulphide concentrations above 7.5 mg S^{2-}·L^{-1}. Similar results were obtained for AOB, in which inhibition remained constant for sulphide concentrations over 5 mg S^{2}·L^{-1}. Beristain-Cardoso et al., (2010) who fed a floating biofilm reactor with thiosulphide and ammonium in order to adapt the sludge, reported a 50% inhibition for AOB at a sulphide concentration of 13 mg S^{2-}·L^{-1}.Comparing the

348 sulphide inhibition of both bacterial groups, NOB were more sensitive to high sulphide
349 concentrations since their activity was reduced by 75%, while this was only 50% in AOB.

350 In the case of the sulphate-exposed sludge, NOB were also more sensitive to the presence
351 of sulphide than AOB. However, the percentages of inhibition were higher for both types
352 of bacteria. For a concentration of 5 mg $S^{2-} \cdot L^{-1}$, inhibition was 88% and 60% for NOB
353 and AOB, respectively. Bejarano-Ortiz et al., (2015) obtained values of 82% and 76%
354 for NOB and AOB, respectively, at a sulphide concentration of 5 mg $S^{2-} \cdot L^{-1}$ in non-
355 adapted sludge. These results are similar to those obtained in the present study for NOB
356 in sulphate-exposed sludge, although AOB were less sensitive to the presence of sulphide
357 than in the above-cited study. However, a wide range of values can be found in the
358 literature. For example, Bejarano Ortiz et al., (2013) reported a 50% inhibition working
359 at a sulphide concentration of 2.6 and 1.2 for AOB and NOB, respectively, which
360 represents a higher inhibition than the obtained in this work.

361 Several authors have reported that bacteria acclimatisation could reduce the inhibiting
362 effect on bacterial metabolism (Wan et al., 2017; Wang et al., 2010). This could explain
363 the fact that sludge which is usually in contact with sulphide is less inhibited while the
364 other one presents similar values to those in the bibliography for non-adapted sludge,
365 indicating that adaptability can reduce the inhibition of the nitrification process in both
366 NOB and AOB.

367

368 **3.3. Modelling and calibration of sulphide inhibition.**

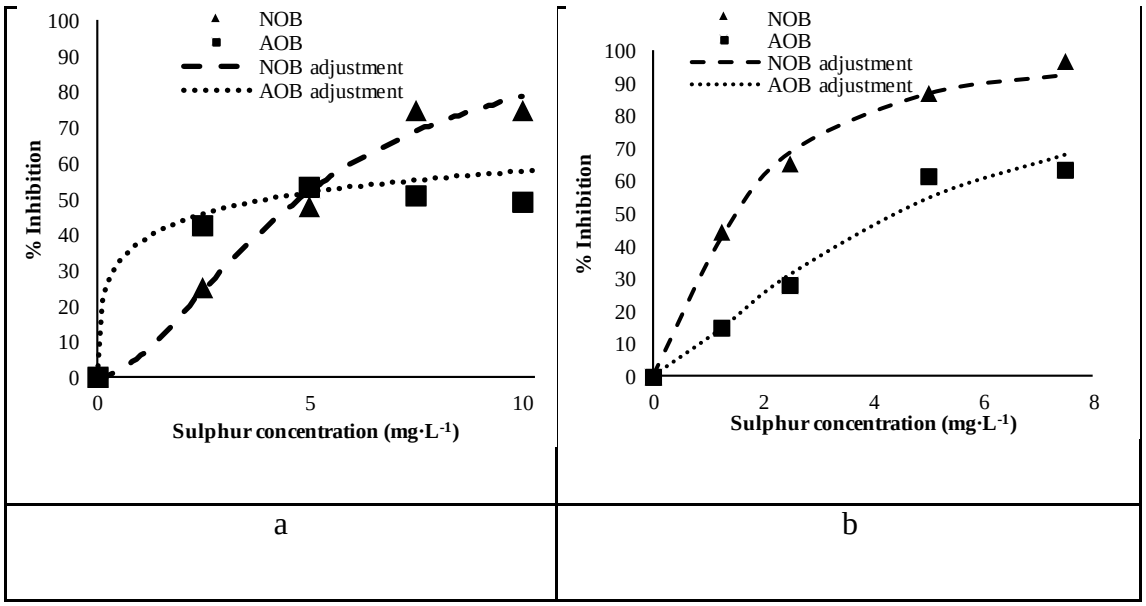
369 Different inhibition functions were evaluated to model the sulphide inhibition observed
370 in both sludges. The non-competitive inhibition equation is the most frequently used to
371 describe inhibitory effects in wastewater treatment, although its predictions were not

372 accurate in these cases (data not shown). Other inhibition functions were therefore
373 considered. As Claros et al., (2010) used the Hill function to evaluate the salinity effect
374 on AOB successfully, this equation was applied (Eq. (14)) to model the sulphide
375 inhibition effect.

376 $$\frac{K_I^n}{K_I^n + I^n} \quad (14)$$

377 K_I being the 50% inhibitor concentration ($mg \cdot L^{-1}$); n the adjustment parameter and I the
378 inhibitor concentration ($mg \cdot L^{-1}$).

379 Figure 4 shows the model predictions applying the Hill equation jointly with the
380 experimental values. The model parameters obtained are shown in Table 5. As can be
381 seen in this figure, the Hill equation accurately reproduced the behaviour observed
382 experimentally in both bacterial groups.



383

384 *Figure 4. Experimental and modelled Inhibition percentages for: a) sulphide exposed sludge; b) sulphate exposed*
385 *sludge*

386 The setting values of the Hill equation obtained for sulphide-rich sludge present similar
387 values of K_I for both types of bacteria. At around 4 mg $S^{2-} \cdot L^{-1}$ inhibition is 50%,
388 suggesting a similar sulphide effect on each type of bacteria. Considering that the non-
389 ionized form (H_2S) is responsible for the inhibition, this would mean approximately 1 mg
390 $S\text{-}H_2S \cdot L^{-1}$. However, in the case of the sulphate-exposed sludge, the results are quite
391 different. Although AOB also present a K_I of approximately 4 mg $S^{2-} \cdot L^{-1}$ (1 mg $S\text{-}H_2S \cdot L^{-1}$),
392 the value of this constant for NOB is 1.51 mg $S^{2-} \cdot L^{-1}$ (0.38 mg $S\text{-}H_2S \cdot L^{-1}$), confirming
393 that this bacterial group is extremely sensitive to the presence of sulphide.

394 *Table 5. Setting values of Hill equation*

	Calibration	Process	K_I (mg·L^{-1})	n	Squared Error
Sulphide-exposed sludge	Ec. Hill	NOB	4.79	1.76	0.0060
		AOB	4.15	0.35	0.0024
Sulphate-exposed sludge	Ec. Hill	NOB	1.51	1.56	0.0033
		AOB	4.39	1.40	0.0081

395

396 Furthermore, pH variation can have an effect on sulphide inhibition. In this way, a pH
397 reduction from 7.5 to 6.5 would increase the inhibition for both NOB and AOB in both
398 sludges. In the case of sulphide exposed sludge, the 50% inhibition would decrease to
399 approximately 1.5 mg $S^{2-} \cdot L^{-1}$ for each type of bacteria. On the other hand, for the sulphate
400 exposed sludge, it would be approximately 1.5 mg $S^{2-} \cdot L^{-1}$ for AOB and 0.5 mg $S^{2-} \cdot L^{-1}$ for
401 NOB. Therefore, it is recommended to operate the WWTPs at high pH values that allow

a reduction of the non-ionized species (H_2S) responsible for the inhibition and compatible with an optimal level of activity of the groups of microorganisms present in the sludge.

4. Conclusions

The main conclusions obtained from this study are as follows:

- The method proposed here based on a modification of the Successive Additions Method accurately calibrated the effect of sulphide on AOB and NOB.
- Sulphide exposed sludge is less inhibited, indicating that the continuous exposure to sulphide may promote the biomass acclimation and thus reduce inhibition of the nitrification process for NOB and AOB.
- The sulphide inhibitory effect was greater in NOB than in AOB. The inhibition in adapted sludge (sulphide exposed) was 75% and 51% for NOB and AOB, respectively at a sulphide concentration of 7.5 ppm. For non-adapted sludge, the inhibition degree was 88% and 60% for NOB and AOB at a sulphide concentration of 5 ppm.
- Inhibition of NOB and AOB was reduced by 25% and 10%, respectively, in adapted sludge.
- The AOB and NOB activity at different sulphide concentrations can be accurately modelled using the Hill inhibition equation.

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