

# Short and long term orange dye effects over AOB and anammox activities

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**Abstract:** In this research work the effects of orange azo dye over ammonia oxidizing bacteria (AOB) and anammox bacteria activities were tested. Adsorption onto the biomass was detected with both types of biomass however no biological decolourization occurred. Performed batch tests indicated that concentrations lower than 650 mg<sub>orange</sub>/L stimulated AOB activity while anammox bacteria activity was inhibited at concentrations higher than 25 mg<sub>orange</sub>/L. Long-term performance of both processes was tested in the presence of 50 mg<sub>orange</sub>/L. In the case of the partial nitrification process both the biomass concentration and the specific AOB activity increased after 50 days of orange azo dye addition. Regarding the anammox process, specific activity decreased down to 58% after 12 days of operation; however, initial values were restored 54 days after stopping the dye addition.

**Keywords:** Adsorption; Anammox; AOB; nitrogen removal; orange dye; partial nitrification.

## Introduction

Dye compounds are commonly present in the wastewater produced from industrial sectors like paper, textiles, paint or printing production. The azo dyes are the most commonly used organic dyes. They are aromatic compounds provided with nitrogen–nitrogen double bond, which confers them on a complex structure and high molecular weight, and make them difficult to be mineralized. Although the acute toxicity of azo dyes is low, their potential health effects, due to their toxic by-products (e.g. aromatic amines), have been described as mutagenic and carcinogenic (Daverey *et al.* 2014). This makes necessary an efficient treatment to remove these compounds prior to the effluents disposal into the environment.

The treatment methods to achieve the dyes decolourization in wastewater include physico-chemical processes (e.g. membranes, reverse osmosis, coagulation/flocculation, etc.); advanced oxidation processes (e.g. ozonation, Fenton process, electrochemical oxidation, UV/ozone/hydrogen peroxide, etc.) and biological processes (e.g. bacterial, fungal and enzymatic degradation) (Gupta *et al.* 2015). Regarding the biological processes specific isolated strains are used to achieve the efficient dye removal (Gupta *et al.* 2015). For this reason, little attention was paid to the effect of dyes over the conventional microbial populations present in the WWTPs. Li & Bishop (2004) studied the removal of acid orange 7 (azo dye) in conventional wastewater treatment processes and they observed that this dye was recalcitrant to aerobic conditions, while it was decoloured under anaerobic and anoxic conditions with adapted sludge.

The anammox (anaerobic ammonium oxidation) process has been widely studied, since the late 90's, to remove nitrogen from wastewater in a more efficient way than via the conventional nitrification-denitrification processes. The anammox bacteria are known to be very sensitive to operational conditions (temperature, pH, dissolved

oxygen concentration, etc.) and they are slow growing organisms. Furthermore, the presence of toxic compounds can easily provoke the failure of the process involving long recovery periods. For these reasons, an extensive amount of research works has been devoted checking the inhibitory effect of different compounds (e.g. salts, antibiotics, heavy metals, etc.) to broaden the range of possible applications for the anammox process (Jin *et al.* 2012). However, at the moment, little attention has been paid to the effects of azo dyes over anammox based processes.

To perform the anammox process (anaerobic) it is necessary previously to convert half of the ammonium present in the wastewater to nitrite (partial-nitrification, aerobic process). In this way the aim of this study is to evaluate the effects of the presence of orange azo dye over both processes (partial nitrification and anammox) separately, considering the short term effect by batch activity tests and the long-term effect by continuous reactor operation.

## Material and Methods

*Experimental set-up:* A continuous stirred tank reactor (CSTR) of 5 L was used for the partial nitrification process. Peristaltic pumps were used for the feeding and withdrawal. The aeration was provided by a fine diffuser (pores of 100  $\mu\text{m}$ ) placed at the bottom of the reactor. The complete mixing of the liquid media was achieved by the action of a mechanical stirrer at a rotating speed of  $150\pm 20$  rpm. The reactor had a thermal water jacket and the temperature inside it was maintained at  $35 \pm 1$  °C. The inoculum, with a specific ammonia oxidizing activity of  $0.27 \text{ g NH}_4^+\text{-N}/(\text{g VSS}\cdot\text{d})$ , was taken from a nitrifying-denitrifying SBR reactor. The initial concentration in the CSTR was of  $0.043 \text{ g VSS/L}$ . The reactor was fed with a synthetic solution composed by:  $1.91\text{-}1.45 \text{ g NH}_4\text{Cl/L}$ ,  $3.50 \text{ g KHCO}_3\text{/L}$ ,  $0.05 \text{ g NaH}_2\text{PO}_4\text{/L}$ ,  $0.3 \text{ g CaCl}_2\cdot 2\text{H}_2\text{O/L}$ ,  $0.2 \text{ g MgSO}_4\cdot 7\text{H}_2\text{O/L}$ ,  $0.006 \text{ g FeSO}_4\text{/L}$ ,  $0.006 \text{ g EDTA/L}$  and  $1.25 \text{ mL/L}$  of trace element solution according to van de Graaf *et al.* (2015). From day 12 on the feeding was supplemented with  $50 \text{ mg/L}$  of orange azo dye. The operation of the CSTR was divided into four stages according to Table 1.

**Table 1.** Operational stages for CSTR partial nitrification and SBR anammox.

	CSTR: partial nitrification				SBR: anammox	
	Start-up	Stage I	Stage II	Stage III	Stage I	Stage II
Operational days	0-11	12-27	28-32	33-60	0-21	22-75
HRT (d)	$1.5\pm 0.1$	$1.2\pm 0.1$	$0.8\pm 0.0$	$1.0\pm 0.1$	$1.6\pm 0.1$	$1.6\pm 0.1$
$\text{NH}_4^+$ (mg N/L)	380	500	500	500	440	200-520
$\text{NO}_2^-$ (mg N/L)	-	-	-	-	440	200-520
$\text{NH}_4^+/\text{HCO}_3^-$ (mg N/mg C)	0.91	1.20	1.20	1.20	-	-
Orange azo dye (mg/L)	0	50	50	50	50	0
pH	$7.5\pm 0.7$	$6.1\pm 0.1$	$7.4\pm 0.4$	$6.8\pm 0.1$	$8.2\pm 0.3$	$8.1\pm 0.2$

A sequencing batch reactor (SBR) with a working volume of 5 L was used for the anammox process. The SBR was operated in cycles of 6 h comprising the following stages: 315 min of mixing and continuous feeding, 15 min of mixing, 15 min of sedimentation and 15 min of effluent withdrawal. A set of two peristaltic pumps were used for the feeding and withdrawal. A mechanical stirrer operated at a rotating speed of  $180\pm 20$  rpm maintained the complete mixture inside the reactor. A programmable logic controller (PLC, CPU 224 Siemens) controlled the actuation of the pumps, valves and stirrer. The temperature was maintained at  $30\pm 1$  °C with a thermal water jacket around the reactor. To avoid the solubilisation of oxygen, argon gas was continuously flushed

in the headspace of the reactor. The SBR was inoculated with biomass from another laboratory anammox reactor (Fernandez *et al.* 2012). The initial biomass concentration was 4.7 g VSS/L and with a specific anammox activity (SAA) of 0.72 g N/(g VSS·d). The feeding consisted of a synthetic solution according to Dapena-Mora *et al.* (2004): 0.75-2.00 g NH<sub>4</sub>Cl/L, 1.00-2.55 g NaNO<sub>2</sub>/L, 0.8 g KHCO<sub>3</sub>/L, 0.05 g NaH<sub>2</sub>PO<sub>4</sub>/L, 0.3 g CaCl<sub>2</sub>·2H<sub>2</sub>O/L, 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O/L, 0.006 g FeSO<sub>4</sub>/L, 0.006 g EDTA/L and 1.25 mL/L of trace element solution according to van de Graaf *et al.* (2015). The feeding was supplied with 50 mg/L of orange azo dye during the first 21 operational days (Table 1).

*Analytical methods:* The concentrations of ammonium, nitrite, nitrate, biomass as volatile suspended solids (VSS) and total suspended solids (TSS) were determined according to the Standard Methods (APHA-AWWA-WPCF 2005). Inorganic carbon concentration was measured with a Shimadzu analyzer (ASI-5000-S). Orange azo dye concentration was colorimetrically determined using a spectrophotometer (Shimadzu UV-1603, UV-Visible) at 480 nm. The pH measurements were performed with an electrode (Crison Instruments, S.A., 52-03) equipped with an automatic compensatory temperature device (Crison Instruments, S.A., 21-910-01). A dissolved oxygen probe (AQUALITYC, model OXI-921) connected to a meter (M-Design Instruments TM-3659) was used to control DO concentration in the CSTR. Respirometric tests were performed using a Bench-model Oxygen Meter (YSI 5300) with oxygen-selective probes (YSI 5331) for the determination of AOB and nitrite oxidizing bacteria (NOB) activities according to López-Fiuza *et al.* (2002). Batch assays were used to estimate the SAA according to with the procedure described by Dapena-Mora *et al.* (2007).

*Calculation of absorption/desorption:* The dye absorption and desorption (mg<sub>orange</sub>/g VSS) were calculated according to with the procedure described by Feroso *et al.* (2010). The theoretical concentration of dye in the effluent of the reactor ( $C$ ) with the time ( $t$ ) assuming no reaction was determined considering the following equation:

$$C = C_0 \cdot \exp(-t / HRT)$$

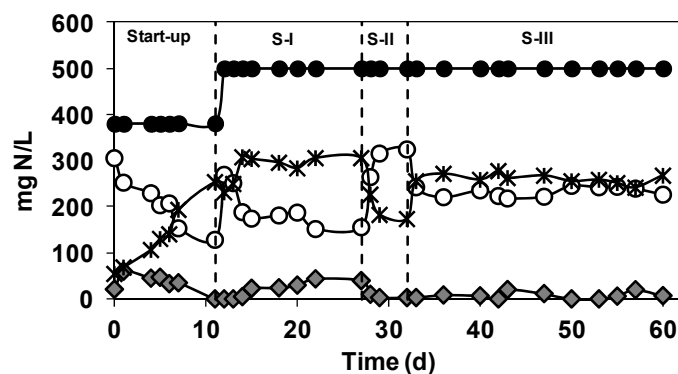
Where  $C_0$  is the dye concentration in the feeding,  $t$  is the operational time since the beginning of the experiment and  $HRT$  is the hydraulic retention time of the reactor. The theoretical concentration obtained was compared with the experimental (concentration measured in the effluent) with the time. The area under both curves as mg<sub>orange</sub>/(L·d) was then divided by the concentration of solids in this operational time (g VSS/L) to obtain the quantity of dye absorbed or desorbed.

## Results and discussion

### *Effect of orange azo dye over the partial nitrification process*

The operation of the CSTR lasted 60 days and was divided into four stages. The first one corresponded with a start-up stage (0-11 days) to promote the partial nitrification process development. For this purpose, the ammonium to bicarbonate (NH<sub>4</sub><sup>+</sup>/HCO<sub>3</sub><sup>-</sup>) ratio was set at 0.91 g N/g C and the temperature was of 35 °C. The ammonium concentration in the feeding was of 380 mg NH<sub>4</sub><sup>+</sup>-N/L and the HRT of 1.5 days, which corresponded with an applied nitrogen load rated (NLR) of 0.25 g N/L·d. In this stage the nitrite concentration in the effluent increased progressively from 54 (day 1) to 253 mg NO<sub>2</sub><sup>-</sup>-N/L (day 11) and the nitrate concentration almost depleted (Figure 1). After 11 operational days, the achieved ammonium oxidation to nitrite was 66%, without nitrite oxidation to nitrate, which indicates the absence of NOB activity, despite that the DO concentration was maintained over 6 mg O<sub>2</sub>/L.

Only between days 15 and 28 a slight increase of nitrate concentration in the effluent was observed due to NOB proliferation, associated with the long sludge retention times of the biomass grown as a biofilm on the reactor walls. Then regular cleaning of the walls (every 2-3 days) was performed in order to avoid this problem, and no more nitrate accumulation inside the reactor was observed. Furthermore, the NOB activity checked regularly by batch ex-situ respirometric assays, was very low (around  $0.014 \pm 0.002$  g N/g VSS·d).



**Figure 1.** Profile of nitrogen compounds in the CSTR:  $\text{NH}_4^+$  in the influent (●),  $\text{NH}_4^+$  in the effluent (○),  $\text{NO}_2^-$  in the effluent (\*) and  $\text{NO}_3^-$  in the effluent (◆).

In order to restrict the ammonium oxidation to a 50% desirable to obtain a nitrite to ammonium ( $\text{NO}_2^-/\text{NH}_4^+$ ) ratio of 1 g N/g N the ammonium concentration in the influent was increased to 500 mg  $\text{NH}_4^+$ -N/L from Stage I on. Furthermore, the HRT was decreased in Stage I to 1.2 days, which corresponded with an increase of the applied NLR to 0.42 g N/L·d. The first three days after these changes the  $\text{NO}_2^-/\text{NH}_4^+$  ratio was close to 1 g N/g N, but then it was stabilized around 1.6 g N/g N. For this reason in Stage II the HRT was decreased to 0.8 days. Consequently, the ammonia concentration in the effluent increased and the nitrite concentration decreased, obtaining an  $\text{NO}_2^-/\text{NH}_4^+$  ratio around 0.5 g N/g N. The decrease of the HRT in Stage II provoked a washout of biomass, as the solids concentration inside the reactor decreased from 0.110 g VSS/L (day 28) to 0.075 g VSS/L (day 33). Finally, in Stage III, the HRT was increased to 1.0 day to improve the retention of the AOB and an adequate  $\text{NO}_2^-/\text{NH}_4^+$  ratio ( $1.12 \pm 0.05$ ) was obtained in the effluent for a subsequent anammox process.

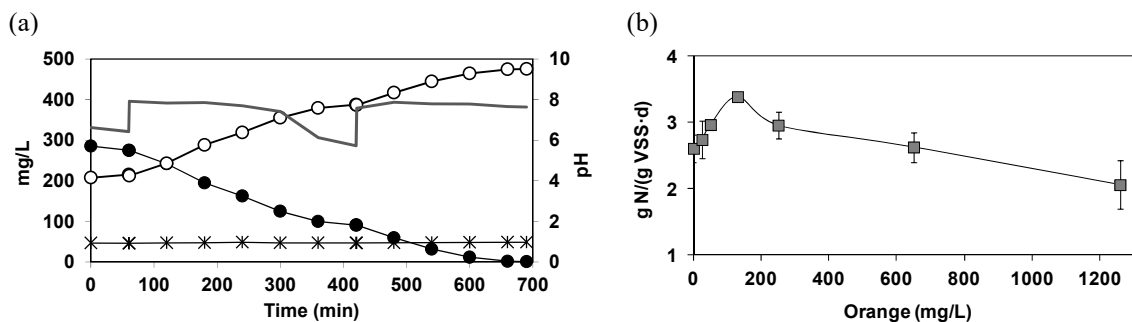
Orange azo dye was supplied to the feeding in Stage I and it took three days to measure the concentration in the effluent similar to that present in the influent. The difference between both was around  $2.1 \pm 0.9$  %, probably due to the adsorption of some amount of dye by the biomass that continuously growth. This adsorption was calculated to be  $9.7$  mg<sub>orange</sub>/g VSS for the dye concentration fed ( $50$  mg<sub>orange</sub>/L). Orange concentration in the effluent was also maintained the same as in the influent irrespective of HRT changes, during the rest of the experiment, indicating the absence of biological decolourization or significant removal by adsorption by the AOB. The absence of biological orange azo dye removal by the AOB is in agreement with the results obtained by Li & Bishop (2004) with nitrifying aerobic biofilm. These authors observed that the small orange dye concentration removed corresponded to the adsorption by the biofilm matrix, which was for a concentration of dye of  $50$  mg<sub>orange</sub>/L around  $12$  mg<sub>orange</sub>/g VSS, similar to the value obtained in this work.

To check the absence of biological decolourization by AOB a batch in-situ activity test was performed at the end of the operational period (day 61) inside the reactor (Figure 2.a). The influent and effluent pumps were switched off and the initial

concentrations of nitrogen compounds were the corresponding to the reactor operation in that moment (285 mg  $\text{NH}_4^+\text{-N/L}$  and 210 mg  $\text{NO}_2^-\text{-N/L}$ ). The pH was low (6.62) at the initial moment and after one hour it decreased to 6.42 due to the bicarbonate consumption by the AOB activity, with an ammonium oxidation rate of 0.25 g N/L·d. To maximize the AOB activity inside the reactor bicarbonate was added (650 mg  $\text{HCO}_3^-/\text{L}$ ) in a concentration enough to increase the pH to 7.92. Due to this bicarbonate addition, the ammonium oxidizing rate increased (Figure 2.b) to 0.91 g N/L·d and the pH slowly started to decrease (0.12 pH/hour) until minute 300. Then the pH strongly decreased (0.84 pH/hour), which indicated that bicarbonate concentration was depleted and, consequently, the ammonium oxidation rate was low. Again bicarbonate was added (390 mg  $\text{HCO}_3^-/\text{L}$ ) on minute 420 to increase the ammonium oxidation rate to 0.71 g N/L·d. Through all batch experimental time the orange concentration was monitored and it was maintained at 50  $\text{mg}_{\text{orange}}/\text{L}$  without changes (Figure 2.a), despite the promotion of a high ammonium oxidation rate. This experiment showed that AOB and/or the intermediates formed during the ammonium oxidation have not the ability to decolorize the orange azo dye.

For the 49 operational days with continuous orange azo dye addition (50 mg/L) no significant effect of this compound on the AOB activity was detected inside the reactor, which was maintained around 3.10 g  $\text{NH}_4^+\text{-N}/(\text{g VSS}\cdot\text{d})$ . Furthermore, the biomass concentration in the CSTR increased during the whole operational period from 0.043 g VSS/L (day 0) to 0.112 g VSS/L (day 60), except in Stage II due to the low HRT. This indicated that at long term operation the orange azo dye at a concentration of 50 mg/L has not a negative effect on AOB activity. This observation was corroborated by batch ex-situ activity tests.

In these tests, the biomass collected on day 61 from the CSTR (adapted to an orange concentration of 50 mg/L) was submitted to different orange azo dye concentrations. The results obtained (Figure 2.b) indicated a stimulation effect over AOB activity with concentrations of orange azo dye between 0 and 650 mg/L, the maximal activity being 3.40 g  $\text{NH}_4^+\text{-N}/(\text{g VSS}\cdot\text{d})$  at 130 mg/L of orange azo dye. He & Bishop (1994) reported that the orange azo dye inhibits all stages of the nitrification process, although they observed that NOB were more sensitive than AOB. Li & Bishop (2002) observed that biofilm nitrifying activity was inhibited by orange azo dye at the tested concentrations from 1 to 25 mg/L. Comparing the results of these authors with the ones obtained in this work can be concluded that AOB can be adapted to treat the orange azo dye without loss of activity.

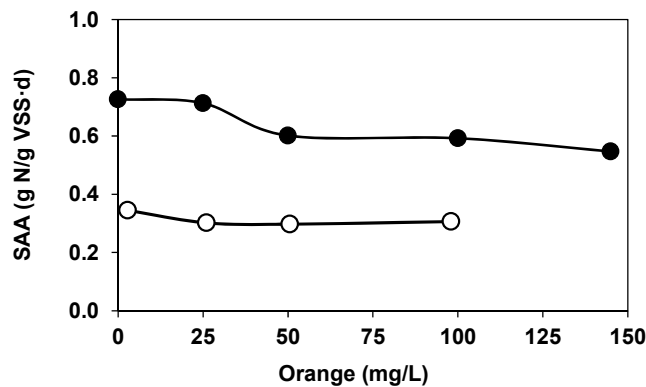


**Figure 2.** (a) Batch experiment inside the reactor on day 61: ammonium (●), nitrite (○) and orange dye (\*) concentrations and value of pH (—); (b) Batch ex-situ activity tests for CSTR biomass on day 61 at different orange dye concentrations.

### Effect of orange azo dye over anammox process

A set of SAA tests were performed previously to the anammox SBR operation to check the effect of different orange azo dye concentrations over the anammox activity. The results obtained (Figure 3) indicated that for concentrations lower 25 mg/L no inhibition occurred, while at 50 mg/L inhibition of around 17% took place, with no significant differences at higher orange azo dye concentrations. These tests served to predict the loss of activity in the anammox SBR operation with orange azo dye addition and to design an operational strategy to avoid the failure of the process.

The anammox SBR was operated during 75 days and the orange azo dye was supplied during the first 21 days at a concentration of 50 mg/L (Stage I). The nitrogen removal efficiency was 70-80% during the overall operational period (Figure 4.a) in spite of the strong decrease of the SAA due to the presence of the orange azo dye. This was attributed to the fact that the applied specific nitrogen loading rate (SNLR) was lower than the maximal specific capacity of the system, corresponding to the value of the SAA checked by batch tests (Figure 4.b). Therefore, the extra anammox capacity was able to cope with the inhibitory effect of orange azo dye and to maintain the nitrogen removal efficiency. This operational strategy was followed during the whole operation.



**Figure 3.** Maximal specific anammox activity at different orange azo dye concentrations for the biomass at: day 0 (●) and day 14 (○) determined by ex-situ activity tests.

The concentration of orange in the effluent was lower than in the influent the first 10 days of operation (Figure 4.a), probably due to absorption process. The calculated absorption was 6.4 mg<sub>orange</sub>/g VSS. After four days of orange addition, a decrease of 33% in the maximal anammox activity was observed (from 0.72 to 0.49 g N/(g VSS·d)). At the same time, nitrite concentration slightly increased from not detectable value to 2.6±0.3 mg NO<sub>2</sub><sup>-</sup>-N/L. After 12 days anammox activity decreased around 58% (0.30 g N/(g VSS·d)). Since nitrite concentration increased up to around 80 mg NO<sub>2</sub><sup>-</sup>-N/L on day 20, to avoid an inhibitory effect on anammox bacteria the inlet nitrogen concentration was decreased. Simultaneously the orange azo dye addition was stopped (Stage II) to promote the recovery of the process. When no nitrite concentration was measured in the effluent (day 31), the inlet nitrogen concentrations of ammonium and nitrite were increased to the initial values.

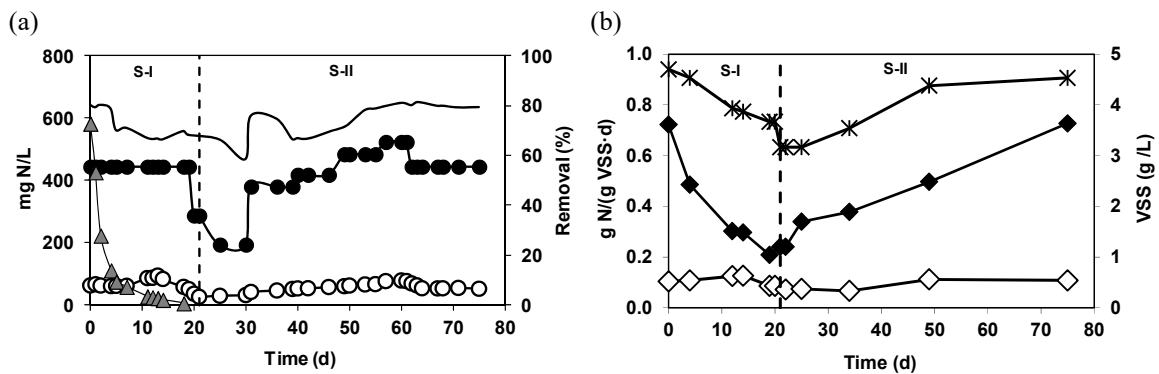
The results showed that a recovery of 47%, 69% and 100% of initial activity was achieved after 4, 28 and 54 days to stop the orange azo dye addition, respectively. Desorption process took place during 9 days after the dye removal from the feeding and was calculated as 27.2 mg<sub>orange</sub>/g VSS. This means that the anammox process after exposition to orange azo dye can be recovered in a reasonable period of time. Although

there are no other studies about the effect and recovery of anammox bacteria to orange azo dye, the studies with another type of toxic compounds indicates long recovery periods of anammox bacteria activity. For example Zhang et al. (2015) proposed a novel strategy to accelerate the recovery of anammox activity after to Cu(II) exposition based on the use of a pre-treatment with EDTA washing combined with ultrasonic irradiation. With this strategy, they lasted 64 days to recover the complete anammox activity inside the reactor.

In Stage I slowly decrease of biomass was observed from 4.70 to 3.93 g VSS/L, due to the washout of biomass in the effluent, with an average solids concentration of  $27.7 \pm 7.0$  mg VSS/L, and the biomass used in the SAA experiments. This loss of biomass was not compensated by the growth due to the inhibitory effect of the orange azo dye addition. In Stage II, without of dye addition, the biomass concentration increased progressively due to the recovery of the anammox activity (Figure 4.b).

To check the inhibitory effect of the orange azo dye over anammox bacteria the SAA of adapted biomass to the orange azo dye (day 14) was measured at different orange dye concentrations (from 0 to 150 mg/L) and compared to the values obtained for non-adapted biomass (day 0). The results showed that the SAA was lower than that of the non-adapted biomass for all the tested dye concentrations (Figure 3). Furthermore, the values of SAA for adapted biomass remained practically constant independently of the orange azo dye concentrations. No stimulating effect was observed contrary that occurred with the AOB. Although the profiles obtained in Figure 3 indicated not an adaptation of anammox bacteria to the orange azo dye, the activity not decreased with the increase of the dye concentration for adapted biomass.

Mechanism of gradually anammox inhibition is not known but the strong anammox activity decrease could be correlated with the dye adsorption. Adsorption could be responsible for inhibition effect.



**Figure 4.** Profiles of different parameters in the anammox SBR: (a) Concentrations (mg N/L) of  $\text{NH}_4^+$  and  $\text{NO}_2^-$  in the influent (●) and  $\text{NO}_3^-$  in the effluent (○); percentage of orange azo dye removal (▲) and nitrogen removal percentage (—). (b) Maximal SAA (◆); applied SNLR (◇) and solids concentration inside the reactor (\*).

## Conclusions

- Biological decolourization of orange azo dye was not possible by AOB or anammox bacteria.
- The orange azo dye was absorbed onto both microbial populations at a similar rate:  $9.7 \text{ mg}_{\text{orange}}/\text{g VSS}$  for AOB and  $6.4 \text{ mg}_{\text{orange}}/\text{g VSS}$  for anammox bacteria.

- A concentration of 50 mg/L of dye provoked a stimulatory effect over AOB (15%) and an inhibitory effect on anammox bacteria: low at short term (15-20%) but important for long term operation (58% in 12 days).
- Despite the inhibition of anammox bacteria by the orange azo dye, the recovery of their activity was possible after to stop the dye addition.

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