Inhibition of the anammox activity by aromatic compounds

Carlos Ramos, Isaac Fernández, María Eugenia Suárez-Ojeda*, Julián Carrera

GENOCOV research group. Department of Chemical Engineering, School of Engineering, Edifici Q, Universitat Autònoma de Barcelona, , 08193 Bellaterra, Barcelona, Spain

*Corresponding author: María Eugenia Suárez-Ojeda. mariaeugenia.suarez@uab.cat. Department of Chemical Engineering, School of Engineering, Edifici Q, Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain.

Abstract

The short-term effect of several aromatic compounds (*o*-cresol, *p*-nitrophenol, *o*-chlorophenol and quinoline) was evaluated over granular anammox sludge cultivated over 2 years in a Sequencing Batch Reactor (SBR). The anammox granular sludge had an average size of 1.0 ± 0.2 mm and was enriched in *Brocadia* sp. Specific Anammox Activity (SAA) batch tests with this granular biomass were carried out in the presence of *o*-cresol, *p*-nitrophenol, *o*-chlorophenol, quinoline and their mixtures. The anammox biomass was never exposed to the tested aromatic compounds, prior to the SAA tests. The concentration and the mixture of aromatic compounds had a strong effect over the loss of the anammox activity. The higher the concentrations of the aromatic compounds, the higher the reduction of the SAA. Quinoline and *p*-nitrophenol have a lower negative effect compared to *o*-cresol and *o*-chlorophenol. The Luong inhibition model seems to adjust better the inhibition of anammox biomass by the tested aromatic compounds. Depending on the aromatic compound, toxic or inhibitory effect was measured. *o*-Cresol and *o*-chlorophenol caused a toxic effect whereas *p*-nitrophenol and quinoline produced an inhibitory effect. In general, synergistic effects were observed when mixtures of aromatic compounds were studied.

Keywords: Inhibition, Anammox, phenols, quinoline.

1. Introduction

Complex industrial wastewaters composed by a mixture of high-strength ammonium and toxic/recalcitrant organic compounds are produced by industries like coking, petrochemical, waste management, steel manufacturing and coal gasification factories [1-8]. The main recalcitrant/toxic organic compounds present in these wastewaters are aromatic hydrocarbons, which include phenolic compounds, poly-aromatic hydrocarbons and mono- and poly-cyclic nitrogen-containing aromatics. Phenolic compounds, such as phenol, nitrophenols, methylphenols (cresols) and chlorophenols are commonly found. Others important aromatic compounds frequently found are heterocyclic compounds, such as quinoline. These industrial wastewaters are often treated by physic-chemical processes. However, these technologies have serious drawbacks such as: (i) high costs (due high temperature and pressure conditions and chemical requirements (for acid/base conditions and chemical oxidizing agents) [2, 9-11]), (ii) do not allow complete degradation of the aromatic compounds and (iii) they can generate other hazardous by-products (secondary pollutants) [11, 12]. On the contrary, biological treatments can satisfactorily overcome the disadvantages of physic-chemical treatments but they are known to be sensitive to the aromatic compounds, mainly due to inhibitory effects [11]. Nevertheless, biological systems based on granular biomass can solve the problem associated with these inhibitory effects [13].

The application of a two-step granular process to treat high-strength ammonium and aromatic compounds wastewaters has been recently proposed [14-16]. In that configuration, the first aerobic granular reactor allows ammonium to be oxidized to nitrite by ammonia-oxidising bacteria and aromatic compounds to be totally biodegraded by specialized heterotrophic biomass [14-16]. In this sense, a suitable effluent for a subsequent anammox reactor can be achieved: (i) a nitrite/ammonium ratio close to 1.0 and (ii) no presence of organic matter [14, 15]. In this way, the anammox reactor would only receive aromatic compounds, if the aerobic granular reactor is affected by shock-loads or sequentially alternating pollutants events [14, 15].

The specific anammox activity (SAA) has been demonstrated to be a very useful tool to assess the behaviour of anammox biomass at short-term under different conditions, including exposition to potentially endogenous or exogenous inhibitory compounds such as ammonia, nitrite, nitrate, inorganic salts and organic carbon sources [17-22]. Therefore, SAA tests seem to be appropriated to get some light about the behaviour of the anammox process in the presence of aromatic compounds. Nowadays, few references are reported regarding the effect of aromatic compounds over the anammox bacteria being phenol [20, 23-27] and toluene [28] the only compounds that have been evaluated. Both aromatic

compounds produce inhibition of the anammox biomass but the activity can be recovered (reversible inhibition) [20, 23-28]. To the best of our knowledge, there are no references regarding the effect of other aromatic compounds over the anammox process. Therefore, this study aims to quantify, at short-term, the effect of several aromatic compounds: *o*-cresol, *p*-nitrophenol, *o*-chlorophenol and quinoline over a granular anammox biomass.

2. Materials and methods

2.1. Granular anammox biomass

A Sequencing Batch Reactor (SBR) of 10 L of effective volume was employed to enrich the anammox biomass during more than 2 years. The reactor has a diameter of 20 cm and a height of 61 cm. The temperature was controlled at 35 °C. pH of the SBR varied between 7.5-8.5 while the pH of the influent was between 6.5-7.0. Complete mixing was achieved using a mechanical stirrer with a rotating speed between 75 and 110 rpm. The reactor was operated in 6 h cycles distributed in four periods: mixed fill (300 min), mix (30 min), settle (15 min) and draw (15 min). The exchange volume was fixed at 25%, thus, the hydraulic retention time was 1 d. Low dissolved oxygen concentration was assured in the SBR by continuous addition of N_2 to the SBR headspace at a constant flow of 300 mL min^{-1}. Control of the pumps and different periods of the operational cycles was performed with a PLC system (Siemens LOGO! 230RC).

The SBR was fed with the following synthetic autotrophic medium [29] (in mg L^{-1}): 100 of $KHCO_3$, 50 of H_2PO_4, 100 of $CaCl_2x2H_2O$, 200 of $MgSO_4x7H_20$, 6.3 of EDTA and 1.25 mL L^{-1} of a trace elements solution. The ammonium to nitrite ratio in the feeding media was kept ca. 1.0 to operate in ammonium excess, avoiding nitrite accumulation in the reaction medium. The nitrogen loading rate (NLR) applied was 0.5 g N L^{-1} d^{-1} with a total nitrogen removal efficiency about 90%. The biomass concentration was 1.2 g VSS L^{-1}. The sludge volumetric index at 5 min (SVI_5) remained stable around 30 mL g^{-1} VSS, with a SVI ratio at 5 and 30 min (SVI_5/SVI_{30}) close to one during the whole operational period. The average particle size was also stable at 1.0 ± 0.2 mm.

2.2. Specific Anammox Activity (SAA) tests

The specific anammox activity has been demonstrated to be a very useful tool to assess the behaviour of anammox biomass at short-term under different conditions, including exposition to potentially endogenous or exogenous inhibitory compounds [17]. Therefore SAA tests seem appropriated to get

some light about the behaviour of the anammox process in the presence of aromatic compounds. Batch experiments were employed to determine the SAA and to study the short-term effects of the chosen monosubstituted phenols (*o*-cresol, *p*-nitrophenol and *o*-chlorophenol) and a heterocyclic compound (quinoline). The applied methodology for the SAA determination was according to Dapena-Mora et al. [17], based on the measurement along time of the overpressure generated by the nitrogen gas produced by the anammox culture in closed bottles.

Completely closed bottles with a total volume of 60 mL (50 mL of reaction volume) were used to conduct the specific anammox activities. The bottles were inoculated with granular anammox biomass from the SBR described in section 2.1. Previous to each test, the biomass was washed and re-suspended in phosphate buffer (0.14 g L^{-1} KH_2PO_4 and 0.75 g L^{-1} K_2HPO_4). The initial pH and biomass concentration in each vial were fixed at 7.8 and 1.0 g L^{-1}, respectively. Bottles were sealed tightly with butyl rubber caps. The headspace of the vial was gasified and purged with nitrogen gas to remove the oxygen. The bottles were placed in a thermostatic shaker, at 150 rpm and 30 °C until stable conditions were reached. Finally, the substrates ($(NH_4)_2SO_4$ and $NaNO_2$ at 35 mg N L^{-1} each one) and eventually, the aromatic compounds were added. Pressure was equalized to the atmospheric one prior to start the measurements. The batch tests were based on the measurement of nitrogen gas production and were tracked by measuring the overpressure in the headspace with a certain time frequency, by means of a portable transducer (range 0-5 psi; Centrepoint electronics, Ireland). The length of the test was established by the nitrogen production rate of the control test (without the presence of the aromatic compounds), which happened not later than 5-6 h after the start of each test (see Figure 1). In each case, at least three bottles without any aromatic compound were used as control tests to assess the maximum specific activity of the biomass (SAA_{max}), while two other bottles were used for each tested concentration of aromatic compounds to measure its short-term effect over the anammox activity. The SAA presented in the results were denoted as relative SAA, referred to the SAA in the presence of the aromatic compounds over the SAA_{max} and the data are given in percentage (Equation (1)):

$$\text{relative SAA} = \frac{\text{SAA}_{\text{in the presence of aromatic compound(s)}}}{\text{SAA}_{\text{max}}} \cdot 100 \qquad \text{Equation (1)}$$

In a first set of experiments, single aromatic compounds were added to the flasks. The range of the concentrations tested was 5-25 mg L^{-1} for *o*-cresol, *p*-nitrophenol and quinoline and 5-9 mg L^{-1} for *o*-chlorophenol. These concentrations ranges were tested taken into consideration two facts: i) low

concentrations can enter to an anammox reactor if a two-step granular process is applied (as is indicated in the introduction) and ii) the effects obtained in the current study (see section 3 of Results and discussion). Then, a second set of experiments was carried out to evaluate the effects of mixture of aromatic compounds (as shown Table 1). The selection of the mixtures was done according to the behaviour obtained with the single aromatic compounds in the first set of experiments. Finally, a third set of experiments was performed with a slightly different methodology. In this case, after about 6 h of SAA test in presence of a single or a mixture of aromatic compounds, the bottles were opened and the biomass was carefully washed with the phosphate buffer. Subsequently, the bottles were closed again and flushed with nitrogen gas and a new SAA test was performed with the same biomass but without the addition of aromatic compound(s). Consequently, the comparison of both SAA tests should provide information on whether the effect of the aromatic compounds is inhibitory or toxic, depending on the recovery of the SAA. The tested conditions are present in Table 2.

2.3. Inhibition kinetic modelling

Inhibition kinetic models can be considered to describe the inhibition of anammox activity by aromatic compounds, where the inhibiting compound is not a substrate. Non-competitive, extended non-competitive, Aiba and Luong inhibition kinetic model has been proposed to autotrophic biological nitrogen removal processes [30, 31]:

Non-competitive inhibition kinetic model:

$$\text{relative SAA} = \frac{K_I}{I+K_I} \qquad \text{Equation (2)}$$

Extended non-competitive inhibition kinetic model:

$$\text{relative SAA} = \frac{K_I}{I^m+K_I} \qquad \text{Equation (3)}$$

Aiba inhibition kinetic model:

$$\text{relative SAA} = \exp(-K_{IA} \cdot I) \qquad \text{Equation (4)}$$

Luong inhibition kinetic model:

$$\text{relative SAA} = \left(1 - \left(\frac{I}{K_{IL}}\right)^{n}\right) \qquad \text{Equation (5)}$$

Where, K_I is the non-competitive inhibition constant, K_{IA} is the Aiba inhibition constant, K_{IL} is the Luong inhibition constant, m is the extended non-competitive constant, n is the Luong constant and I is the inhibitor concentration.

2.4. Analytical methods

Total and volatile suspended solids (TSS and VSS), as well as, Sludge Volume Index after 5 min and 30 min of settling (SVI_5 and SVI_{30}) were determined according to Standard Methods [32]. Particle sizes were measured with a Malvern Mastersizer 2000 particle size analyser.

2.5. Molecular biology

Fluorescence In-Situ Hybridization (FISH) identification with confocal laser scanning microscopy (CLSM) was performed according to the methodology described by Suárez-Ojeda et al. [33]. The specific probes employed in this study were targeting all anammox microorganisms (AMX368: CCTTTCGGGCATTGCGAA) [34] and the species *Candidatus Brocadia anammoxidans* (BAN162: CGGTAGCCCCAATTGCTT) and *Candidatus Kuenenia stuttgartiensis* (KST157: GTTCCGATTGCTCGAAAC) [35].

Pyrosequencing was used to analyse the microbial community composition of the granular biomass used in this study. Genomic DNA was extracted and purified using a PowerBiofilm™ DNA Isolation Kit (MoBio Laboratories, USA); in accordance with the manufacturer's instructions. Purified community DNA samples were sent to the Research and Testing Laboratory (Lubbock, TX, USA) for Bacterial Tag-Encoded FLX-Titanium Amplicon Pyrosequencing (bTEFAP) in a Roche 454 GS-FLX+ and data processing. The forward primer selected for bacteria diversity assay was the 530F (GTGCCAGCMGCNGCGG) and the reverse primer was the 1100R (GGGTTNCGNTCGTTR). For anammox specific diversity assay, forward primer was the Amx368F (TTCGCAATGCCCGAAAGG) and the reverse primer the Amx820R (AAAACCCCTCTACTTAGTGCCC). Detailed information can be found in Isanta et al. [36].

3. Results and discussion

3.1 Bacterial community diversity in the anammox granules used in the SAA tests

Before doing the SAA tests, the presence and type of anammox bacteria was assessed using pyrosequencing and FISH-CLSM. Pyrosequencing as a second-generation sequencing technology provide comprehensive information about microbial communities since it can detect microorganisms at low abundance [25]. Pyrosequencing results using the general primers showed that Plantomycetia was the most abundant class (46 % of the total reads), followed by β-proteobacteria (9 % of the total reads) whereas 39 % of the total reads were unclassified bacteria. The coexistence of anammox and heterotrophic denitrifiers in granular/biofilm reactors has been previously described [37]. Planctomycetia class contains all known anammox bacteria, while β-proteobacteria class contains several groups of nitrifying, denitrifying and other N-cycle related microorganisms. Therefore, these results indicated that the granular biomass was enriched in anammox bacteria. At genus level, using the anammox specific primers, *Brocadia* predominated (99 % of the reads) over Candidatus *Kuenenia* (1 % of the reads). FISH results also confirm enrichment of anammox bacteria in the granular biomass, because 72 ± 9% of the active bacteria present in the granules were anammox as shown by positive hybridization with AMX368 probe (see Figure S1 in Supporting Information) and were arranged as the typical anammox compact cauliflower-shaped colony [34]. The most part of the microorganisms were also giving positives when the probe BAN162 (Candidatus *Brocadia anammoxidans*) was used, while no positives were obtained with the probe KST157 (Candidatus *Kuenenia stuttgartiensis*).

3.2. Specific Anammox Activity tests in the presence of single aromatic compounds

The anammox granular biomass was used to perform a series of SAA tests with four selected aromatic compounds (*o*-cresol, *p*-nitrophenol, *o*-chlorophenol and quinoline) at different concentrations. Figure 1 shows an example of a typical curve of nitrogen gas production of the control test (without the present of the aromatic compounds) and with the presence of an aromatic compound, which produced a decrease in the nitrogen gas production compared to the control test.

The relative SAA achieved with the monosubstituted phenols (*o*-cresol, *p*-nitrophenol and *o*-chlorophenol) and the heterocyclic compound (quinoline) showed a decreasing tendency when the concentrations of aromatic compounds were increased (Figure 2). The effect caused by *p*-nitrophenol was relatively small (71 ± 1 % of relative SAA with 25 mg L^{-1}) compared to the others compounds, even at the highest concentration tested. A stronger negative effect was obtained with *o*-cresol (5 ± 1 % of relative SAA at 25 mg L^{-1}) than with *p*-nitrophenol at the same concentration. Quinoline had lower

negative effect than *o*-cresol, but higher than that of *p*-nitrophenol (54 ± 8 % of relative SAA with 25 mg L^{-1} of quinoline). *o*-Chlorophenol caused the loss of more than 60 % of the SAA_{max} at only 9 mg L^{-1} suggesting that the *o*-chlorophenol has the stronger negative effect of the aromatic compounds studied. Therefore, the negative effect of the studied aromatic compounds over the granular anammox biomass would depend on the specific compound and on the concentrations applied. The aromatic compounds tested can be ordered from lowest to highest negative effect as follow: *p*-nitrophenol, quinoline, *o*-cresol and *o*-chlorophenol. To better describe the achieved behaviour, several kinetic models were applied as described in the next section.

Two possible mechanisms can be related to the inhibition/toxicity of the monosubstituted phenol (*o*-cresol, *p*-nitrophenol and *o*-chlorophenol in this case): i) preservation of the cell membrane integrity and ii) diffusion. For the first postulate, the available data show that as a result of the lipophilic character of the aromatics tested, interactions with hydrophobic parts of the cell membrane might play an important role in the mechanism of the toxic action. The accumulation of lipophilic compounds into the lipid bilayer of the cytoplasmic membrane may cause toxicity problems [38], which are related to the structural and functional properties of this membrane. As a result of accumulated lipophilic molecules, the membrane loses its integrity, and an increase in permeability of protons and ions can be observed [38]. The lipophilic character of a compound is equivalent to its hydrophobic character [39], which is normally represented by the logarithm of the octanol/water partition coefficient (pK_{OW}) [40]. So that, the higher the hydrophobicity of a particular monosubstituted phenol, the higher its toxic effect on bacteria is [41]. From the SAA results, the studied monosubstituted phenols can be ordered from lowest to highest negative effect over anammox bacteria as follow: *p*-nitrophenol, *o*-cresol and *o*-chlorophenol. While, the hydrophobicity of these compounds can be ordered from lowest to highest as follow: *p*-nitrophenol (pK_{OW}=1.91), *o*-cresol (pK_{OW}=1.95) and *o*-chlorophenol (pK_{OW}=2.15). Therefore, the loss of the membrane integrity by the accumulation of hydrophobic compounds can be postulated as the mechanism explaining the increasing toxicity of *p*-nitrophenol, *o*-cresol and *o*-chlorophenol for the anammox biomass.

The second postulate is related to the diffusion of the monosubstituted phenols from outside the cell to the bacterial cytoplasm. It is well established that the toxicity of several phenolic compounds to different organisms is dependent on pH, like: *Poecilia reticulata* [42], *Daphnia magna* [43], *Tetrahymena pyriformis* [40], mixed bacterial cultures degrading phenolic compounds [44], etc. In general, these toxicity studies concluded that different diffusion rates through the cell membrane of the ionised and nonionised forms of the phenolic compounds resulted in different contributions to toxicity.

The diffusion of the nonionised forms from the medium to the cell was generally faster than the ionised forms and consequently, the nonionised forms were the most toxic compounds to the cells [45]. The ionized or nonionized form of these compounds is related to the logarithm of the acid dissociation constant (pKa) and pH of the media. The pKa of the monosubstituted phenol studied in this paper are: *p*-nitrophenol (pKa=7.15), *o*-cresol (pKa=10.29) and *o*-chlorophenol (pKa=8.56) [46]. The tests were performed at pH 7.8, therefore, *p*-nitrophenol was mainly in its ionised form, producing lower toxicity compared to *o*-cresol and *o*-chlorophenol, which were mainly in their nonionized forms. Thus, the toxicity/inhibition of the studied monosubstituted phenol over the anammox bacteria can also be related to the diffusion through the cell membrane of ionised and nonionised forms.

For the case of quinoline, both postulates do not fit properly, possible due the higher complexity of the molecule compared to the monosubstituted phenols. Therefore, further research is needed for this compound.

3.3. Inhibition kinetic modelling

Figure 3 shows the adjustment of the kinetic equations with the experimental relative SAA data achieved in the presence of single aromatic compounds. The inhibition kinetic coefficients, statistical value for Normality and Constant Variance (*P* values) and regression coefficient (R^2) are shown on Table 3. The *P* value is the probability of being wrong in concluding that the coefficient is not zero. The smaller the *P* value, the greater is the probability that the coefficient is not zero. Traditionally when $P \leq 0.05$, it can be concluded that the independent variable can be used to predict the dependent variable [47]. Consequently, extended non-competitive and non-competitive inhibition models for *o*-cresol and extended non-competitive and Luong models for quinoline should not be taken into consideration, because they are not statistically valid to describe the inhibition.

For *o*-cresol and *p*-nitrophenol, Luong inhibition model presented better adjustment than the other two models (highest R^2 value). Modelling the *o*-cresol data with extended non-competitive model presented very high R^2 value, however, *P* values for this model were over 0.05. For quinoline, the adjustment obtained with the three models was very similar. However, *P* values for Luong model were over 0.05. *o*-Chlorophenol was not adjusted due to the limited amount of data. *p*-Nitrophenol was fitted with extended non-competitive model, however the information obtained of the adjustment was very poor and therefore is not shown.

Therefore, based on the comparison of the regression coefficients and the *P* values, different inhibition kinetic models can be applied depending on the aromatic compound in contact with the anammox biomass ,. In consequence, Luong model can be used for the presence of *o*-cresol and *p*-nitrophenol and Aiba model for quinoline. The calibrated models obtained in the present study could have practical applications. For short-term exposure of the anammox biomass to aromatic compounds, the kinetic models could predict the decrease of the anammox activity as function of the concentration of the aromatic compound in the reactor and therefore, safety actions could be applied prior to totally losing the stability of the anammox reactor.

3.4. Specific Anammox Activity tests in the presence of mixtures of aromatic compounds

Several SAA tests were performed with different mixtures of selected aromatic compounds to assess their joint effects on the anammox activity (Table 1). Joint effects can generally be divided into four categories: antagonistic, synergistic, additive and irrelevant effects [20]. An antagonistic effect means that the joint effect is less than the sum of the individual effects of the inhibitory compounds. A synergistic effect indicates that the sum of the individual effects is less than the joint effect. An additive effect means that the joint effect is equal to the sum of the individual effects and finally, an irrelevant effect means that the joint effect is the same as the strongest individual effect.

First, mixtures of *o*-cresol with *o*-chlorophenol and *p*-nitrophenol with *o*-chlorophenol were tested at low concentrations (experiments I and II in Table 1), because the relative SAAs were close to 100 % when each compound was tested alone (Figure 2). However, the relative SAAs achieved with the mixtures (between 22 and 55 %, Table 1) were significantly lower than the relative SAAs of the individual compounds when were tested at 5 mg L^{-1} (Figure 2). This fact, clearly shows a synergistic effect over the anammox biomass when mixtures of aromatics were applied, similar results have been reported for oxytetracycline with sulphide and for phenol with sulphide [20].

Later, different mixtures with higher concentrations of quinoline, *p*-nitrophenol and *o*-cresol than the previous experiments were tested (experiments III-VI in Table 1). *o*-Chlorophenol was not tested due to the inhibition obtained when tested alone at 9 mg L^{-1}. When *o*-cresol, quinoline and *p*-nitrophenol were individually tested at 12 mg L^{-1}, the relative SAA were around 50, 70 and 90 %, respectively (Figure 2). If there was an additive effect with the mixture of these compounds, the expected relative SAA would be 20% for quinoline with *o*-cresol (experiment III), 40% for *p*-nitrophenol with *o*-cresol (experiment IV) and 60% for *p*-nitrophenol with quinoline (experiment V). However, the relative SAA

achieved with the mixtures of quinoline with *o*-cresol and *p*-nitrophenol with *o*-cresol were 2 ± 3 % and 13 ± 6 %, respectively (Table 1). On the other hand, the relative SAA achieved with the mixture of *p*-nitrophenol with quinoline was 89 ± 9 % (Table 1). In view of these results, the effect of quinoline with *o*-cresol and *p*-nitrophenol with *o*-cresol can be described as synergistic while the effect of *p*-nitrophenol with quinoline can be described as antagonistic. Finally, a mixture of three compounds (*p*-nitrophenol, *o*-cresol and quinoline) was tested (experiment VI) and the relative SAA achieved (10%, Table 1) also presented a synergistic effect, because the relative SAA that would be expected if an additive effect is considered is of 65%. These results reveal that the presence of *o*-cresol in the mixture of several aromatic compounds produce a synergic inhibitory effect over the anammox activity.

3.5. Inhibition versus toxicity caused by aromatic compounds on anammox biomass

Inhibition and toxicity are two different phenomena, but both depend on the exposure time and the concentration of the inhibitory or toxic compound [19]. Inhibition is defined as a reversible phenomenon and toxicity is stated as an irreversible process. Reversible inhibition refers to a reversible decrease of the catabolic activity during exposure, while toxicity (or irreversible inhibition) indicates damages to the microorganisms associated with an irreversible decrease of the microbial activity [19]. Therefore, it was investigated if the loss of SAA by the tested aromatic compounds was permanent (toxicity) or transitory (inhibition). SAA tests were carried out with two sequential additions of substrates to the same biomass, removing the aromatic compounds after the second substrate addition by washing the biomass. The second addition of substrates (only ammonium and nitrite without the aromatic compounds) was carried out as soon as the first part of the test was finished to avoid starvation effects. This strategy was successful since no decrease of the SAA_{max} was detected in the control tests.

Recovery of relative SAA was observed in the case of *p*-nitrophenol 10 and 25 mg L^{-1} (Table 2), where almost 100 % activity was recovered after the washing of the biomass. Consequently, *p*-nitrophenol can be considered as an inhibitory compound for anammox biomass at the tested conditions (concentrations up to 25 mg L^{-1} and 6 h of exposure time). However, quinoline, *o*-cresol and *o*-chlorophenol produced a certain loss in the SAA activity. For the cases of *o*-cresol at 10 mg L^{-1} and quinoline at 10 and 25 mg L^{-1}, these tests shows a partial loss of the relative SAA. However, for the cases of 10 mg L^{-1} of *o*-chlorophenol and 25 mg L^{-1} of *o*-cresol the anammox activity did not recover at all after the washing of the biomass. Thus, quinoline, *o*-chlorophenol and *o*-cresol can be classified as toxic compounds for

anammox biomass at the tested conditions (concentrations between 10-25 mg L^{-1} and 6 h of exposure time).

Finally, the toxic or inhibitory effect was tested for mixtures of two different aromatic compounds (Table 2). The mixture of *p*-nitrophenol and quinoline (12 mg L^{-1} each one) presented an inhibitory effect. Nevertheless, the mixtures of *o*-cresol and quinoline (12 mg L^{-1} each one) and *p*-nitrophenol and *o*-cresol (12 mg L^{-1} each one) presented a clear toxic effect. These results seem to indicate that depending of the mixture of aromatic compounds, toxicity or inhibition to anammox biomass can be obtained.

3.6. Future research and applications

The next step after this research should be to perform long-term experiments with an enriched anammox reactor receiving an influent with low concentrations (up to 25 mg L^{-1}) of one or two aromatic compounds, for example *p*-nitrophenol and quinoline. Taking into account previous results of the effect of aromatic compounds on anammox, two different effects can be expected: the loss of activity can be accumulative with time or the anammox biomass can be progressively adapted. The first behaviour was observed when operating an anammox reactor in presence of antibiotics [48], while the latter was reported for an anammox reactor receiving phenol [5]. Considering the results of toxicity obtained for *o*-chlorophenol and *o*-cresol, adaptation to these compounds might be unlikely. In any case, it is clear that these aromatic compounds are detrimental for the performance of the anammox process.

4. Conclusions

A granular anammox biomass enriched in *Brocadia* sp. was used to test the short-term effects of several aromatic compounds (*o*-cresol, *p*-nitrophenol, *o*-chlorophenol and quinoline) on the anammox activity. The concentration of aromatic compounds and their mixture had a strong detrimental effect over the anammox activity. The higher the concentrations of the aromatic compounds, the higher the reduction of the relative SAA. Quinoline and *p*-nitrophenol have lower negative effect than that *o*-cresol and *o*-chlorophenol. The Luong inhibition model adjusts better the inhibition of anammox biomass by the tested aromatic compounds than other kinetic models. The negative effect can be toxic or inhibitory depending on the aromatic compound. Toxic effect was described for *o*-cresol and *o*-

chlorophenol whereas inhibitory effect was described for *p*-nitrophenol and quinoline. In general, synergistic effects were observed when mixtures of the aromatic compounds were tested.

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FIGURE CAPTIONS

Figure 1. Example of the obtained nitrogen gas production curve of the control (without the presence of any aromatic compound) and with the presence of *o*-cresol.

Figure 2. Relative Specific Anammox Activities (SAA) of different aromatic compounds at several concentrations. Relative SAA is referred to the SAA in the presence of the aromatic compounds over the SAA of the control.

Figure 3. Fitting of the inhibition kinetic models for several aromatic compounds over granular anammox biomass.

Table 1

Table 1. Specific Anammox Activity (SAA) tests in the presence of mixtures of aromatic compounds. Relative SAA is referred to the SAA in the presence of the aromatic compounds over the SAA without the studied compounds (blank).

Experiment	Concentration (mg L^{-1})				Relative SAA (%)
	p-nitrophenol	*o*-cresol	*o*-chlorophenol	quinoline	
I	4	-	2	-	55 ± 12
II	-	4	2	-	22 ± 8
III	-	12	-	12	2 ± 3
IV	12	12	-	-	13 ± 6
V	12	-	-	12	89 ± 9
VI	8	8	-	8	10 ± 8

Table 2

Table 2. Specific Anammox Activity (SAA) recovery tests in the presence of single and mixed aromatic compounds at different concentrations before and after washing the biomass. Relative SAA is referred to the SAA in the presence of the aromatic compound over the SAA without the studied compound (control).

Concentration (mg L^{-1})				Relative SAA (%)	
p-nitrophenol	*o*-cresol	*o*-chlorophenol	quinoline	Before washing	After washing
-	10	-	-	69 ± 8	56 ± 1
10	-	-	-	95 ± 4	96 ± 1
-	-	10	-	41 ± 12	7 ± 1
-	-	-	10	86 ± 9	89 ± 1
-	25	-	-	5 ± 1	0 ± 0
25	-	-	-	71 ± 1	96 ± 2
-	-	-	25	54 ± 8	80 ± 2
-	12	-	12	2 ± 3	20 ± 6
12	12	-	-	13 ± 6	0 ± 0
12	-	-	12	89 ± 9	100 ± 1

Table 3

Table 3. Kinetic constants of the inhibition models for several aromatic compounds over granular anammox biomass. K_i: non-competitive inhibition constant, K_{iA}: Aiba inhibition constant, K_{iL}: Luong inhibition constant, I: inhibitor concentration, n: Luong constant, *P*: statistical value for normality and constant variance and R^2: adjusted regression coefficient.

Aromatic compound	Inhibition model	Constant	Units	All data		
				Value	*P*	R^2
o-cresol	Non-competitive	K_i	mg L^{-1}	15 ± 7	0.07	0.67
	Extended non-competitive	K_i	mg L^{-1}	27274 ± 24444	0.33	0.99
		m	dimensionless	4 ± 0.4	0.00	
	Aiba	K_{iA}	(mg L^{-1})$^{-1}$	0.05 ± 0.0	0.01	0.77
	Luong	K_{iL}	mg L^{-1}	25 ± 2	0.00	0.93
		n	dimensionless	1 ± 0	0.01	
p-nitrophenol	Non-competitive	K_i	mg L^{-1}	82 ± 17	0.01	0.81
	Aiba	K_{iA}	(mg L^{-1})$^{-1}$	0.01 ± 0.0	0.00	0.83
	Luong	K_{iL}	mg L^{-1}	54 ± 11	0.01	0.92
		n	dimensionless	2 ± 0	0.01	
quinoline	Non-competitive	K_i	mg L^{-1}	31 ± 6	0.01	0.86
	Extended non-competitive	K_i	mg L^{-1}	93 ± 120	0.33	0.86
		m	dimensionless	1 ± 0.5	0.05	
	Aiba	K_{iA}	(mg L^{-1})$^{-1}$	0.03 ± 0.0	0.00	0.88
	Luong	K_{iL}	mg L^{-1}	53 ± 22	0.09	0.83
		n	dimensionless	1 ± 0	0.07	

Figure 1

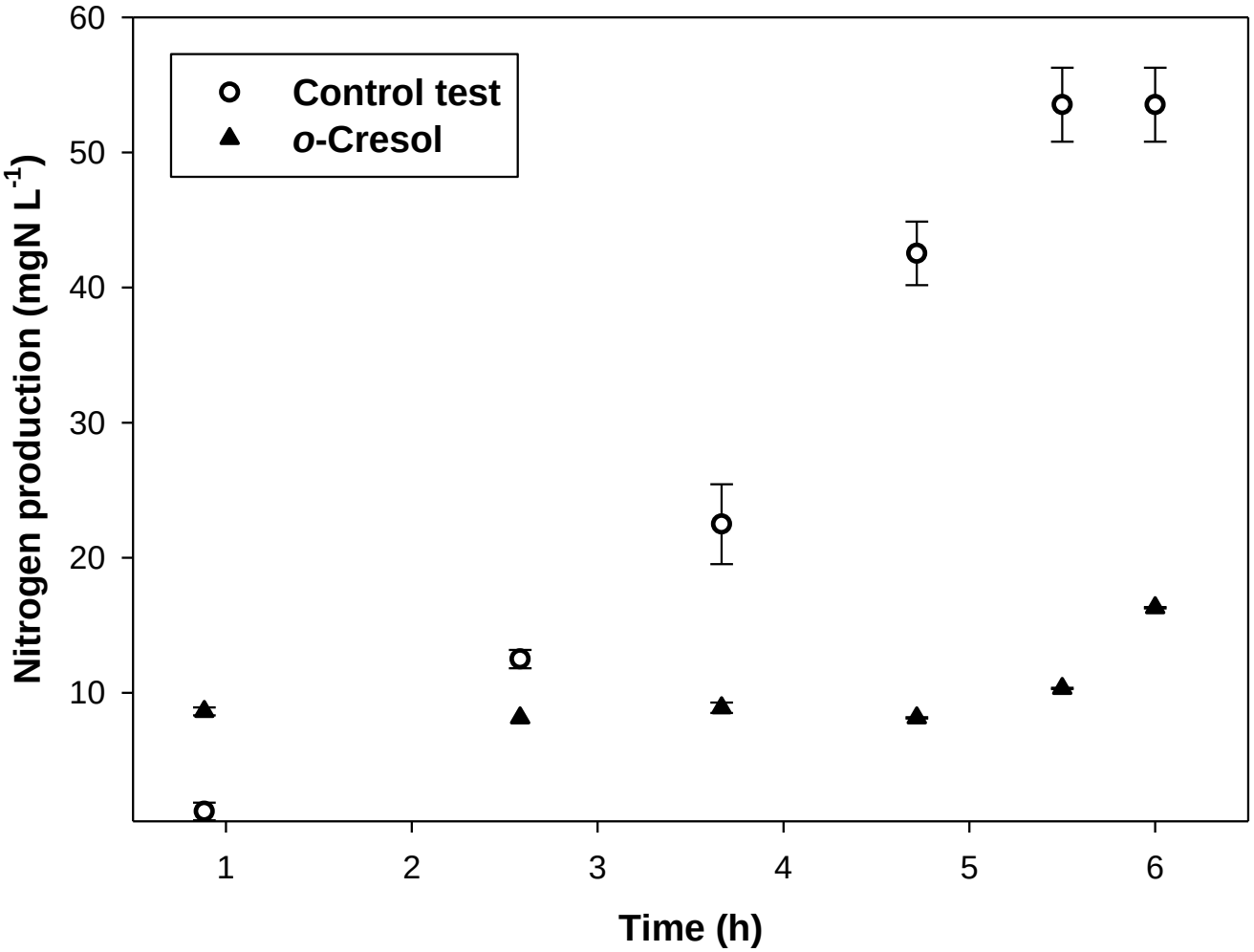

Figure 1.

Figure 2

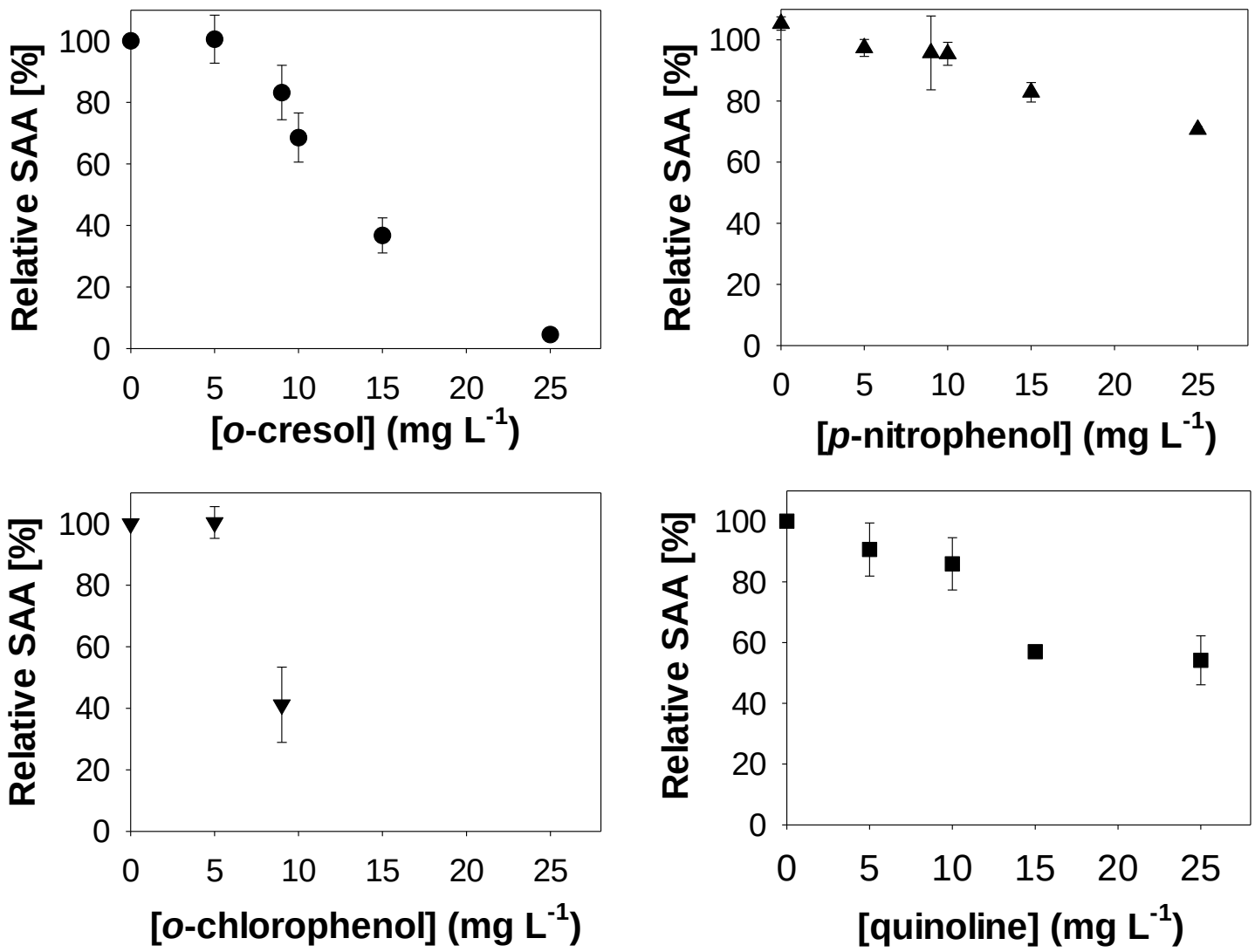

Figure 2.

Figure 3

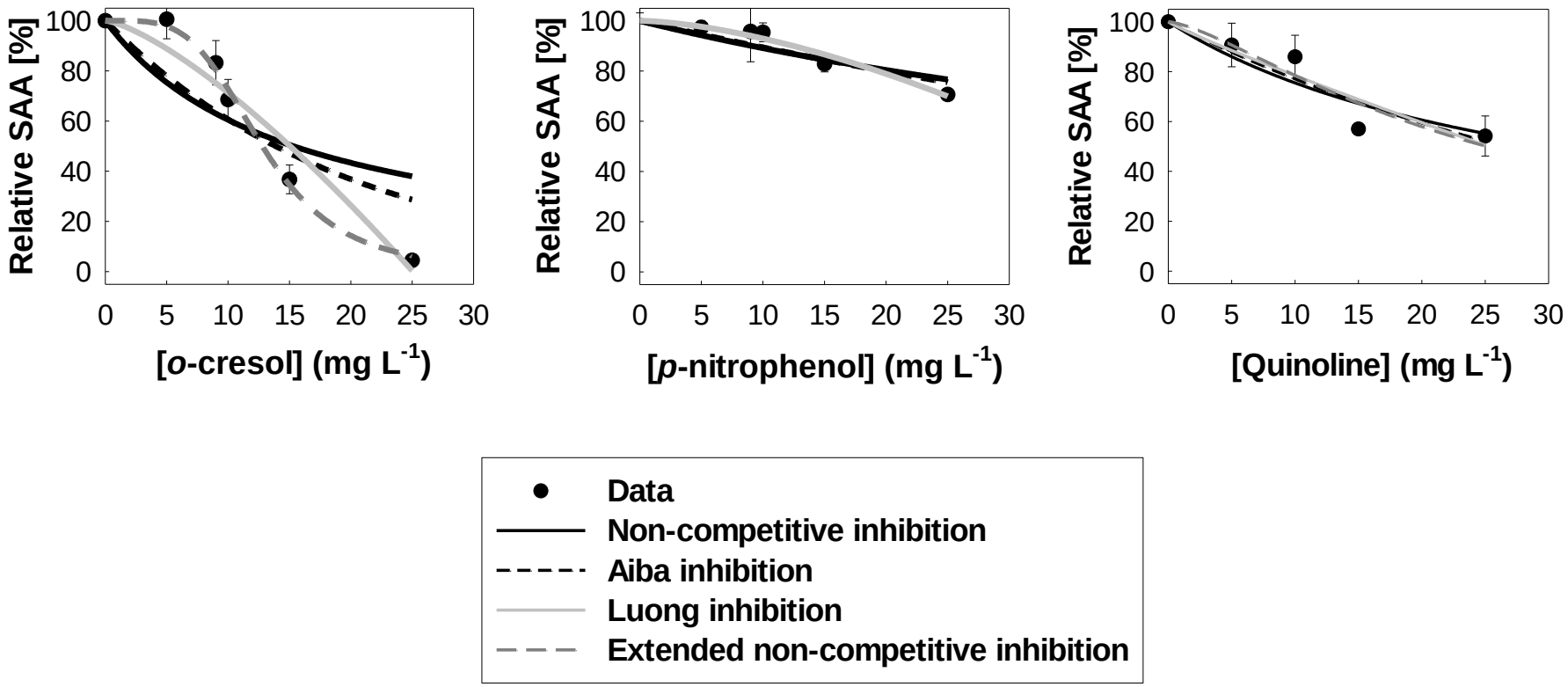

Figure 3

Supplementary Material

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