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BIOLOGICAL REMOVAL OF MIXTURES OF AMMONIUM, PHENOL, CRESOL ISOMERS, AND SULFIDE IN A SEQUENCING BATCH REACTOR

ELIMINACIÓN BIOLÓGICA DE MEZCLAS DE AMONIO, FENOL, ISÓMEROS DE CRESOL Y SULFURO EN UN REACTOR DE LOTE SECUENCIADO

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Abstract

The kinetic and metabolic ability of a nitrifying sludge to simultaneously oxidize ammonium and mixtures of phenol, *p*-cresol, *m*-cresol, *o*-cresol, and sulfide was evaluated throughout the operation cycles of a sequencing batch reactor (SBR). In the nitrifying SBR, it was possible to remove ammonium (150 mg N/L) by nitrification, mineralize the mixtures of phenolic compounds (up to 120 mg C/L), and oxidize sulfide (15 mg S/L) to sulfate. The addition of mixed phenolic compounds and sulfide into the reactor provoked a decrease in specific rates of the nitrifying processes, however, the use of SBR system allowed a decrease of the inhibitory effects along the cycles. The sludge showed a metabolic adaptation to consume the mixtures of phenolic compounds and sulfide throughout the cycles with increasing specific rates of removal of these contaminants. The obtained results showed that the nitrifying SBR can be used for the treatment of wastewaters contaminated with ammonium, mixtures of phenolic compounds, and sulfide.

Keywords: ammonium, mixtures, phenolic compounds, sequencing batch reactor, sulfide.

Resumen

La capacidad cinética y metabólica de un lodo nitrificante para oxidar simultáneamente amonio y mezclas de fenol, *p*-cresol, *m*-cresol, *o*-cresol y sulfuro fue evaluada a través de los ciclos de operación de un reactor de lote secuenciado (SBR). En el reactor SBR nitrificante, fue posible eliminar amonio (150 mg N/L) por nitrificación, mineralizar los compuestos fenólicos en mezcla (hasta 120 mg C/L) y oxidar sulfuro (15 mg S/L) a sulfato. La adición de compuestos fenólicos en mezclas y sulfuro en el reactor provocó una disminución en las velocidades específicas de los procesos nitrificantes, sin embargo, el uso del sistema SBR permitió una disminución de los efectos inhibitorios a lo largo de los ciclos. El lodo mostró una adaptación metabólica para consumir las mezclas de compuestos fenólicos y sulfuro a través de los ciclos con incrementos en las velocidades específicas de eliminación de estos contaminantes. Los resultados obtenidos mostraron que el reactor SBR nitrificante puede ser usado para el tratamiento de aguas residuales contaminadas con amonio, mezclas de compuestos fenólicos y sulfuro.

Palabras clave: amonio, compuestos fenólicos, mezclas, reactor de lote secuenciado, sulfuro.

1 Introduction

Ammonium, phenolic compounds, and sulfide are contaminants found in mixture in the effluents of diverse industries such as those generated by chemical, pharmaceutical, and petrochemical activities (Show *et al*., 2013; Jemaat *et al*., 2014; Huiliñir *et al*., 2018). Some petrochemical streams have been reported to contain ammonium at 710 mg N/L, phenol at 120 mg/L, total cresols at 80 mg/L, and sulfide at 320 mg/L (Olmos *et al*., 2004). Ammonium pollution in the receiving water bodies can lead to serious environmental problems such as eutrophication, hypoxia, and loss of biodiversity (Chen *et al*., 2018). Total nitrogen levels lower than 0.5-1.0 mg/L may prevent aquatic ecosystems from developing acidification and eutrophication, at least by inorganic nitrogen pollution (Camargo and Alonso, 2006). Phenolic compounds cause undesirable severe effects on the environment and human health, such as bioaccumulation and toxicity (Divate and Hinge, 2014). Phenolic compounds are toxic to humans and lethal to aquatic organisms at concentration of 5

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mg/L (De la Torre-Velasco *et al*., 2013). Sulfide is also a well-known highly toxic compound to living organisms and plants (Nielsen *et al*., 2006). Sulfide levels greater than 10 mg/L can affect human health while levels of 500-1000 mg/L can cause death (De la Torre-Velasco *et al*., 2013). Due to their chemical complexity, the effluents with nitrogen-, carbon-, and sulfur-containing contaminants constitute a great challenge for their treatment before their discharge in the environment. Biological treatment is an attractive alternative to the physicochemical processes because of various advantages such as low costs and release of innocuous products in the environment (Tomei and Annesini, 2008). Nitrification coupled to denitrification has potential for the simultaneous removal of nitrogen-, carbon-, and sulfur-containing contaminants from wastewater (Beristain-Cardoso *et al*., 2009; Show *et al*., 2013). In batch experiments, De la Torre-Velasco *et al*. (2013) showed the ability of an aerobic sludge to remove simultaneously ammonium, *p*-cresol, and sulfide by sequential nitrification-denitrification process. Such information can be applied for the simultaneous removal of nitrogenous, phenolic, and sulfurous compounds in biological reactors but more research is needed.

Nitrification is an aerobic respiratory process carried out by lithoautotrophic bacteria to obtain energy (Bernet and Spérandio, 2009). Nitrifying bacteria use inorganic carbon as carbon source for cellular growth and ammonium and nitrite as energy sources. In a first step, the ammonia-oxidizing bacteria oxidize ammonium to nitrite via hydroxylamine by using two enzymes: the ammonium monooxygenase (AMO) (Eq. 1) and the hydroxylamine oxidoreductase (Eq. 2). In a second step, the formed nitrite is oxidized to nitrate by the nitrite-oxidizing bacteria (Eq. 3). The nitrite oxidation process is catalyzed by the enzyme nitrite oxidoreductase. Nitrification is used in wastewaters biological treatment for transforming ammonium into nitrite or nitrate and coupling this process to the denitrification process where the resulting nitrite or nitrate are reduced to nitrogen gas (Kuenen and Robertson, 1994).

$$NH_4^+ + 0.5O_2 \rightarrow NH_2OH + H^+ \quad (1)$$

$$NH_2OH + O_2 \rightarrow NO_2^- + H^+ + H_2O \quad (2)$$

$$NO_2^- + 0.5O_2 \rightarrow NO_3^- \quad (3)$$

The use of nitrifying consortia has been proposed to achieve the simultaneous oxidation of ammonium and aromatic compounds (Zepeda *et al*., 2006). In nitrifying consortia, the oxidation of organic compounds can be obtained by the action of the enzyme AMO which has a wide range of substrates (chlorinated and aromatic compounds) and/or the organotrophic microflora of the sludge (Tran *et al*., 2013; Silva *et al*., 2014). The simultaneous removal of 2-chlorophenol, phenol, *p*-cresol and *p*-hydroxybenzaldehyde was evaluated under nitrifying conditions by Silva *et al*. (2011). In this study, the authors reported that the microbial consortium mineralized all the phenolic compounds either individual or in mixture with the concomitant oxidation of ammonium, suggesting that such information might be useful for coupling the nitrification-denitrification for treating wastewaters of chemical complexity. They also reported that the kinetic behavior of the nitrifying consortium was different when it was exposed to a single phenolic compound with regard to mixture, being the 2-chlorophenol and ammonium oxidation processes improved in the presence of the mixed phenolic compounds. This previous work was performed in 12 h batch assays and more studies in biological reactors with different mixtures of contaminants are needed. Beristain-Cardoso *et al*. (2011) showed that the simultaneous oxidation of ammonium, *p*-cresol, and sulfide could be obtained using a nitrifying sludge in a continuous stirred tank bioreactor. In this work, the consumption efficiencies for all substrates were of 100%, being nitrate, bicarbonate, and sulfate the end products. More investigation is required in bioreactors with different configurations and different mixtures of nitrogenous, phenolic, and sulfurous compounds for evaluating the simultaneous removal of various contaminants through nitrification processes and its potential applications. Kinetic and metabolic information on the behavior of nitrifying sludge in bioreactors may contribute to the design and control of nitrifying processes for the treatment of wastewaters with ammonium and mixed contaminants.

SBR technology is considered as a good alternative to the continuous-flow systems for nitrogen removal through the coupled biological processes of nitrification and denitrification (Guerrero *et al*., 2016; Liu *et al*., 2017; Li *et al*., 2018). SBR bioreactors have been also successfully used to remove mixtures of phenolic compounds (Tomei and Annesini, 2008; Fernández *et al*., 2013). Various studies reported that nitrifying SBR could be a good alternative for the simultaneous removal of ammonium and recalcitrant phenolic compounds such as *p*-hydroxybenzaldehyde (Téllez-Pérez *et al*., 2013), *m*-cresol (Zepeda *et al*., 2013), *p*-cresol (Silva *et al*., 2014) or 2-chlorophenol

(Martínez-Jardines *et al*., 2018). In spite of the inhibitory effects caused by organic matter, the nitrification process can be completed with high values of efficiencies and nitrate yields. It has been evidenced that the inhibition decreased along the operation cycles in the SBR system due to a metabolic adaptation of the sludge. Recently, Sekine *et al*. (2018) proposed the use of a SBR with a long filling period for treating an effluent containing ammonium and sulfide. However, these studies were carried out with a unique phenolic compound or sulfide in mixture with ammonium. Salas-Cortés *et al*. (2017) reported that the use of nitrifying SBR allowed the simultaneous oxidation of ammonium and a mixture of the three cresol isomers. These authors emphasized on the fact that the strategy and sequence of the addition of ammonium and cresols in mixture were decisive in the success and control of the multi-functional biological process, influencing on both recalcitrance and inhibitory impact of cresols in the nitrifying SBR culture. More information is needed to better understand how using SBR systems could help to obtain sludge metabolically able to perform simultaneously nitrification and oxidation of mixtures of inhibitory compounds present in wastewaters. Phenol and sulfide are both commonly found and highly toxic contaminants from industrial wastewaters but their coordinated effects on nitrifying processes have been scarcely studied, in particular when they are present in mixtures with ammonium and other phenolic compounds. Such information can be useful for further realistic applications in wastewaters treatment plants.

The objective of the present study was to evaluate the removal of ammonium, a mixture of phenol, *p*-cresol, *m*-cresol, *o*-cresol, and sulfide in a SBR reactor inoculated with a nitrifying sludge. The nitrification process and the capability of the microbial sludge to remove phenolic compounds and sulfide were evaluated at different initial concentrations of the contaminants and throughout the operation cycles of the SBR system.

2 Materials and methods

2.1 Nitrifying sludge

A mixture (50:50, w:w) of two nitrifying sludges were used to inoculate the SBR. The inoculum 1 was previously exposed to cresols according to the methodology described by Salas-Cortés *et al*. (2017) while the inoculum 2 was previously exposed to sulfide under the conditions reported by Bejarano *et al*. (2013). Before inoculating the SBR, the nitrifying activities of both inocula were evaluated in sequential 24 h batch cultures under nitrification conditions: nitrifying culture medium (Silva *et al*., 2011) with 100 ± 3 mg NH_4^+-N/L, agitation of 200 rpm, continuous aeration, and pH of 7.4 ± 0.6. The inoculum 1 maintained a nitrifying activity during five consecutive batch cultures with average values of 92 ± 8% for ammonium consumption efficiency and 0.92 ± 0.07 mg NO_3^--N/mg NH_4^+-N consumed for nitrate production. Under the same conditions, the inoculum 2 maintained a nitrifying activity with average values of 92 ± 8% for ammonium consumption efficiency and 0.96 ± 0.02 mg NO_3^--N/mg NH_4^+-N consumed for nitrate production. In all cases, there was no significant accumulation of nitrite at the end of the cultures. These results confirmed that both inocula performed a stable nitrifying respiratory process. Then, these sludges were used for inoculating the reactor at an initial concentration of 305 mg microbial protein/L (equivalent to 0.46 g volatile suspended solids (VSS)/L).

2.2 Sequencing batch reactor

A 2-L laboratory-scale SBR was operated under nitrifying conditions (Applikon, model p100). The reactor was fed with a synthetic medium containing the following nutrients (g/L): $(NH_4)_2SO_4$ (0.71), NH_4Cl (0.57), KH_2PO_4 (0.46), $MgSO_4$ (0.35), NaCl (0.54), $NaHCO_3$ (5.45), and $CaCl_2$ (0.03), and giving the C/N, C/S, and C/P ratios values of 2.6, 6.8, and 6.2, respectively. At the beginning of each cycle, the initial concentration of ammonium was 154.9 ± 10.3 mg NH_4^+-N/L. Each 24 h operation cycle consisted of the following phases: filling (0.08 h), biological reaction (23 h), settling (0.42 h), and withdrawal (0.50 h). All phases of the SBR were controlled electronically by timers. The reactor was continuously aerated (dissolved oxygen concentration of 4.0 ± 0.2 mg/L) and operated at 250 rpm, 25 ± 5°C, and pH of 8.0 ± 0.3. The volumetric exchange ratio of liquid was 92% and the hydraulic retention time (HRT) was 1.1 d.

Firstly, control abiotic assays without biomass (12 h) were performed in the SBR to discard possible loss of phenol and cresols due to adsorption onto the experimental system, chemical reaction with components from the medium and/or volatilization.

Table 1. Operation phases of the SBR.

Phase	Cycles	Compound	Concentration (mg N, C, or S/L)
I	1-71 (Control)	Ammonium	150
II	72-125	Ammonium Phenol	150 10, 20, 30
III	126-195	Ammonium Phenol *p*-Cresol	150 30 10, 20, 30
IV	196-218	Ammonium Phenol *p*-Cresol *m*-Cresol	150 30 30 10, 20, 30
V	219-243	Ammonium Phenol *p*-Cresol *m*-Cresol *o*-Cresol	150 30 30 30 10, 20, 30
VI	244-299	Ammonium Phenol + 3 cresols Sulfide	150 30 each one 5, 10, 15

Then, the SBR was inoculated and operated under nitrifying conditions for 71 cycles. Each phenolic compound and sulfide were then added into the reactor according to the procedure presented in Table 1. In the case of phases V and VI with addition of *o*-cresol and sulfide, the biological reaction time of some cycles was extended to obtain a complete nitrification (reaction times are indicated in Table 2). Samples were taken periodically in the influent and effluent of the SBR, filtered (0.2 μm), and analyzed for ammonium, nitrite, nitrate, phenol, cresols, sulfide, sulfate, and thiosulfate. Samples were also withdrawn at different times during the cycles for conducting kinetic studies. The capability of the sludge to remove the different components was evaluated in terms of consumption efficiencies (E, [mg N, C, or S consumed/mg N, C, or S fed] $\times$100). The nitrifying activity was evaluated in terms of production yields (Y, mg NO_3^--N, NO_2^--N, or biomass-N produced/mg NH_4^+-N consumed). To determine the biomass yield, it was considered that 16% of microbial protein is nitrogen (Bailey and Ollis, 1986). Profiles of substrates consumption and products formation were established at different cycles and the specific rates (q, mg N or C/mg microbial protein.h) were calculated from the slope observed in linear regressions. In all cases, the coefficient of determination (R^2) was higher than 0.96. Increments in specific rates values along the operation cycles were associated with the metabolic adaptation of the sludge.

2.3 *Analytical methods*

Ammonium concentration was determined with a selective electrode (Phoenix Electrode Company). Nitrite and nitrate concentrations were determined by HPLC (Perkin Elmer series 200) using an ion exchange column (IC-Pak Anion HC, 4.6 x 150 mm, Waters) and a UV detector at 214 nm. The mobile phase was composed of (mL/L): borate-gluconate solution (20), *n*-butanol (20), and acetonitrile (120) at 2 mL/min. The composition of the borate-gluconate solution (g/L) was: sodium gluconate (16), boric acid (18), and sodium tetraborate decahydrate (25). Phenol, *p*-cresol, *m*-cresol, and *o*-cresol were monitored by HPLC (Perkin Elmer series 200) using a C18 reverse-phase column (Bondclone, 3.9 x 300 mm, Phenomenex,) and a UV detector at 254 nm. The mobile phase was acetonitrile-water (50:50, v/v) at 1.0 mL/min. These experimental conditions allowed the *o*-cresol separation (retention time (RT) of 5.5 min) from other cresol isomers but did not allow the *m*-cresol separation from *p*-cresol (RT of 5.2 min). Phenol was detected at a RT of 4.4 min. Consequently, when the SBR was fed with a mixture of *m*-cresol and *p*-cresol, results are reported as the sum of *m*-cresol and *p*-cresol consumption. Sulfide concentration in aqueous phase was determined by an iodometric method (Bartlett and Skoog, 1954). Sulfate and thiosulfate concentrations were determined by HPLC (Waters Series 600) with an ion exchange column (IonoSpher A, 4.6 x 250 mm, Varian) and a UV-visible detector at 308 nm. The mobile phase consisted of a potassium hydrogen phthalate solution (0.04 M) at 0.8 mL/min. Total Organic Carbon (TOC) was measured using a TOC meter (TOC-5000, Shimadzu). Modified Lowry's method was employed to measure microbial protein concentration (Lowry *et al.*, 1951; Martínez *et al.*, 2000). VSS were determined according to standard methods (APHA, 1998). pH and dissolved oxygen concentration were determined by selective electrodes. Standard curves were drawn in triplicates for each analytical method. In all cases, the coefficient of variation in the slope value was less than 8% and the coefficient of determination (R^2) was higher than 0.98, showing a high reproducibility and linearity of the methods.

Table 2. Efficiencies (E) and yields (Y) of nitrification in the SBR fed with NH_4^+ in mixture with phenol, cresols, and sulfide.

Operation cycles	Concn. (mg C or S/L)[a]	Reaction time (h)[b]	Lag phase (h)[c]	E_{NH4+} (%)	$E_{Phenols}$[d], $E_{sulfide}$ (%)	Y_{NO2-}[e]	Y_{NO3-}[e]	Y_{BM}[e]
1-71 (Control)	150 mg NH_4^+-N/L	23	0	100.0 ± 0.1	-	0	0.89 ± 0.02	0.02 ± 0.01
72-90	Phenol (10)	23	3	97.5 ± 2.8	98.1 ± 0.6	0	0.90 ± 0.01	0.01 ± 0.01
91-106	Phenol (20)	23	3	100.0 ± 0.1	89.0 ± 1.4	0	0.90 ± 0.01	0.02 ± 0.02
107-125	Phenol (30)	23	1	100.0 ± 0.1	76.6 ± 10.8	0	0.90 ± 0.01	0.03 ± 0.01
126-153	Phenol (30) + *p*-cr (10)	23	2	100.0 ± 0.1	91.8 ± 5.5	0	0.80 ± 0.16	0.03 ± 0.01
154-174	Phenol (30) + *p*-cr (20)	23	0	100.0 ± 0.1	94.3 ± 5.5	0	0.90 ± 0.01	0.03 ± 0.01
175-195	Phenol (30) + *p*-cr (30)	23	0	100.0 ± 0.1	99.0 ± 0.1	0	0.90 ± 0.01	0.01 ± 0.02
196-212	Phenol (30) + *p*-cr (30) + *m*-cr (10)	23	3	100.0 ± 0.1	92.3 ± 7.5	0	0.80 ± 0.04	0.02 ± 0.02
213-215	Phenol (30) + *p*-cr (30) + *m*-cr (20)	23	0	100.0 ± 0.1	97.0 ± 2.1	0	0.90 ± 0.01	0.04 ± 0.01
216-218	Phenol (30) + *p*-cr (30) + *m*-cr (30)	23	0	100.0 ± 0.1	98.8 ± 2.1	0	0.90 ± 0.01	0.04 ± 0.01
219-224	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (10)	32	7	100.0 ± 0.1	98.0 ± 0.7	0	0.90 ± 0.01	0.04 ± 0.01
225-231	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (20)	25	2	100.0 ± 0.1	94.0 ± 0.1	0	0.80 ± 0.06	0.01 ± 0.01
232-243	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30)	24	1	100.0 ± 0.1	90.5 ± 3.9	0	0.90 ± 0.03	0.03 ± 0.01
244-255	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30) + sulfide (5)	52	9	100.0 ± 0.1	90.7 ± 8.1 100.0 ± 0.1	0.10 ± 0.01	0.80 ± 0.02	0.01 ± 0.01
256-269	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30) + sulfide (10)	30	2	97.3 ± 2.6	97.0 ± 2.8 100.0 ± 0.1	0.10 ± 0.04	0.80 ± 0.02	0.02 ± 0.03
270-299	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30) + sulfide (15)	24	0	100.0 ± 0.1	100.0 ± 0.1 100.0 ± 0.1	0.20 ± 0.02	0.70 ± 0.01	0.05 ± 0.01

[a] *p*-cr: *p*-cresol, *m*-cr: *m*-cresol, and *o*-cr: *o*-cresol. [b] Reaction time of the operation cycles. [c] Lag phase for ammonium oxidation at the end of different feeding periods. [d] $E_{Phenols}$: efficiency of consumption for all phenolic compounds. [e] All yields values are expressed as mg N/mg NH_4^+-N consumed.

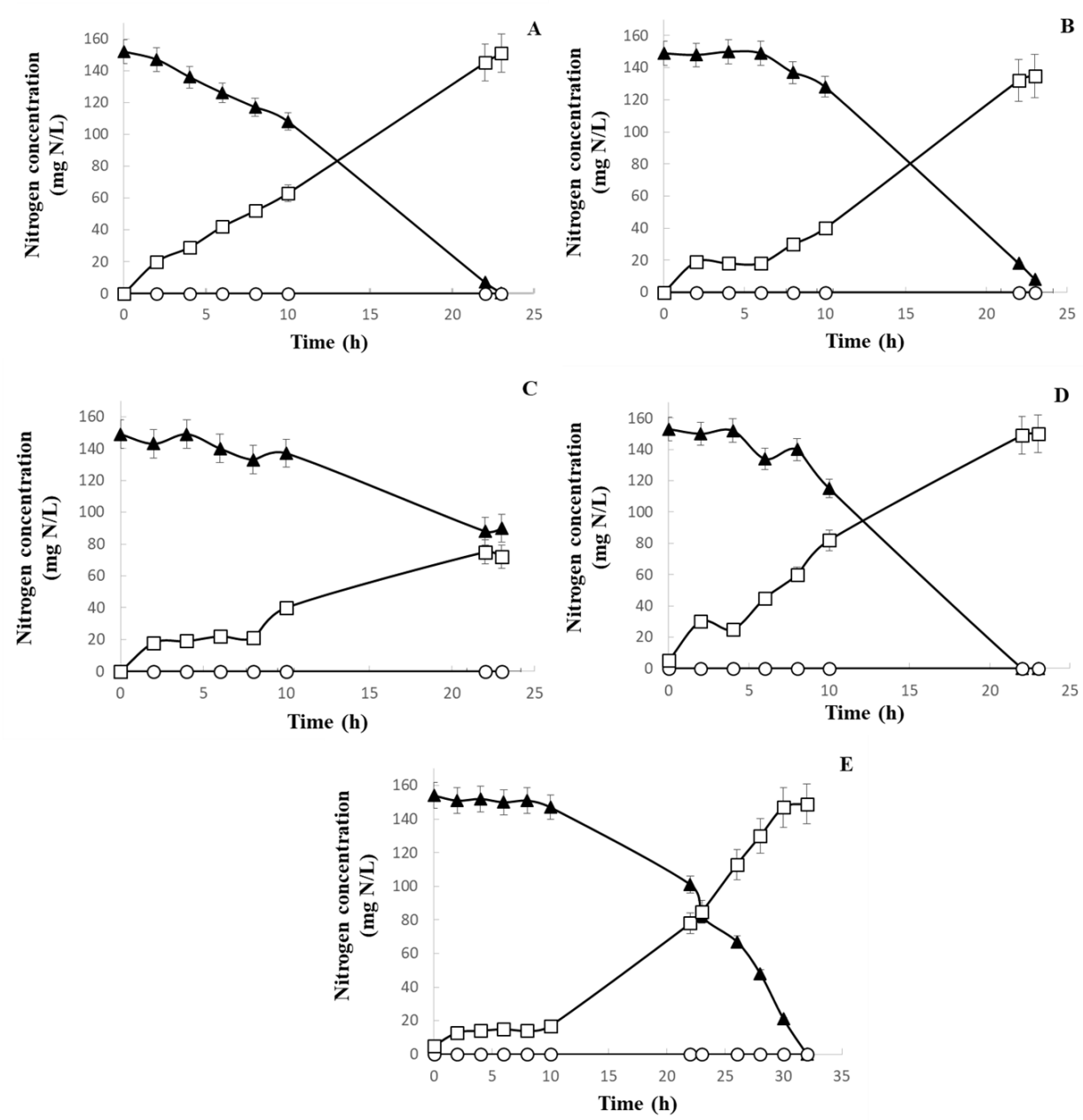

Fig. 1. Kinetic profiles of nitrification in the SBR fed with: A) ammonium (control) (cycle 71), B) ammonium and phenol (10 mg C/L) (cycle 72), C) ammonium, phenol (30 mg C/L), and *p*-cresol (10 mg C/L) (cycle 126), D) ammonium, phenol (30 mg C/L), *p*-cresol (30 mg C/L), and *m*-cresol (10 mg C/L) (cycle 196), E) ammonium, phenol (30 mg C/L), *p*-cresol (30 mg C/L), *m*-cresol (30 mg C/L), and *o*-cresol (10 mg C/L) (cycle 219). (▲) Ammonium, (o) Nitrite, (□) Nitrate.

3 Results and discussion

3.1 Abiotic assays and nitrification process in the control SBR

In the abiotic assays performed in the SBR without biomass, concentrations of ammonium, phenol, *p*-cresol + *m*-cresol, and *o*-cresol were kept constant at average values of 155.1 ± 2.2 mg N/L, 27.0 ± 1.4, 56.0 ± 1.8, and 31.0 ± 0.9 mg C/L, respectively. These results allowed to discard possible loss of ammonium, phenol, and cresols due to adsorption onto the experimental system, chemical reaction with components from the medium and/or volatilization.

During the control operation phase of the reactor fed with ammonium (cycles 1 to 71), a total oxidation of ammonium was obtained as the concentrations of residual ammonium were very low in the effluent. Since the first operation cycle of the SBR, the sludge was able to oxidize ammonium into nitrate as the final product of the nitrifying respiratory process. An example of the kinetic profiles of nitrification is shown in Fig. 1A for the cycle 71. Under these conditions, a complete and stable nitrifying process was obtained with the total conversion of ammonium ($E_{NH_4^+}$ = 100%) into nitrate ($Y_{NO_3^-} = 0.89 \pm 0.02$) without nitrite accumulation ($Y_{NO_2^-}$ of zero) in the effluent (Table 2). The specific rates of nitrification were determined during various operation cycles for using them as reference values (Table 3).

3.2 Nitrification process in the SBR fed with ammonium and phenol

When 10 mg C/L of phenol were added into the SBR (cycle 72), a 6 h lag phase appeared for the ammonium oxidation but the nitrification process was completed after 23 h with nitrate as the majority end product (Fig. 1B). These results showed that phenol affected mainly the ammonium oxidation process, the first step of nitrification, because nitrite was never accumulated in the SBR culture. The lag phase was decreasing throughout the cycles, obtaining a value of 3 h at cycle 90 (Table 2). According to the values of specific rates, a decrease of 11% was observed for $q_{NH_4^+}$ and 7% for $q_{NO_3^-}$ comparing with their values obtained in the control phase (Table 3). When the phenol concentration was increased to 20 mg C/L (cycles 91 to 106), the lag phase for ammonium oxidation was maintained at a value of 3 h, ammonium consumption efficiency and nitrate production yield were maintained at high values, and the specific rates did not significantly change respect to the phase with 10 mg C/L (Tables 2 and 3). At 30 mg C/L of phenol (cycles 107-125), the lag phase for nitrification was only of 1 h and the values for ammonium consumption efficiency, nitrate yield, and specific rates did not change significantly.

In Fig. 2A, it can be observed the consumption of phenol by the nitrifying sludge throughout the cycles and at different initial concentrations. In all cases, after a lag phase for phenol consumption, the sludge was able to oxidize the phenolic compound, obtaining efficiencies higher than 76.6 ± 10.8% after 23 h of culture (Table 2). The specific rates of phenol consumption increased from 0.001 to 0.002 mg C/mg microbial protein.h throughout the cycles (Table 3). These results evidenced the metabolic adaptation of the sludge to the presence of phenol and coincide with previous reports where the consumption of phenol by nitrifying sludge has been observed (Beristain-Cardoso *et al*., 2009). The nitrifying and/or heterotrophic bacteria of the sludge could adapt to the presence of phenol for consuming it and this would contribute to reach no inhibitory concentrations for nitrification (Silva *et al*., 2014). In the other hand, in some works, it has been reported that phenolic compounds can inhibit the nitrification process by altering the enzyme AMO activity and/or the cellular membrane as well as the consumption of the phenolic compounds can be slow (Martínez-Jardines *et al*., 2018). However, in the present study, under the experimental conditions used in the SBR, results show that the nitrifying sludge was able to completely oxidize ammonium to nitrate and consume phenol.

3.3 Nitrification process in the SBR fed with ammonium and a mixture of phenol and cresols

p-Cresol (10 mg C/L) was added into the reactor in mixture with ammonium and phenol for the first time at cycle 126 (Fig. 1C). During this cycle, the lag phase for ammonium oxidation was only of 4 h and once ammonium consumption had initiated, it was oxidized into nitrate without nitrite accumulation. After 23 h, the $E_{NH_4^+}$ was only of 40% but the $Y_{NO_3^-}$ was close to 1.0, showing that *p*-cresol affected mainly the ammonium oxidation process as previously observed in the case of phenol.

Table 3. Specific rates (q) of nitrification and oxidation of phenolic compounds and sulfide along the operation cycles of the SBR.

Cycles	Concn. (mg C or S/L)[a]	$q^b_{NH_4^+}$	$q^b_{NO_3^-}$	$q^c_{Phenols}$	$q^d_{HS^-}$
1-71 (Control)	150 mg NH_4^+-N/L	0.018±0.001	0.015±0.001	—	—
72-90	Phenol (10)	0.016±0.001	0.014±0.001	0.001±0.001	—
91-106	Phenol (20)	0.016±0.001	0.014±0.001	0.002±0.001	—
107-125	Phenol (30)	0.016±0.001	0.013±0.001	0.002±0.001	—
126-153	Phenol (30) + p-cr (10)	0.013±0.001	0.010±0.002	0.002±0.001	—
154-174	Phenol (30) + *p*-cr (20)	0.015±0.001	0.013±0.001	0.010±0.002	—
175-195	Phenol (30) + *p*-cr (30)	0.015±0.001	0.012±0.001	0.016±0.001	—
196-212	Phenol (30) + *p*-cr (30) + *m*-cr (10)	0.018±0.001	0.014±0.001	0.023±0.003	—
213-215	Phenol (30) + *p*-cr (30) + *m*-cr (20)	0.016±0.001	0.012±0.001	0.027±0.005	—
216-218	Phenol (30) + *p*-cr (30) + *m*-cr (30)	0.015±0.001	0.013±0.001	0.034±0.003	—
219-224	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (10)	0.013±0.001	0.012±0.001	0.003±0.002	—
225-231	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (20)	0.013±0.001	0.010±0.001	0.011±0.001	—
232-243	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30)	0.015±0.001	0.012±0.001	Cycle 243: $q_{Phenol} = 0.011 \pm 0.002$ $q_{m-cr+p-cr} = 0.029 \pm 0.003$ $q_{o-cr} = 0.023 \pm 0.002$	—
244-255	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30) + sulfide (5)	0.015±0.001	0.015±0.001	ND	0.003±0.001
256-269	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30) + sulfide (10)	0.012±0.002	0.010±0.002	ND	0.009±0.003
270-299	Phenol (30) + *p*-cr (30) + *m*-cr (30) + *o*-cr (30) + sulfide (15)	0.014±0.003	0.014±0.001	Cycle 270: $q_{Phenol} = 0.004 \pm 0.001$ $q_{m-cr+p-cr} = 0.008 \pm 0.002$ $q_{o-cr} = 0.003 \pm 0.001$	0.008±0.001

[a] *p*-cr: *p*-cresol, *m*-cr: *m*-cresol, and *o*-cr: *o*-cresol. [b] $q_{NH_4^+}$ and $q_{NO_3^-}$ expressed as mg N/mg microbial protein.h. [c] $q_{Phenols}$ expressed as mg C/mg microbial protein.h is q_{Phenol} for cycles 72-125; q_{p-cr} for cycles 126-195; $q_{m-cr+p-cr}$ for cycles 196-218 and q_{o-cr} for cycles 219-231. [d] q_{HS^-} expressed as mg S/mg microbial protein.h. ND = Not determined.

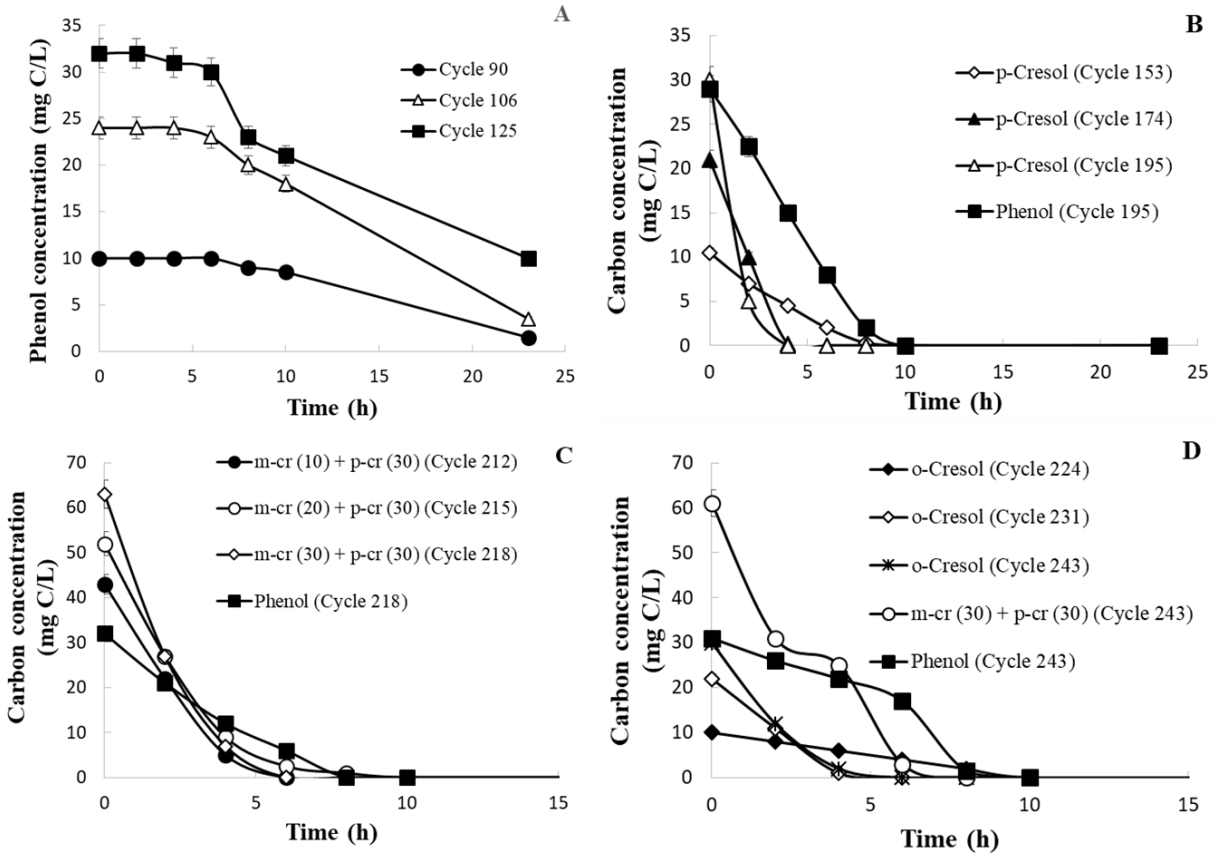

Fig. 2. Kinetic profiles of consumption in the SBR for: A) phenol at (mg C/L): 10 (cycle 90), 20 (cycle 106), and 30 (cycle 125), B) *p*-cresol at (mg C/L): 10 (cycle 153), 20 (cycle 174), 30 (cycle 195), and phenol (30 mg C/L) (cycle 195), C) sum of *p*-cresol (*p*-cr) (30 mg C/L) and *m*-cresol (*m*-cr) at (mg C/L): 10 (cycle 212), 20 (cycle 215), 30 (cycle 218), and phenol (30 mg C/L) (cycle 218), D) *o*-cresol at (mg C/L): 10 (cycle 224), 20 (cycle 231), 30 (cycle 243), phenol (30 mg C/L) (cycle 243), and sum of *p*-cresol (*p*-cr) (30 mg C/L) and *m*-cresol (*m*-cr) (30 mg C/L) (cycle 243).

As operation cycles went by under these experimental conditions (cycles 126-153), the nitrifying process improved, obtaining high average values for efficiency (100.0 ± 0.1%) and nitrate yield (0.80 ± 0.16) (Table 2). Addition of *p*-cresol provoked an additional inhibitory effect on nitrification with a decrease of 28% for $q_{NH_4^+}$ and 33% for $q_{NO_3^-}$ comparing with the values obtained in the control phase (Table 3). However, in spite of the consecutive increases of the *p*-cresol concentration at 20 mg C/L (cycles 154-174) and 30 mg C/L (cycles 175-195), the lag phase for ammonium oxidation disappeared and the specific rates of nitrification increased throughout the cycles, showing the capability of the microbial sludge to metabolically adapt to the presence of the mixture of phenol and *p*-cresol (Tables 2 and 3). During the feeding period (cycles 175-195) with phenol (30 mg C/L) and *p*-cresol (30 mg C/L), the $E_{NH_4^+}$ was of 100.0 ± 0.1% and the $Y_{NO_3^-}$ of 0.90 ± 0.01. As shown in Fig. 2B, the consumption of *p*-cresol improved throughout the cycles as previously reported by Silva *et al*. (2014) in a SBR reactor fed with *p*-cresol as unique phenolic compound. In the present study, the results showed that the sludge acquired a major ability for consuming *p*-cresol in mixture with phenol and throughout the cycles with an increase of the specific rates of *p*-cresol consumption (Table 3).

During the first cycle (cycle 196) of *m*-cresol (10 mg C/L) addition in mixture with phenol (30 mg C/L) and *p*-cresol (30 mg C/L), nitrification was completed after 22 h with an efficiency of 100% and a nitrate yield of 0.98 (Fig. 1D). These results contrast with those previously obtained with the first cycle of *p*-cresol addition (cycle 126) (Fig. 1C) where an accumulation

of ammonium was observed after 23 h of culture. These results show that the nitrifying sludge was more tolerant to the addition of *m*-cresol that *p*-cresol. Actually, when *m*-cresol was added, the specific rates of nitrification did not suffer any inhibitory effect, on the contrary, they tended to increase respect to their values from the previous phase with phenol and *p*-cresol (Table 3). These results did not coincide with previous results presented by Zepeda *et al*. (2013). The study of these authors was carried out in a SBR fed with 50 mg NH_4^+-N/L where a significant decrease was observed for $q_{NH_4^+}$ (- 74%) and $q_{NO_3^-}$ (- 59%) when *m*-cresol (12.5 mg C/L) was added for the first time in the reactor. According to the results from Salas-Cortés *et al*. (2017), the strategy and sequence of the addition of ammonium and cresols in mixture at different concentrations in a SBR system were decisive in the capability of metabolic adaptation of the sludge for simultaneously oxidizing ammonium and a mixture of cresols. In the present work, the previous feeding of the SBR culture with phenol and *p*-cresol may have contributed to a higher tolerance of the sludge to the presence of *m*-cresol. When *m*-cresol concentration was increased to 20 mg C/L (cycles 213-215) and 30 mg C/L (cycles 216-218), the lag phase for ammonium consumption disappeared and average values of $E_{NH_4^+}$ close to 100% and $Y_{NO_3^-}$ of 0.90 ± 0.01 were achieved during both periods (Table 2). Kinetic results showed that the sludge acquired a higher capability to consume the mixture of *p*-cresol and *m*-cresol throughout the operation cycles with increasing specific rates values (Fig. 2C, Table 3).

When the following phenolic compound (*o*-cresol) was fed into the reactor at 10 mg C/L (cycle 219), the nitrification process was successfully performed but after a lag phase of 8 h for ammonium oxidation and a longer total time of 32 h (Fig. 1E). As cycles went by and *o*-cresol concentrations were increased in the feeding mixture of phenol, *p*-cresol, *m*-cresol, and *o*-cresol at 30 mg C/L each one, the nitrifying activity improved as the lag phase for ammonium oxidation decreased and high values of efficiency and nitrate yield were determined after only 24 h of culture (Table 2). These results showed that, in spite of the addition of phenol and the three isomers of cresol up to 120 mg C/L in the SBR, nitrification was successfully completed converting all the ammonium into nitrate. The sludge also acquired a major metabolic capability for oxidizing *o*-cresol along the operation cycles with an increase of the specific rates of consumption (Fig. 2D, Table 3). The metabolic adaptation of the sludge to the repeatedly exposure to the phenolic compounds can be related to changes in its microbial population and the synthesis and kinetic of enzymes involved in the nitrification and/or the inhibitor consumption pathways (Kjeldal *et al*., 2014; Silva *et al*., 2014).

It is noteworthy that at the end of the feeding period with phenol and the three isomers at 30 mg C/L, there was no presence of these compounds in the effluent, obtaining a high efficiency of consumption of 90.5 ± 3.9% (Table 2). Analysis of TOC evidenced the absence of organic compounds in the effluent, discarding the possible accumulation of organic intermediaries. The biomass yields were lower than 0.04 mg biomass-N/mg NH_4^+-N consumed (Table 2), indicating that the oxidation of the phenolic compounds was mainly through a dissimilative process. Then, it can be assumed that phenol and cresols were mainly mineralized by the sludge. At cycle 243, the specific consumption rates for phenol, *m*-cresol + *p*-cresol, and *o*-cresol reached values of 0.011 ± 0.002, 0.029 ± 0.003, and 0.023 ± 0.002 mg C/mg microbial protein.h, respectively (Table 3). These results show that under the experimental conditions used in the SBR, *o*-cresol was less recalcitrant than phenol. This is contrary to previously reported results where *o*-cresol appeared as the most recalcitrant phenolic compound (Lee *et al*., 2011; Salas-Cortés *et al*., 2017). The higher capacity of the sludge obtained in the present study for oxidizing *o*-cresol was probably linked to the sequential feeding used in the SBR of the different mixed contaminants. Different substrate interaction patterns (no interaction, inhibition, cometabolism, induction…) have been previously reported in the literature for mixtures of compounds (Deeb and Alvarez-Cohen, 1999). However, kinetic studies focused on the effect of the feeding sequence of mixed contaminants on their consumption along the operation cycles of SBR systems are lacking in the literature. The present study gives novel kinetic information for a quaternary mixture of phenolic compounds added into a nitrifying SBR, evidencing that the feeding sequence strategy used was successful in favouring *o*-cresol consumption by the sludge.

3.4 Nitrification process in the SBR fed with ammonium and a mixture of phenol, cresols, and sulfide

The SBR operation continued with the addition of sulfide at 5 mg S/L (cycle 244). The nitrification process was successfully performed but after a lag

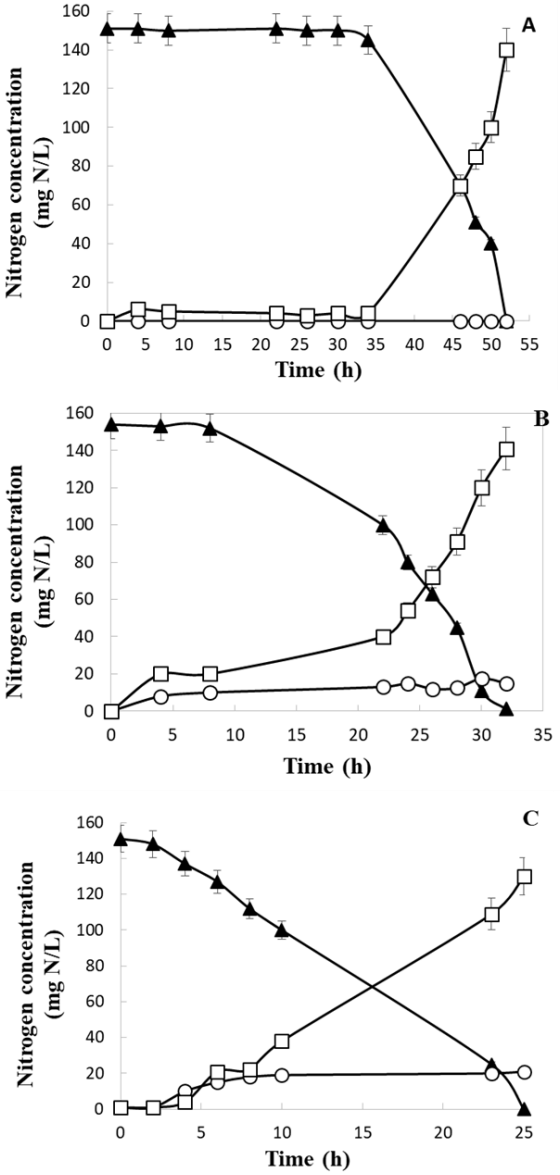

Fig. 3. Kinetic profiles of nitrification in the SBR fed with ammonium, a mixture of phenol, *p*-cresol, *m*-cresol, *o*-cresol (30 mg C/L each one), and sulfide at (mg S/L): A) 5 (cycle 244), B) 10 (cycle 256), C) 15 (cycle 270). (▲) Ammonium, (o) Nitrite, (□) Nitrate.

phase of 34 h for ammonium oxidation and a longer total time of 52 h (Fig. 3A). Under these conditions, the ammonium oxidation was the most altered process as the nitrite oxidation process seemed not to be modified with a high production of nitrate and no nitrite accumulation in the culture. As cycles went by and sulfide concentrations were increased, the ammonium oxidizing activity improved as the lag

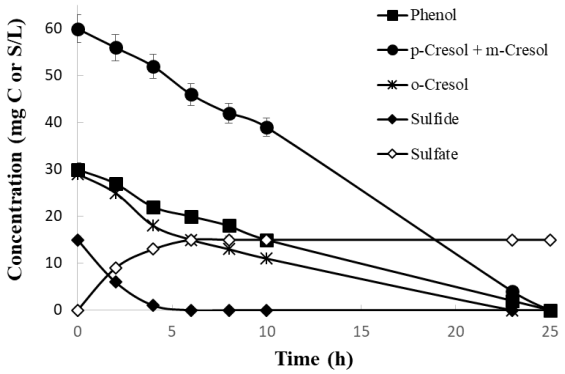

Fig. 4. Kinetic profiles of oxidation of phenol, *p*-cresol, *m*-cresol, *o*-cresol, and sulfide in the SBR reactor (Cycle 270).

phase for ammonium oxidation decreased. In fact, an $E_{NH_4^+}$ of 100% was determined after only 24 h of culture during the period of feeding with sulfide at 15 mg S/L (Fig. 3C, Table 2). However, at cycle 256 with 10 mg S/L, nitrite began to accumulate and at the end of cycle 270 with 15 mg S/L, a $Y_{NO_3^-}$ of 0.86 and a $Y_{NO_2^-}$ of 0.14 were determined (Fig. 3B and 3C). These results showed that despite sulfide addition, the ammonium oxidation process was carried out efficiently, however there was an accumulation of nitrite in the medium, indicating that the nitrite oxidation process was affected by the addition of sulfide along the cycles. These results corroborate those reported by Erguder *et al*. (2008) who observed that the addition of sulfide in a nitrifying SBR caused nitrite accumulation. It has been well documented that sulfide is a strong inhibitor of the nitrification processes, being the nitrite oxidation process the most sensible step according to Bejarano-Ortiz *et al*. (2013, 2015).

Fig. 4 presents the kinetic consumption profiles of the different phenolic compounds and sulfide during the cycle 270. Total removal of all contaminants was obtained after 25 h. Sulfide was mainly oxidized to sulfate. According to the kinetic evaluation, the specific rates of sulfide oxidation increased along the cycles (Table 3). It was also evidenced that sulfide at 15 mg S/L caused inhibitory effects on the phenolic compounds oxidation with decreases of 64, 72, and 87% for specific rates of phenol, *m*-cresol + *p*-cresol, and *o*-cresol consumption, respectively at cycle 270 in comparison with results from cycle 243 (Table 3). The same experimental conditions were maintained from cycle 270 at the end of the reactor operation, and the simultaneous removal of ammonium (150 mg

N/L), phenol (30 mg C/L), *p*-cresol (30 mg C/L), *m*-cresol (30 mg C/L), *o*-cresol (30 mg C/L), and sulfide (15 mg S/L) occurred with high values of efficiencies (Table 2). The oxidation of ammonium by nitrification led to the formation of nitrite ($Y_{NO_2^-}$ = 0.20 ± 0.02) and nitrate ($Y_{NO_3^-}$ = 0.70 ± 0.01) while the phenolic compounds were mainly mineralized and sulfide oxidized to sulfate.

Conclusions

In spite of the negative effects of the mixtures of phenolic compounds and sulfide on nitrification, it was possible by using a SBR to maintain a stable and efficient nitrifying respiratory process with high production of nitrate. Up to 10 mg S/L of sulfide, the nitrifying sludge was able to mineralize a quaternary mixture of phenol, *p*-cresol, *m*-cresol, and *o*-cresol (total of 120 mg C/L), oxidize 150 mg N/L of ammonium into nitrate ($Y_{NO_3^-}$ = 0.80) while sulfide was totally oxidized to sulfate. These results give novel kinetic and metabolic information in order to use nitrifying SBR to remove simultaneously ammonium, mixed phenolic compounds, and sulfide in one reactor.

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