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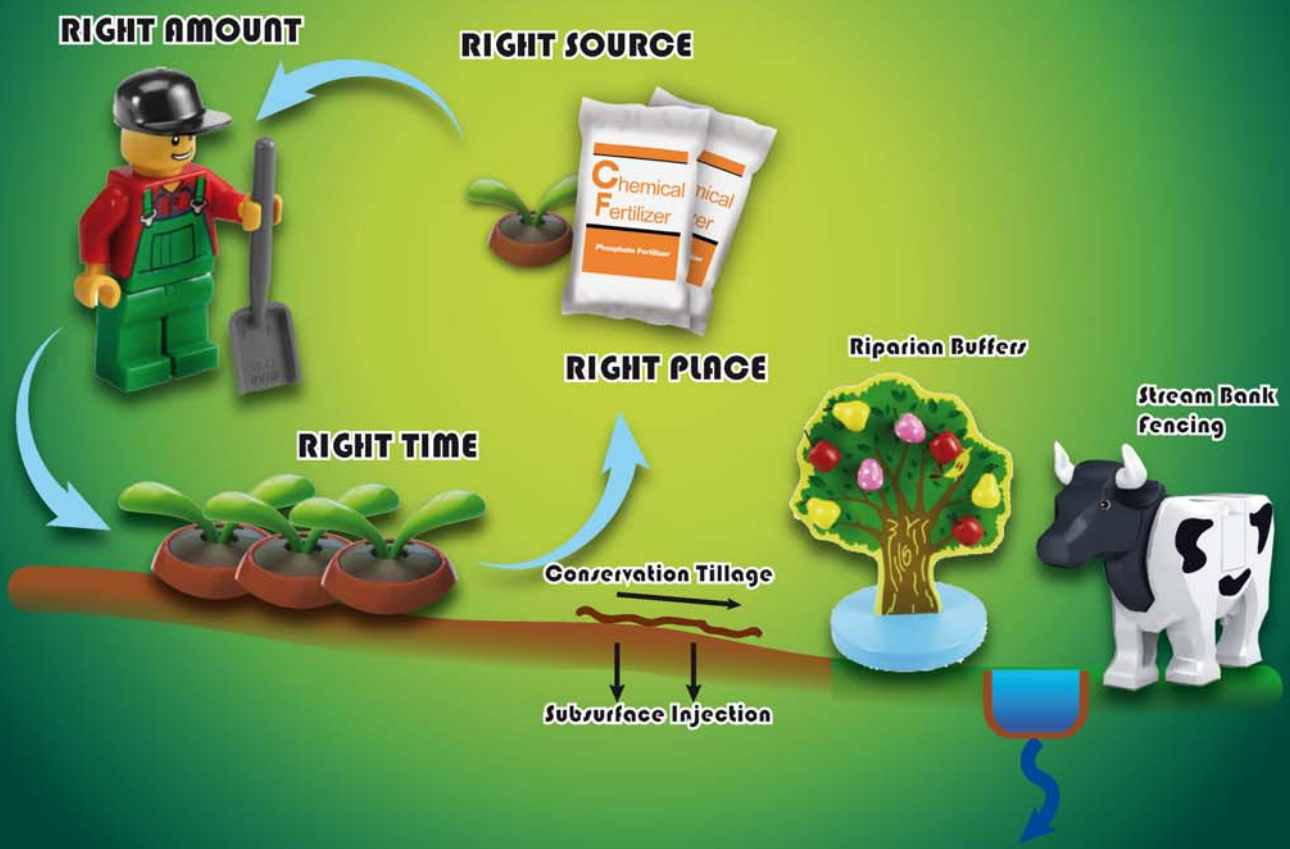

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p-Cresol mineralization and bacterial population dynamics in a nitrifying sequential batch reactor

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ABSTRACT

The ability of a nitrifying sludge to oxidize *p*-cresol was evaluated in a sequential batch reactor (SBR). *p*-Cresol was first transformed to *p*-hydroxybenzaldehyde and *p*-hydroxybenzoate, which were later mineralized. The specific rates of *p*-cresol consumption increased throughout the cycles. The bacterial population dynamics were monitored by using denaturing gradient gel electrophoresis (DGGE) and sequencing of DGGE fragments. The ability of the sludge to consume *p*-cresol and intermediates might be related to the presence of species such as *Variovorax paradoxus* and *Thauera mechernichensis*. *p*-Cresol (25 to 200 mg C/L) did not affect the nitrifying SBR performance (ammonium consumption efficiency and nitrate production yield were close to 100% and 1, respectively). This may be related to the high stability observed in the nitrifying communities. It was shown that a nitrifying SBR may be a good alternative to eliminate simultaneously ammonium and *p*-cresol, maintaining stable the respiratory process as the bacterial community.

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Introduction

The use of sequential batch reactors (SBRs) in the biological treatment of wastewaters has been widely extended from lab-scale studies to wastewater treatment plants. The major advantages of SBR systems over traditional continuous flow systems are the design simplicity, the absence of a separate clarifier, the operating flexibility and the low cost for operation. The SBR technology can be used for eliminating ammonium nitrogen from the water through the coupled respiratory processes of nitrification and denitrification (Puig et al., 2004). Nitrification is an aerobic respiratory process carried out by two groups of gram negative lithoautotrophic bacteria that are phylogenetically unrelated, where ammonium is oxidized to nitrate. Nitrate is subsequently reduced to molecular nitrogen by denitrification. Nitrification is the succession of the oxidation of ammonium to nitrite by the ammonia-oxidizing bacteria (AOB) and the subsequent oxidation of nitrite to nitrate by the nitrite-oxidizing bacteria (NOB).

It is well known that a wide variety of organic compounds could be toxic or provoke inhibitory effects on nitrification (McCarty, 1999). Inhibitory effects of phenolic compounds on the nitrification process have been previously reported (Kim et al., 2008). However, knowledge on the inhibitory effects of cresols on the nitrification respiratory process is still limited (Silva et al., 2009). Ammonium and cresols can be found in various industrial effluents (petrochemical, pharmaceutical, chemical, coke wastewater, synthetic resin wastes) (Olmos et al., 2004; Kim et al., 2008). Phenolic compounds including cresols are toxic and can cause persistence and bioaccumulation effects in animal and vegetable

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organisms. Many of these organic chemicals might be also carcinogenic, mutagenic and teratogenic (Davì and Gnudi, 1999).

Several studies have reported the ability of nitrifying consortia to oxidize various organic compounds (Silva et al., 2011). These compounds include recalcitrant aromatic compounds such as benzene, toluene, *m*-xylene and phenol (Amor et al., 2005; Zepeda et al., 2006). Previous results showed that nitrifying SBR might be a good alternative to eliminate simultaneously ammonium and inhibitory aromatic compounds from wastewaters (Texier and Gomez, 2007). Nevertheless, further work is required to kinetically characterize the ability of the nitrifying SBR culture for oxidizing inhibitory compounds such as *p*-cresol throughout the cycles and evaluate the accumulation of intermediates.

Nowadays, attempts are made to relate the physiological response of microbial consortia with their structure and population dynamics (Wittebolle et al., 2008). Several molecular techniques have been developed to evaluate microbial communities in natural samples and bioreactors (Talbot et al., 2008). Denaturing gradient gel electrophoresis (DGGE) is a powerful tool to determine the genetic diversity of microbial communities and to identify the phylogenetic affiliation of community members in bioreactors operated under different experimental conditions (Xia et al., 2005; Martínez-Hernández et al., 2009). Based on a DGGE analysis and sequencing of the microbial community structure of cresol-degrading aerobic granules, Lee et al. (2011) found 15 predominant bacterial species, including the genera *Bacillus*, *Acinetobacter*, *Corynebacterium*, and *Nocardioides*. Other bacteria, including *Pseudomonas*, *Arthrobacter*, *Alcaligenes*, and *Rhodococcus* metabolized *p*-cresol as carbon and energy source (Kang et al., 1998; Gallego et al., 2008; Wang et al., 2009; Ho et al., 2010; Lee et al., 2011).

Therefore, the aim of this study was to evaluate the ability of a nitrifying sludge to consume *p*-cresol and its intermediates as well as to monitor the population dynamics of the bacterial community throughout the operation cycles in a SBR system.

1. Materials and methods

1.1. Nitrifying sequential batch reactors

The sludge used for inoculating the SBRs was obtained from a continuous stirred tank reactor in steady-state nitrification. The system was operated continuously at 300 r/min, 25 ± 2°C, and pH of 8.0 ± 0.5. The dissolved oxygen concentration was maintained at 4.3 ± 0.1 mg/L. The hydraulic retention time was 1.8 days. The reactor was fed with a medium containing the following nutrients (g/L): $(NH_4)_2SO_4$ (1.18), NH_4Cl (0.94), KH_2PO_4 (1.40), $MgSO_4$ (0.60), NaCl (1.0), $NaHCO_3$ (9.33) and $CaCl_2$ (0.05). The loading rate of NH_4^+-N was 141 ± 6 mg/(L·day). Under steady state conditions, the ammonium consumption efficiency was close to 100% while nitrate was the main product from ammonium oxidation.

The initial concentration of volatile suspended solids (VSSs) in the SBRs was of 1 g/L. Two laboratory-scale SBRs (Model p100, Applikon, Dover, NJ, USA) were operated with cycles of 12 hr for more than 5 months. The 2 L SBR_A was fed with culture medium and used as control while the 1.7 L SBR_B was fed with different initial *p*-cresol concentrations. Each cycle consisted of the four following periods: fill (0.25 hr), reaction (11.25 hr), settle (0.25 hr) and draw (0.25 hr). All of the periods of the SBRs were controlled electronically by timers. The volumetric exchange ratio of liquid was 90% and the hydraulic retention time was 0.56 days. The medium used for the SBR cultures was the mixture of two media as nitrogen (A) and carbon (B) sources. The chemical composition of medium A was (g/L): $(NH_4)Cl$ 0.45, $(NH_4)_2SO_4$ 0.54, KH_2PO_4 0.65, $MgSO_4$ 0.5, and NaCl 0.5. Medium B consisted (g/L) of $NaHCO_3$ 4.5 and $CaCl_2$ 0.03. Media A and B were added at 60 and 40 mL/min, respectively. At the beginning of each cycle, the initial concentration of NH_4^+-N was 100 mg/L, corresponding to 200 mg NH_4^+-N/(L·day). $FeSO_4·7H_2O$ was added to the reactors daily at 0.01 g/L. A *p*-cresol solution (536–4288 mg/L) was added to the SBR_B at 17 mL/min for 6 min to obtain initial *p*-cresol concentrations ranging from 25 to 200 mg *p*-cresol-C/L (50 to 400 mg *p*-cresol-C/(L·day)). A constant aeration and agitation of 225 r/min were maintained into the cultures to give a dissolved oxygen concentration of 5.3 ± 0.1 mg/L at the beginning of each cycle. Samples were withdrawn at different times over 12 hr cycles for conducting kinetic studies. Additionally, the possible adsorption of *p*-cresol onto the sludge was evaluated by sterile controls (the sludge was sterilized at 120°C for 20 min) in serological bottles according to the methodology described by Silva et al. (2009). Furthermore, abiotic batch cultures (in the absence of sludge) were conducted to discard the possible loss of *p*-cresol by volatilization or chemical reaction.

1.2. Analytical methods

Ammonium nitrogen was analyzed by a selective electrode (Ammonia gas sensing ISE, Cole-Parmer, Equipar, Mexico), total organic carbon (TOC) by using a TOC-meter (TOC-5000A, Shimadzu, Columbia, Maryland, USA), and VSSs were determined according to standard methods (APHA, 1998). Nitrite, nitrate, *p*-cresol, *p*-hydroxybenzaldehyde (pOHBD), and *p*-hydroxybenzoate (pOHBT) concentrations were determined by HPLC as previously described by Silva et al. (2009). Oxygen concentration was determined by a dissolved oxygen meter (HI 98186, Hanna, Romania) with a polarographic probe (HI76407/4F, Hanna, Romania). Analytical methods had a variation coefficient of less than 10%.

1.3. Culture evaluation

The ammonium consumption efficiency (E_{NH_4} was expressed as (g NH_4^+-N consumed/g NH_4^+-N initial) × 100 and nitrate production yield (Y_{NO_3}) as g NO_3^--N/g NH_4^+-N consumed. To determine the biomass yield (Y_{BM}, mg biomass-C produced/mg consumed-C), it was considered that 50% of the microbial biomass (VSS) is carbon. The Gompertz model was used to analyze the kinetic data of *p*-cresol consumption (Draper and Smith, 1981). In this model, the consumed *p*-cresol concentration (S_c) may be expressed as a function of the time according to the following Eq. (1):

$$S_c = \alpha \exp(-\beta \exp(-kt)) \quad (1)$$

where, $S_c = (S_0 - S)$, S_0 (mg/L) is the initial substrate concentration, S (mg/L) is the substrate concentration, α (mg *p*-cresol/L) is

the maximum *p*-cresol concentration consumed, β is a parameter related to the initial conditions, k (hr^{-1}) is the specific rate of *p*-cresol consumption, and t (hr) is the time. The parameters α, β, and k were adjusted from experimental data using the analysis program Origin 8.0 (OriginLab Inc.). The maximum rate of *p*-cresol consumption (V_{max}, mg/(L·hr)) was calculated according to Eq. (2).

$$V_{max} = 0.368\alpha k \quad (2)$$

Finally, specific rates were determined by dividing the volumetric rates (V_{max}) between the VSS concentration. The coefficient of determination was higher than 0.95 for all cases.

1.4. Molecular analysis

DGGE and sequencing methods were used to determine the diversity of the bacterial communities present in the control reactor and the reactor fed with *p*-cresol.

1.4.1. DNA extraction and 16S rDNA gene amplification

Duplicate samples were taken (0.5 g of sludge) from both reactors at the first and the last cycle of operation for each *p*-cresol concentration tested in SBR_B. The samples were centrifuged at 8000 ×*g* for 10 min and then the pellets were frozen at −20°C until DNA extraction.

DNA was extracted using the UltraCleanTM Soil DNA Isolation kit (MO BIO Laboratories, Carlsbad, CA, USA), following the manufacturer's recommendations. The amount of DNA was verified by electrophoresis on 2% (W/V) agarose gel at 90 V for 1 hr. The DNA was stored at −20°C until further use.

The V6–V8 regions of the 16S rDNA gene were amplified using the bacterial primers 968F (5′-GAACGCGAAGAACCTTACC-3′) with clamp and 1401R (5′-CGGTGTGTACAAGACCC-3′) (Großkopf et al., 1998). The clamp consisted of a 40 bp sequence (5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG-3′). The polymerase chain reaction (PCR) amplification was performed in a thermocycler CG1-96 (Corbett Research, Sydney, Australia) as follows: pre-denaturation at 94°C for 2 min; 30 cycles of denaturation at 94°C for 45 sec, annealing at 60°C for 30 sec and elongation at 72°C for 90 sec; post-elongation at 72°C for 10 min (García-Saucedo et al., 2008). The reaction mixture for PCR consisted of (μL): 2.5 of each primer (20 mmol/L), 1 of nucleotide mixture (10 mmol/L per nucleotide), 10 of buffer (10×), 5 of $MgCl_2$ (25 mmol/L), 0.5 of Taq DNA polymerase (5 U/μL), 1 of sludge DNA and 27.5 of sterile water.

1.4.2. Denaturing gradient gel electrophoresis

Amplification products were separated by DGGE in a universal DCode mutation detection system (Bio-Rad Laboratories, Hercules, USA) at 60°C. Polyacrylamide gels were used at 6% (W/V) and the denaturant gradients varied from 30% to 38% and from 45% to 60% (100% denaturant stock solution was made of 7 mol/L urea and 40% formamide). The gel was run for 5 min at 200 V and then for 16 hr at 85 V, according to the procedure described by García-Saucedo et al. (2008). DGGE gels were stained with silver nitrate and scanned in an Epichemi3 photodocumented Darkroom (UVP Biolmaging Systems, Upland, CA, USA).

Species richness (S) and evenness (J) of the microbial community were estimated. Structural changes were analyzed by means of the J index because it reflects an overview of the predominance of species within a microbial community. The J index was calculated as $J = H / \ln S$. The term H is defined as $H = -\Sigma P_i \ln P_i$, where P_i is the relative abundance of the bands in a lane and is calculated as $P_i = n_i / N$, n_i is the band intensity for individual bands, and N is the sum of intensities of bands in a lane. The richness index S represents the total number of bands in a lane. Determination of all indices was based on the DGGE profiles. Each band within a profile was considered a fragment of a different microbial population, while bands with similar migration positions in different profiles were considered fragments of the same population (Martínez-Hernández et al., 2009). The relation between the surface area of a band and the mean pixel intensity of that area was considered the intensity of the band and calculated using the image analysis program Image J (public-domain image processing and analyzer program), developed at the National Institutes of Health.

1.4.3. Purification and sequencing of 16S rDNA bands

The bands from DGGE gels were excised and reamplified using the primers listed above. The PCR products were purified (Wizard ® SV gel and PCR clean-up system, Promega, Madison, WI, USA), visualized by agarose gel electrophoresis and sequenced in the Genetic Analyzer ABI Prism 3100 (Applied Biosystems, Warrington, UK) using ABI Prism Big-Dye Terminator 3.1 ready reaction cycle sequencing. The DNA sequences were analyzed using Chromas (http://technelysium.com.au/?page_id=13) and BioEdit (http://www.mbio.ncsu.edu/bioedit/bioedit.html) programs. The sequences were checked for chimeric artifacts with the Check-chimera service from the ribosomal Database Project, comparing the alignments at the beginning and the end of each sequence, and alignment of the entire sequence. Phylogenetic affiliations of the partial sequences were estimated using the program Basic Local Alignment Search Tool (McGinnis and Madden, 2004). Phylogenic trees of the 16S rDNA gene sequences of DGGE fragments (*Escherichia coli* positions 110 to 482 and 969 to 1367) were constructed using the neighbor-joining method as implemented in the MEGA5 software (Tamura et al., 2011).

2. Results and discussion

2.1. p-Cresol oxidation by nitrifying SBR culture

Results from the nitrifying SBR_B showed that *p*-cresol was totally eliminated from the culture (Fig. 1). This occurred since the first cycle of the aromatic compound addition (cycle 106) where *p*-cresol was completely consumed before 2 hr (Fig. 2). The specific rate of *p*-cresol consumption (q_{pcr}) increased from 30 ± 4 mg *p*-cresol-C/(g VSS·hr) at cycle 106 to 160 ± 10 mg *p*-cresol-C/(g VSS·hr) at cycle 226. When *p*-cresol was added into the SBR_B for the first time at 200 mg C/L (cycle 296), the q_{pcr} decreased by 94% (10 ± 2 mg *p*-cresol-C/(g VSS·hr)), but 26 cycles later the q_{pcr} increased again, reaching a value of 40 ± 4 mg *p*-cresol-C/(g VSS·hr). The high decrease in q_{pcr} at cycle 296 was probably due to the high volumetric loading rate of the inhibitory compound added to the culture (400 mg *p*-cresol-C/(L·day)). These results showed that the metabolic capacity of the nitrifying consortium to oxidize *p*-cresol increased throughout the

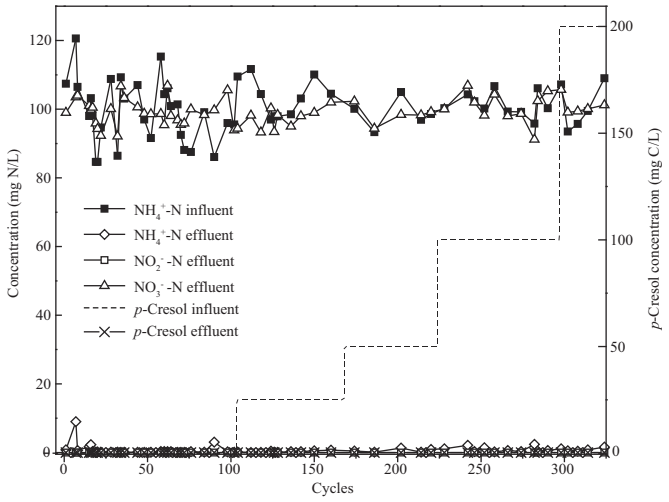

Fig. 1 – *p*-Cresol consumption and nitrification process in a SBR fed with *p*-cresol. NH_4^+-N influent: ammonium concentration in the influent; NH_4^+-N effluent: ammonium concentration in the effluent; NO_2^--N effluent: nitrite concentration in the effluent; NO_3^--N effluent: nitrate concentration in the effluent.

operation cycles. Silva et al. (2009) observed in batch nitrifying cultures that a consortium produced in steady-state nitrification was able to oxidize *p*-cresol (25 mg C/L) at a specific rate of 0.17 ± 0.06 mg *p*-cresol-C/(mg microbial protein·hr) (30 mg *p*-cresol-C/(g VSS·hr)). In the nitrifying SBR_B, higher values for q_{pcr} were obtained, showing that the SBR system allowed an increase in the metabolic capacity of the sludge to oxidize the phenolic compound. A similar behavior for the ammonium and nitrite oxidizing processes has been previously reported by Texier and Gomez (2004) in a SBR. Our results are also in accordance with Zhuang et al. (2005) which suggest that the repetitive stable and selective conditions of the SBR would be conducive to an increase in the specific rates.

p-Cresol was oxidized to the following intermediates: pOHBD and pOHBT, by the nitrifying culture (Fig. 2). As shown in Fig. 3, when *p*-cresol was added at 25 mg C/L, the intermediates pOHBD and pOHBT were accumulated in the culture medium during 26 cycles. pOHBD and pOHBT were also accumulated when 50 mg C/L of *p*-cresol was added, however the nitrifying sludge was able to eliminate both intermediates almost completely after 58 operation cycles. When the *p*-cresol concentration was increased to 100 mg C/L, the intermediates were not significantly accumulated in the culture. At the highest *p*-cresol concentration (200 mg C/L), an important accumulation of both intermediates was observed at the first cycle of addition (cycle 296) where pOHBD reached the highest concentration of about 125 mg C/L and accumulated for 8 hr. However, the nitrifying sludge could recuperate its oxidizing ability as 26 cycles later the accumulation of intermediates was low. These results showed the increase in metabolic capacity of the nitrifying sludge for oxidizing *p*-cresol intermediates. It was also observed that pOHBD tended to accumulate more than pOHBT in the SBR culture, suggesting that pOHBD was the most recalcitrant of the *p*-cresol intermediates. The nitrifying sludge was able to oxidize rapidly *p*-cresol and it did it faster and faster throughout the operation cycles. The consortium showed also a higher metabolic capacity for oxidizing the intermediates produced which appeared to be more recalcitrant than *p*-cresol. These results are relevant because they can contribute to establish suitable strategies in the operation of nitrifying SBRs fed with effluents contaminated with cresols.

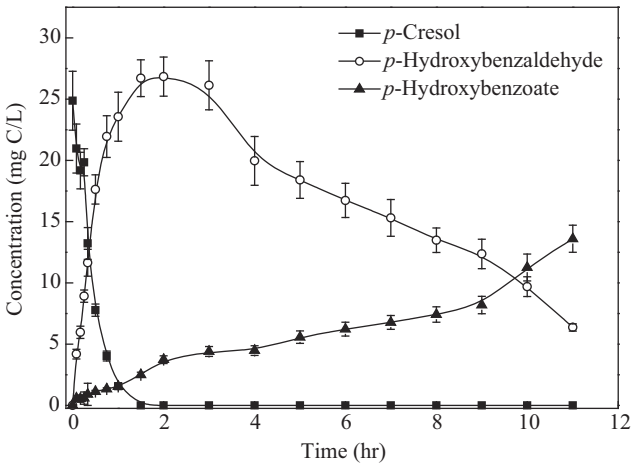

Fig. 2 – *p*-Cresol consumption and intermediates production at the first cycle of *p*-cresol addition (cycle 106) in the nitrifying SBR.

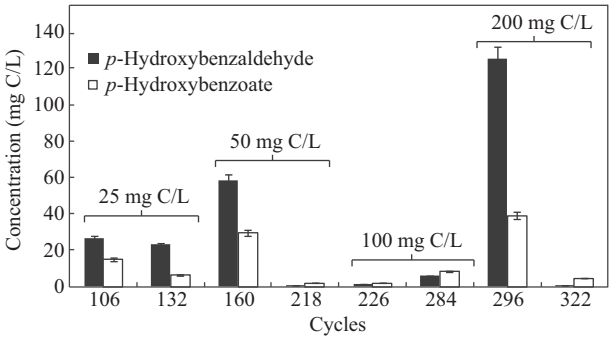

Fig. 3 – Maximum accumulation of *p*-cresol intermediates in the nitrifying SBR culture fed at different *p*-cresol concentrations (25–200 mg C/L).

TOC concentration in the effluent was negligible (1.3 ± 1.3 mg TOC/L) for each initial *p*-cresol concentration tested (except during the first *p*-cresol addition corresponding to cycle 106), indicating that *p*-cresol, pOHBD, and pOHBT were completely removed from the liquid medium and that there was no accumulation of other carbonaceous products. Furthermore, it was determined that the yield of biomass production (Y_{BM}) was only of 0.01 mg biomass-C produced/mg consumed-C, showing that the process was clearly dissimilative. These results suggested that the main end product from *p*-cresol, pOHBD, and pOHBT oxidation was CO_2. On the other hand, according to the abiotic and sterile controls, volatilization of *p*-cresol was not significant, while its adsorption onto the sludge was less than 7%.

2.2. Effect of p-cresol on nitrifying process

In spite of the *p*-cresol addition, the nitrification performance did not change significantly in the SBR_B (Fig. 1). The ammonium concentration at the end of culture was 0.5 ± 1.6 mg N/L, while the nitrate concentration was 96.6 ± 7.3 mg N/L, giving E_{NH_4} and Y_{NO_3} values of 99.0% ± 0.5% and 0.98 ± 0.07, respectively. Nitrite was not detected at the end of culture in any case. Similar results were obtained in the control SBR_A reactor without *p*-cresol addition, obtaining E_{NH_4} and Y_{NO_3} values of 98.1% ± 3.1% and 0.94 ± 0.18, respectively. These results showed that pseudo steady-state nitrification conditions were maintained through 5 months of operation in both reactors. The high E_{NH_4} and Y_{NO_3} show that there was no significant effect of *p*-cresol on the overall nitrifying performance of the SBR_B culture.

Texier and Gomez (2007) found that a nitrifying sludge tolerated up to 150 mg/L of *p*-cresol at an initial microbial protein concentration of 250 mg/L (1.25 g VSS/L) maintaining high values for E_{NH_4} and Y_{NO_3}. In the present study, in spite of using a lower inoculum concentration (1 g VSS/L), it was found that up to 200 mg C/L (equivalent to 300 mg/L of *p*-cresol) of the phenolic compound can be tolerated by the nitrifying sludge without altering E_{NH_4} and Y_{NO_3}. In terms of volumetric loading rate, up to 400 mg C/(L·day) of *p*-cresol (or 600 mg *p*-cresol/(L·day)) can be tolerated by the sludge. In a continuous activated sludge reactor fed with phenol at loading rates from 14 to 1120 mg phenol/(L·day), Amor et al. (2005) obtained high efficiencies for ammonium and phenol. However, the Y_{NO_3} decreased, suggesting that part of the consumed ammonium could be assimilated by the heterotrophs as the authors reported that the biomass increased substantially in the reactor. Vázquez et al. (2006) determined consumption efficiencies of 71% for ammonium nitrogen and 97% for phenols in a laboratory-scale activated sludge plant, but the Y_{NO_3} values were not reported. There was no evidence that nitrification was the predominant respiratory process responsible for ammonium consumption. In the study of Eiroa et al. (2005) the simultaneous removal of formaldehyde and ammonium in a lab-scale activated sludge unit was investigated. High removal efficiencies were obtained for both ammonium and formaldehyde (99.9% and 99.5%, respectively). However, at formaldehyde loading rates higher than 0.48 g COD/(L·day), the nitrate concentration in the effluent decreased. According to the authors, this decrease can be basically attributed to denitrification and ammonium assimilation by the heterotrophs. However, the authors did not present data of N_2 production or biomass yield. In our work, it is important to note that the Y_{NO_3} always remained high, showing that the main end product was nitrate and nitrification was the predominant respiratory process responsible for ammonium oxidation. This is in agreement with the low value of the yield of biomass production (Y_{BM} = 0.01 mg biomass-C produced/mg consumed-C). Only 1% of the consumed carbon was used for biosynthesis. This was probably due to the low values for C/N ratios (2.4–3.6) and the fact that a consortium with a stabilized nitrifying activity was used as inoculum, in contrast with studies that use activated sludge. Activated sludge processes can be conducive to excess sludge formation. As biomass production was limited under our experimental conditions, the assimilated ammonium was negligible and the majority of the consumed ammonium was oxidized to nitrate. As the excess sludge production in wastewater treatment represents a rising problem due to economic, environmental and regulation factors, it is useful to obtain dissimilative processes as in the present study.

2.3. DGGE profiles of bacterial communities

In order to monitor the changes within the microbial community, DGGE analysis was performed in the SBR_A and SBR_B reactors. Two samples were taken from each reactor at the first and the last cycle of operation corresponding to each initial *p*-cresol concentration tested in the SBR_B. A high similarity was observed between all the duplicates of DGGE profiles. Fig. 4 shows the DGGE profiles in both reactors. Some differences in position, intensity and number of bands were noted.

The banding patterns were analyzed by ecological indices (Table 1). The *S* index reflects the richness of species in the sludge while the *J* index reflects the predominance of species within the microbial community (Martínez-Hernández et al., 2009). The *J* index varies between 0 and 1, where the value 0 represents the dominance of one species and the value 1 represents the perfect evenness in the community. Firstly, it was noted that the sludge presented a low diversity of species as the *S* index comprised between 5 and 9 for both SBRs. This was probably due to the fact that the inoculum was obtained from a continuous reactor operated under steady state nitrification and fed with a synthetic nitrifying medium without a phenolic compound. Results showed that the low diversity in the nitrifying sludge did not affect the nitrification performance in the SBRs (Fig. 1). In this respect, Xia et al. (2005) have previously reported that a higher microbial diversity of AOB does not necessarily mean a higher ammonia oxidation activity. Moreover, Ebie et al. (2004) showed that not all AOB found in wastewater treatment processes are metabolically active as some AOB apparently did not express high levels of *amoA* mRNA. In the present study, both reactors presented a high similarity in their *S* values throughout the operation cycles. Thus, it can be concluded that the addition of *p*-cresol into the SBR_B did not cause a significant change in the richness of the community. In both reactors, up to the operation cycle 295 (lanes 15 and 16), the *J* index presented an average value of 0.68 ± 0.03, indicating that the evenness in both SBRs was similar and constant. At the operation cycle 296 in SBR_B (lane 18), the *J* index value dropped to 0.44, suggesting that certain bacterial populations would predominate when compared with the previous operation period. The same behavior was observed in the SBR_A (cycle 296, lane 17), thus this change in the evenness of the community might not be attributed to the

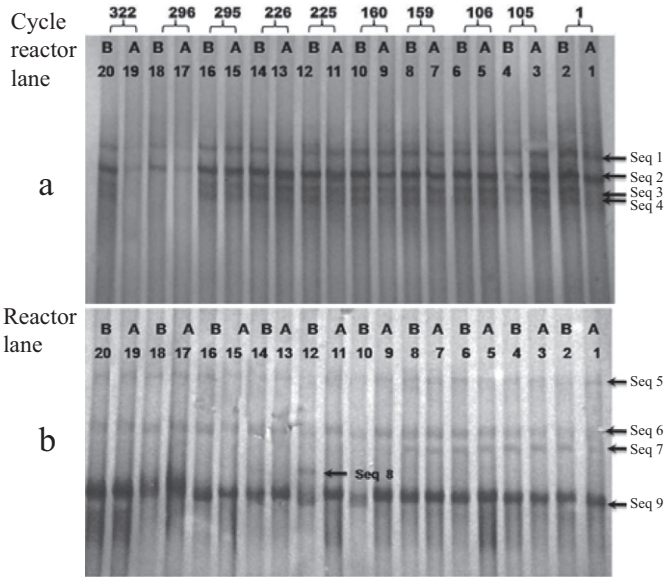

Fig. 4 – DGGE profiles of 16S rDNA from a nitrifying sludge in a SBR fed at different *p*-cresol concentrations. (a): Gradient from 30% to 38%, (b): gradient from 45% to 60%. A: Control SBR_A reactor without *p*-cresol addition and B: SBR_B reactor with *p*-cresol addition: lanes 2 and 4, at 0 mg *p*-cresol-C/L; lanes 6 and 8, at 25 mg *p*-cresol-C/L; lanes 10 and 12, at 50 mg *p*-cresol-C/L; lanes 14 and 16, at 100 mg *p*-cresol-C/L; lanes 18 and 20, at 200 mg *p*-cresol-C/L. Identified DGGE bands are labeled to the right.

addition of *p*-cresol into the reactor. The results indicated that the bacterial communities of both SBRs showed a high stability (richness and evenness) throughout the operation cycles. This could be related to the high stability of the nitrifying performance obtained in SBR_A and SBR_B cultures.

2.4. Sequence analysis of DGGE bands

The nine DGGE bands were sequenced and the phylogenetic tree was constructed (Fig. 5). The majority of the bacterial sequences grouped with members of *Proteobacteria*, with seven in the β subdivision and one in the α subdivision. The remaining one clustered with the *Nitrospira-phylum*. Table 2 shows the identified species in both SBRs at the different cycles of operation. Firstly, it was observed that 44% of the analyzed sequences were associated with AOB (Seq. 1, 2, 3 and 5) and 22% with NOB (Seq. 7 and 9). The bands related to *Nitrosomonas halophila*, the uncultured AOB bacterium and *Nitrosomonas oligotropha* were kept constant during the whole operation of both reactors. In contrast, the sequence 3 related to *Nitrosomonas europaea* only remained until cycle 295 in both reactors (lanes 15 and 16) and was again present in cycle 322 of SBR_B (lane 20). As the disappearance of the band also took place in the reactor control, it is concluded that *p*-cresol was not responsible for the absence of this bacterium. This change in the microbiological composition of the sludge might be associated with another external factor (some isolated variation in dissolved oxygen concentration, pH or temperature) or the number of operation cycles. The NOB found was related to *Nitrospira* sp. (Seq. 7) and *Nitrobacter* sp. (Seq. 9). In both SBRs, the sequence related to *Nitrospira* sp. was only present until cycle 159 (lanes 7 and 8). However, even in the absence of this population, the conversion of nitrite to nitrate was achieved (Y_{NO_3} = 0.98 ± 0.07) during all cycles of the operation probably due to the presence of *Nitrobacter* sp. As previously observed for *N. europaea*, the disappearance of the sequence related to *Nitrospira* sp. cannot be associated with the addition of *p*-cresol in SBR_B as it occurred in both reactors. Texier and Gomez (2004) showed that the kinetic behavior of nitrifying bacteria changed with the number of cycles in SBR systems. These authors observed that after 22 cycles, the activity of the NOB started to decline, whereas the AOB obtained major physiological stability throughout the cycles. These previous results are consistent with those obtained in the present study where the ammonia oxidizing community showed a higher diversity and stability in the SBRs than the nitrite oxidizing community. Our results also showed the high stability in the ammonia oxidizing community throughout the operation cycles of both reactors independent of the *p*-cresol addition. This is consistent with results of previous

Table 1 – Species richness (*S*) and evenness (*J*) indices of the bacterial communities in both SBRs. [a]

Cycles	1		105		106		159		160		225		226		295		296		322	
Lanes [a]	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
S	8	8	8	8	8	8	8	9	6	7	7	7	7	7	7	7	5	5	5	7
J	0.66	0.67	0.67	0.65	0.67	0.67	0.65	0.73	0.60	0.70	0.70	0.70	0.70	0.69	0.71	0.70	0.43	0.44	0.43	0.70

[a] SBR_A: lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19, at 0 mg *p*-cresol-C/L. SBR_B: lanes 2 and 4, without *p*-cresol addition; lanes 6 and 8, at 25 mg *p*-cresol-C/L; lanes 10 and 12, at 50 mg *p*-cresol-C/L; lanes 14 and 16, at 100 mg *p*-cresol-C/L; lanes 18 and 20, at 200 mg *p*-cresol-C/L.

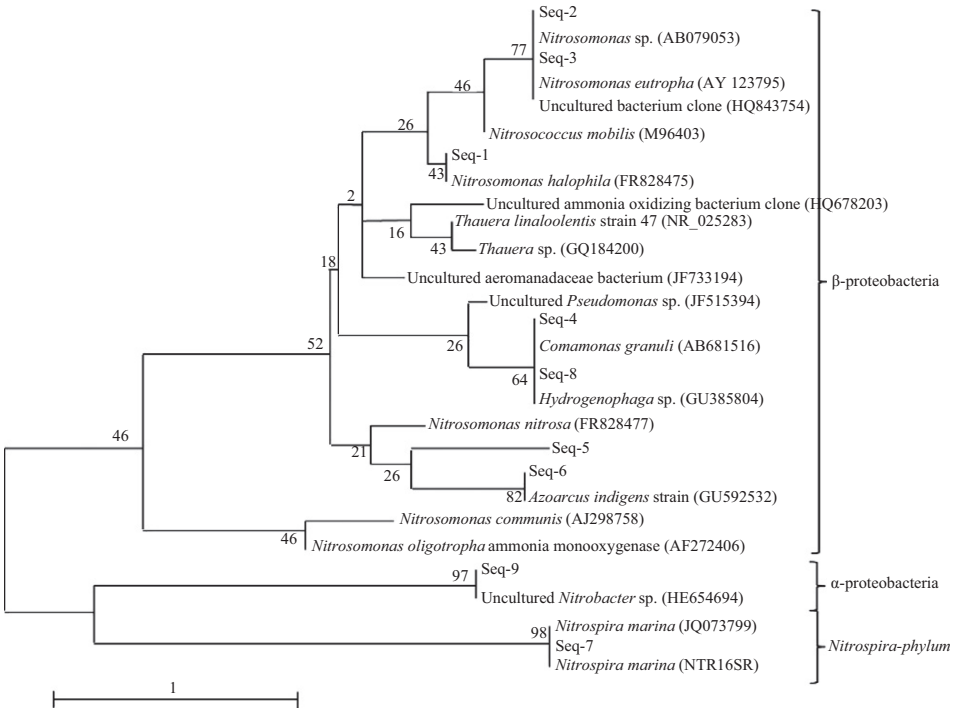

Fig. 5 – Phylogenetic tree of the main bacteria present in both reactors. Phylogenetic trees were generated based on the neighbor-joining method of the sequences of 16S rDNA–DGGE and taking as reference sequences from the databases.

studies, as it was observed that changes in operational conditions did not necessarily provoke significant changes in the community structure and diversity (Xia et al., 2005; Martínez-Hernández et al., 2009). The stability in the nitrifying communities may explain the stability and performance of the nitrification process observed during the operation of the reactors (E_{NH_4} = 98.5% ± 0.5% and Y_{NO_3} = 0.96 ± 0.04). Previous results from Yi et al. (2012) showed that the stable microbial community structure is an important factor for activated sludge performances.

In addition, two sequences were associated with organotrophic microorganisms: *Variovorax paradoxus* and *Thauera mechernichensis* (Seq. 4 and 6). *V. paradoxus* is a nitrate-reducing bacterium known to be able of oxidizing phenolic compounds as sole carbon and energy source (Baek et al., 2003). *T. mechernichensis* is a heterotrophic aerobic denitrifier able to utilize benzoate, pOHBT, leucine and phenylacetate as sole carbon source (Scholten et al., 1999). Furthermore, bacteria of the *Thauera* genus show a versatile degrading capacity of aromatic

Table 2 – Identification of nucleotide sequences analyzed from DGGE bands in both SBRs. Presence (+) and absence (–) of bacteria bands.

Sequence	Closest relatives	Lanes [a]																				%
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Identity
1	*Nitrosomonas halophila* (FR828475)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	91
2	Uncultured ammonia-oxidizing bacterium (JN099282)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	96
3	*Nitrosomonas europaea* (AB07953)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	95
4	*Variovorax paradoxus* (AF508103)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	+	92
5	*Nitrosomonas oligotropha* (EF016119)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	87
6	*Thauera mechernichensis* (NR_026473)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	94
7	*Nitrospira* sp. (EU499598)	+	+	+	+	+	+	+	+	–	–	–	–	–	–	–	–	–	–	–	–	97
8	*Hydrogenophaga bisanensis* (NR_044268)	–	–	–	–	–	–	–	–	–	–	–	+	–	–	–	–	–	–	–	–	99
9	*Nitrobacter* sp. (EF179863)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	90

[a] SBR_A: lanes 1, 3, 5, 7, 9, 11, 13, 15, 17 and 19, at 0 mg *p*-cresol-C/L at the following respective cycles: 1, 105, 106, 159, 160, 225, 226, 295, 296 and 322. SBR_B: lanes 2 and 4, without *p*-cresol addition at cycles 1 and 105; lanes 6 and 8, with 25 mg *p*-cresol-C/L at cycles 106 and 159; lanes 10 and 12, with 50 mg *p*-cresol-C/L at cycles 160 and 225; lanes 14 and 16, with 100 mg *p*-cresol-C/L at cycles 226 and 295; lanes 18 and 20, with 200 mg *p*-cresol-C/L at cycles 296 and 322.

compounds, including phenol and cresols (Mao et al., 2010). Thus, it is likely that these microorganisms would be involved in the *p*-cresol oxidation. It is noteworthy that *N. europaea*, *N. halophila* and *N. oligotropha* might also be involved in the *p*-cresol oxidation as they contain the enzyme ammonia monooxygenase (AMO), the first enzyme in the pathway of ammonia oxidation, which has also been associated with the oxidation of hydrocarbons and aromatic compounds (Keener and Arp, 1994). However, previous results from Silva et al. (2009) obtained in the same nitrifying consortium with a specific inhibitor of AMO indicated that the AMO would not be involved in the *p*-cresol oxidation, suggesting the participation of organotrophs. Both organotrophic microorganisms were present in the sludge since the first cycle of operation of SBR_A and SBR_B. This could explain why the sludge initially had the metabolic ability to consume *p*-cresol. The sequence related to *T. mechernichensis* remained in both cultures during the entire operation period while the sequence related to *V. paradoxus* was observed in both reactors until cycle 295 (lanes 15 and 16). The absence of this population at 200 mg C/L at cycle 296 (lane 18) may be related to the decrease of 94% in the specific rate of *p*-cresol consumption or to the transient accumulation of intermediates observed in cycle 296 (Fig. 3). When it was detected again in cycle 322 in SBR_B (lane 20), the specific rate of *p*-cresol oxidation increased and the intermediates were not significantly accumulated. This would confirm the participation of *V. paradoxus* in the oxidation of *p*-cresol and intermediates. Finally, sequence 8 was related to *Hydrogenophaga bisanensis* which was present only in the SBR_B at cycle 225 (lane 12). Some species of the genus *Hydrogenophaga* are considered heterotrophic denitrifiers. It has been suggested that *Hydrogenophaga* sp. may be present in the simultaneous nitrification and denitrification processes (Bo et al., 2010). However, more studies are still needed to know more on the role of this bacterium in consortia.

Results showed that there were no significant differences between the microbial dynamics in SBR_A and SBR_B. Thus, *p*-cresol did not cause significant changes in the microbiological composition of the nitrifying sludge throughout the cycles. It has been generally observed that functionally stable wastewater bioreactors have stable microbial community structures under normal operating conditions but flexibility can be needed to adapt the community structure when the composition of the influent wastewater changes (LaPara et al., 2002). When an inhibitory or toxic compound is fed into bioreactors, the microbial community structure can be disturbed, the biodiversity can be reduced and in some cases, the selective enrichment of resistant microbes which could cope with the new condition has been reported (Huang et al., 2008). However, the addition of recalcitrant or inhibitory substances into bioreactors has not always been related to significant changes of the microbial community structure (Emanuelsson et al., 2006), as observed in the present study with *p*-cresol addition. Recently, Wang et al. (2011) have reported that the bacterial community composition in a biofilm hydrolytic–aerobic recycling system treating an anthraquinone dye (Reactive Blue 19) was relatively stable at different recycling fluxes and the difference between the dye removal efficiencies cannot be associated with a shift of the microbial community structure in the system. Results from the present study suggested that the specific rate of *p*-cresol consumption was enhanced in SBR_B due to a higher enzymatic capacity rather than changes in the microbial community composition. However, it is clear that more physiological and molecular studies should be carried out in SBR systems to understand better how the microbial dynamics can affect the physiological response of the sludge under different experimental conditions.

3. Conclusions

The mineralization of *p*-cresol and intermediates (pOHBD and pOHBT) might be related to the presence of heterotrophic bacteria as *V. paradoxus* and *T. mechernichensis*. The nitrifying activity of the sludge was probably realized by some AOB related to *N. halophila*, *N. oligotropha*, *N. europaea* and an uncultured AOB and NOB as *Nitrospira* sp. and *Nitrobacter* sp. The nitrifying sludge was able to consume and mineralize up to 400 mg *p*-cresol-C/(L·day) (or 600 mg *p*-cresol/(L·day)). Moreover, the nitrifying performance of the SBR culture was not affected by *p*-cresol addition. The bacterial communities showed a high stability throughout the cycles independent of the *p*-cresol addition. These results showed that SBR might be a good alternative to eliminate simultaneously ammonium and aromatic compounds from wastewaters.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found online at http://dx.doi.org/10.1016/j.jes.2014.06.033.

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