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Stoichiometry and kinetic of the aerobic oxidation of phenolic compounds by activated sludge

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HIGHLIGHTS

- Study of the aerobic degradation of several phenolic compounds by activated sludge.
- Biodegradation rate: catechol > phenol >> pyrogallol ≅ resorcinol > hydroquinone.
- Toxicity: pyrogallol >> catechol ≅ resorcinol >> phenol > hydroquinone.
- Oxidation coefficients are reported.
- The role of some enzymes involved in the degradation pathways is discussed.

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ABSTRACT

The aerobic degradation of phenol (PH), catechol (CA), resorcinol (RE), pyrogallol (PY), and hydroquinone (HY) by phenol-acclimated activated sludge was investigated. A Haldane-type dependence of the respiration rate on PH, RE, and HY was observed; CA and PY exhibited a biphasic respiration pattern. According to the initial biodegradation rate, tested compounds were ordered as follows: CA > PH >> PY RE > HY. Also, they exhibited the following degree of toxicity to their own degradation: PY >> CA RE >> PH > HY. Oxidation coefficients for PH, PY, RE, and HY were constant as a function of the consecutive additions of the compound. Conversely, an increase of $Y_{O/S}$ from 1 to 1.5 $molO_2$ $molCA^{-1}$ was observed during repeated additions of CA. The role of some enzymes involved in the aerobic degradation pathways of the tested compounds is discussed and related to the obtained results.

1. Introduction

Phenolic compounds are considered as priority pollutants by US Environment Protection Agency (US-EPA). Due to the large use of phenol and its derivatives in many industries, phenolic compounds are widespread in the environment. Several industries, such as petroleum processing plants, oil refineries, coke oven, and pharmaceutical generate large amounts of phenolic wastewaters (Lepik and Tenno, 2011; Pramparo et al., 2012). Phenol is used as an intermediate in the production of phenolic resins, which are used in the plywood, adhesive, construction, automotive, and appliance industries, in the production of synthetic fibers, and for epoxy resin precursors (US-EPA, 2000). Hydroxy-derivatives of phenol, such as catechol, resorcinol, hydroquinone, and pyrogallol, are also broadly used in several industrial processes. Catechol and hydroquinone are used as photographic and fur dyes developers, as intermediates for antioxidants in rubber, and lubricating oils, as polymerization inhibitors, and in pharmaceuticals. Resorcinol is an essential component used in the manufacture fiber reinforced rubber mechanical goods. Several adhesives for wood bonding applications are formulated from resorcinol–formaldehyde resins. Resorcinol is a chemical intermediate in the manufacture of UV-light screening agents for the protection of plastics, dye-stuffs, pharmaceuticals, flame retardants, explosive primers, and antioxidants (INDSPEC, 2004). The current main commercial application of pyrogallol is the production of pharmaceuticals, pesticides, and hair dyes. In analytical chemistry, pyrogallol is used as a complexing agent, reducing agent, and, in alkaline solution, as an indicator of gaseous oxygen (NIH, 2012).

The presence of phenols and its derivatives in the environment poses a significant risk to water reservoirs and soils. Phenolic wastewaters may not be conducted into open water without treatment because of the toxicity of phenols. Several physicochemical methods such as ozonation, Fenton's reagent, UV, hydrogen peroxide, or a combination of them can be used to re-

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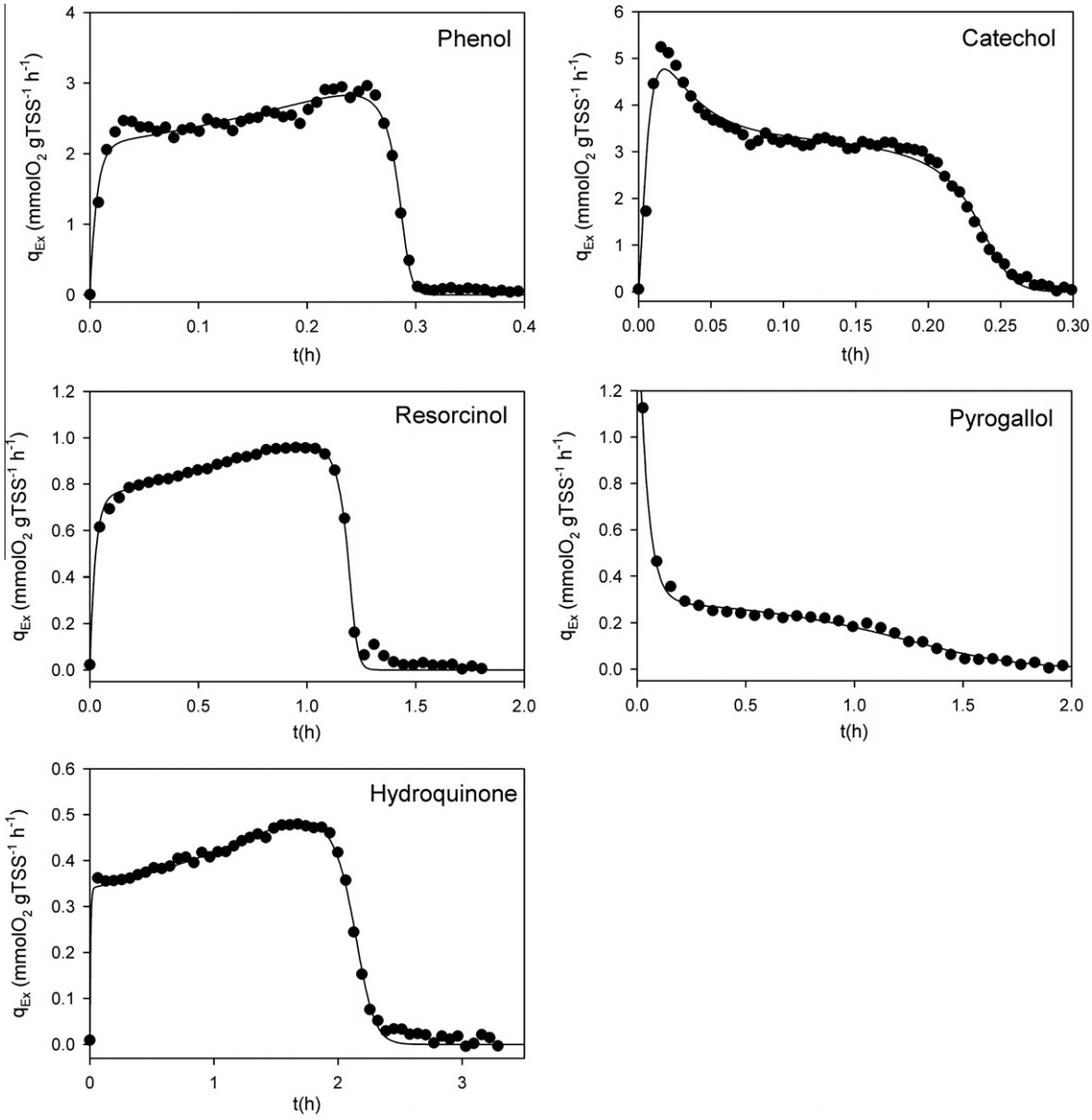

Fig. 1. Examples of the specific exogenous respiration rate (q_{Ex}) as a function of time during the aerobic biodegradation of different phenolic compounds by phenol-acclimated activated sludge.

move phenolic compounds from industrial wastewater. Because these treatments are usually complex and expensive, biological methods are preferred (Busca et al., 2008). A great number of studies on biodegradation of phenolic compounds by pure cultures as well as mixed bacterial consortia have been conducted over the last decades. Several studies have focused attention on the aerobic biodegradation of single and mixtures of phenolic compounds by activated sludge (Orupold et al., 2001; Antizar-Ladislao and Galil, 2004; Bajaj et al., 2008; Lepik and Tenno, 2011; Pramparo et al., 2012). Moreover, control strategies in the removal phenolic compounds by activated sludge have been developed also (Yoong et al., 2000; Buitron et al., 2005). However, detail information concerning the stoichiometry and the stability of the biodegradation ability of activated sludge to remove phenolic compounds has been less studied. Considering that these issues are essential for the understanding of the behavior of the biodegradation process of phenolic compounds, the objective of the present work was to study the stoichiometry and the stability of the biodegradation ability of the aerobic degradation of phenol (PH), catechol (CA), resorcinol (RE), pyrogallol (PY), and hydroquinone (HY) by phenol-acclimated activated sludge.

2. Methods

2.1. Chemicals and reagents

Phenol (PH) (loose crystals, >99%) and resorcinol (RE) (ACS reagent, >99%) were obtained from Sigma (St. Louis, MO, USA). Catechol (CA), pyrogallol (PY), and hydroquinone (HY) were analytical grade from Biomed Inc., (Aurora, Ohio). Dehydrated cheese whey was from Food S.A. (Villa Maipú, Argentina). All inorganic salts were commercial products of reagent grade from Anedra (San Fernando, Argentina).

2.2. Activated sludge and culture conditions

Activated sludge used in this study were cultured in a laboratory scale (2.5 L) cylindrical semicontinuous fill and draw reactor.

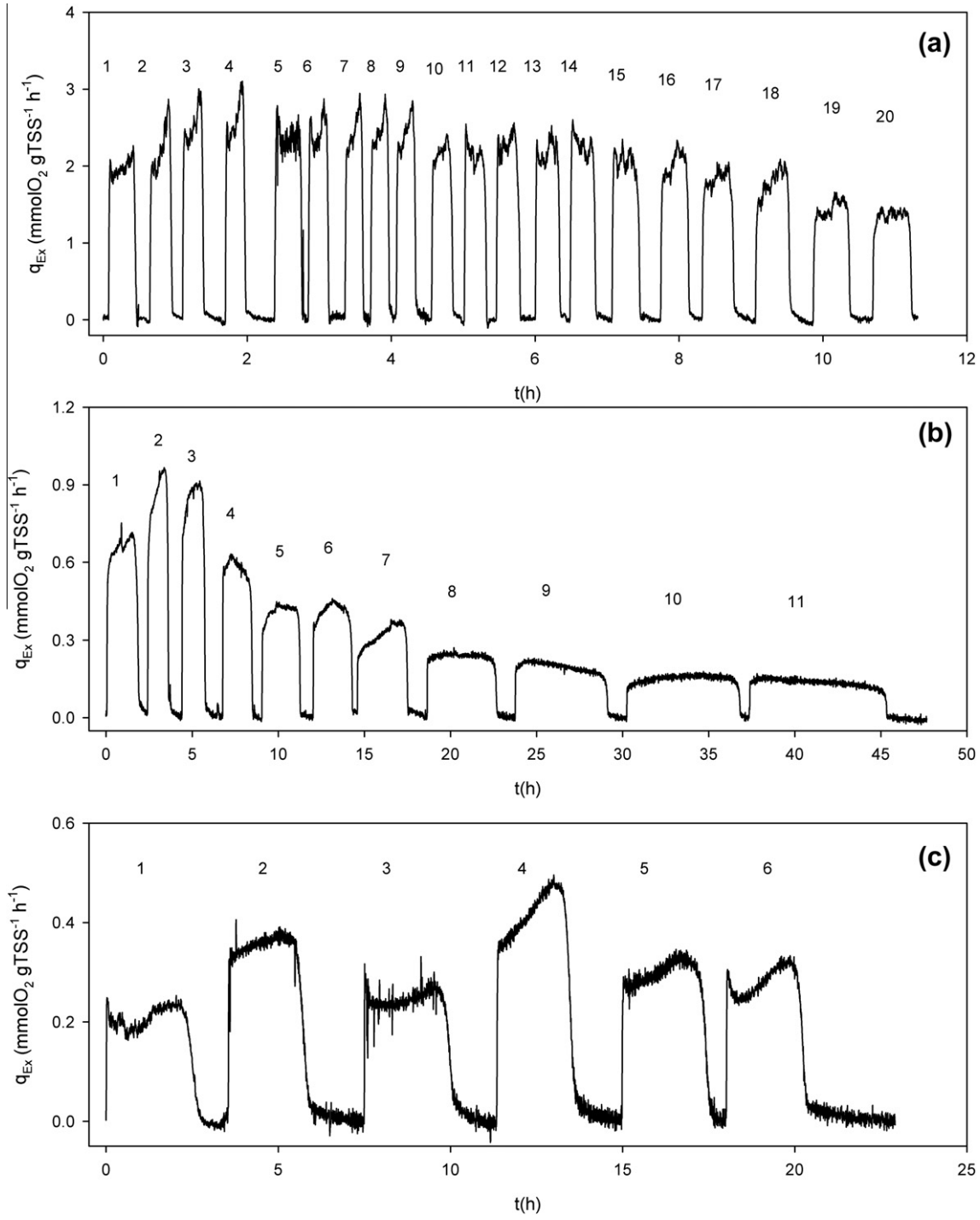

Fig. 2. Specific exogenous respiration rate (q_{Ex}) of phenol-acclimated activated sludge as a function of time during the consecutive addition of (a) phenol, (b) resorcinol, and (c) hydroquinone. Numbers indicate the pulse number.

Aeration was provided at the bottom of the reactor through an airstone using two air pumps at 2 L min^{-1}; dissolved oxygen (DO) concentration was maintained above 4 mgO_2 L^{-1}. Phenol-acclimated activated sludge was obtained as follows. Initially, the reactor was fed with a model wastewater of the dairy industry of the following composition (Ferro Orozco et al., 2008): dehydrated cheese whey 1000 mg, $(NH_4)_2SO_4$ 940 mg, K_2HPO_4 500 mg, and KH_2PO_4 250 mg; all components were diluted in 1 L of tap water. The hydraulic retention time was 80 h and a solids retention time of 30 days was maintained by direct wastage. After 70 days of operation, the model wastewater was replaced by a culture medium with PH as the sole carbon-limiting source (Nuhoglu and Yalcin, 2005): $(NH_4)_2SO_4$ 226 mg L^{-1}, K_2HPO_4 500 mg L^{-1}, KH_2PO_4 250 mg L^{-1} $MgSO_4{\cdot}7H_2O$ 25.2 mg L^{-1}, $MnSO_4{\cdot}H_2O$ 2.52 mg L^{-1}, $CaCl_2$ 2 mg L^{-1}, $FeCl_3$ 1.2 mg L^{-1}, phenol 300 mg L^{-1}. pH was adjusted to 7.0 ± 0.05 by adding a few drops of concentrated solutions of NaOH or HCl. During this phase the solids retention time was increased to 45 days in order to prevent biomass washout. The reactor was monitored periodically by measurements of total suspended solids (TSS), soluble chemical oxygen demand (COD) consumption rate, and total phenols (TPh) consumption rate. Biomass was considered acclimated to phenol when the specific

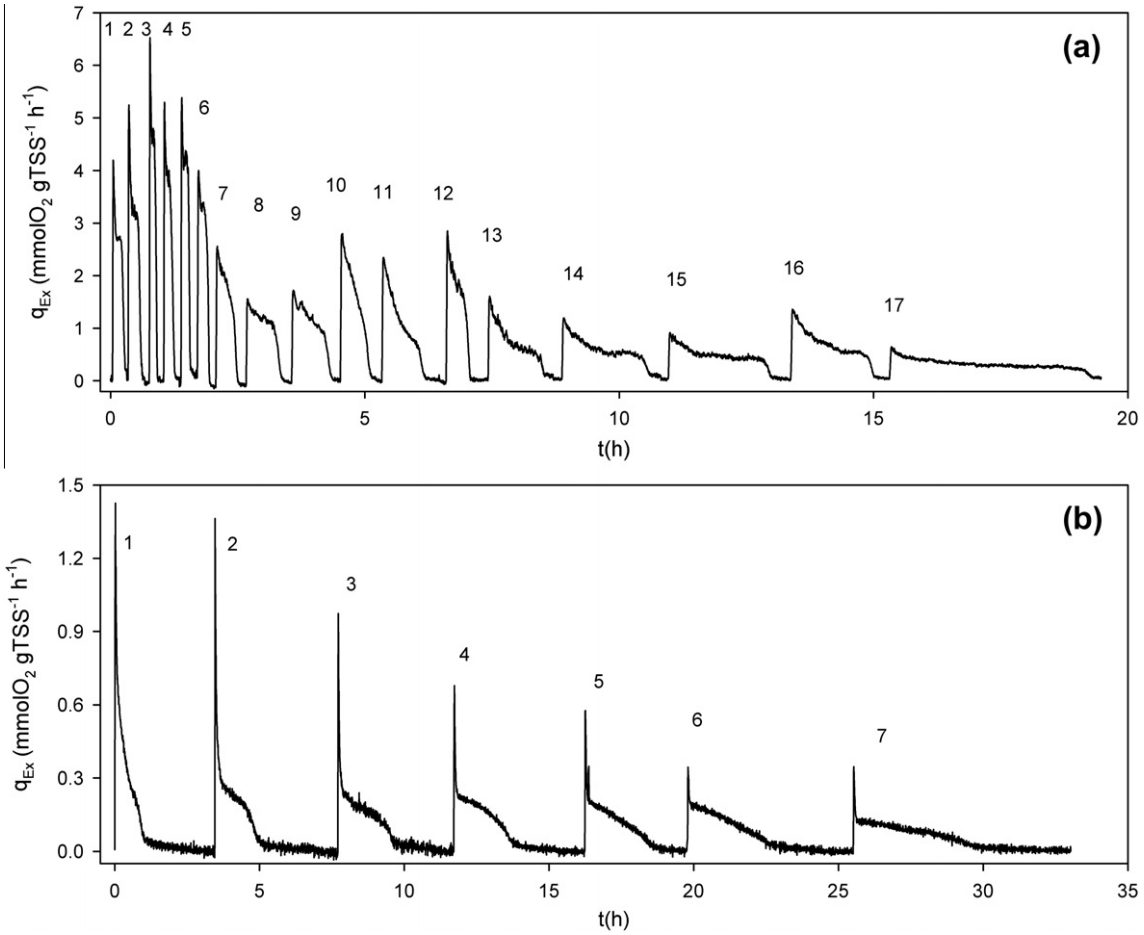

Fig. 3. Specific exogenous respiration rate (q_{Ex}) of phenol-acclimated activated sludge as a function of time during the consecutive addition of (a) catechol, and (b) pyrogallol. Numbers indicate the pulse number.

consumption rates of COD and TPh were constant at least for 2 weeks. These acclimated activated sludges were used in all further experiments.

2.3. Biodegradation of phenolic compounds by phenol-acclimated activated sludge

Aerobic biodegradation kinetics of the tested compounds (phenol, resorcinol, catechol, pyrogallol, and hydroquinone) by phenol-acclimated activated sludge was assessed using an open (flowing gas/static liquid) respirometer. Before performing the assays, activated sludge samples (250–500 mL) were washed three times using a phosphate buffer (15 mM, pH = 7) and resuspended in 500 mL of the above mentioned buffer. Then, 0.5 mL of micronutrient solutions M1 and M2 were added. The composition of M1 was (g/100 mL): $FeSO_4{\cdot}7H_2O$ 1.5, $ZnSO_4{\cdot}7H_2O$ 0.5, $MnSO_4{\cdot}H_2O$ 0.3, $CuSO_4{\cdot}5H_2O$ 0.075, $CoCl_2{\cdot}6H_2O$ 0.015, and citric acid 0.6. Micronutrient solution M2 contained the following (g/100 mL): $(NH_4)_6Mo_{7}O_{24}{\cdot}4H_2O$ 0.05, BO_3H_3 0.01, KI 0.01.

500 mL of washed activated sludge were poured into the open respirometer. Agitation was provided by a magnetic stir-bar; the respirometer was aerated continuously by an air pump. Air was set to a stable flow rate (1.0 L min^{-1}) using a high precision rotameter (Bruno Schilling model MB 60V, Argentina). Operation temperature was 25 ± 0.5 °C. Dissolved oxygen (DO) concentration as a function of time was measured using a polarographic DO probe YSI (model 5739). DO data were acquired by a personal computer interfaced to a DO monitor YSI (model 58) at 1 data/s. Before the addition of the tested compound, the oxygen mass transfer coefficient of the respirometer (k_La) was obtained using a non-steady state procedure. Then, when a stable DO concentration was observed, the respirometer was spiked with the tested compound and the dissolved oxygen (DO) concentration as a function of time was recorded. For PH, CA, and RE, pulses of S_0 = 1 mM were added into the respirometer. Pulses of 0.9 mM were added when HY was tested. The added concentration of PY was 0.7 mM. The oxygen uptake rate (OUR) associated with the substrate oxidation (OUR_{Ex}) was calculated from the DO mass balance in the respirometer:

$$OUR_{Ex} = k_La(C_e - C) - \frac{dC}{dt} \quad (1)$$

Table 1
Specific mean substrate consumption rates during the first pulse (q_{Smean1}), cumulative substrate concentration that produces 50% of inhibition (IC_{50}), and oxidation coefficients ($Y_{O/S}$) corresponding to the aerobic degradation of different phenolic compounds by phenol-acclimated activated sludge.

Phenolic compound	q_{Smean1}[a] (mmolS gTSS^{-1} h^{-1})	IC_{50} (mM)	$Y_{O/S}$ (molO$_2$ molS^{-1})
Catechol	3.44	7.5	0.81–1.70[b]
Phenol	1.21	n.a.	1.57 ± 0.07[c]
Pyrogallol	0.29	2.0	1.49 ± 0.09[c]
Resorcinol	0.27	8.0	2.00 ± 0.11[c]
Hydroquinone	0.13	n.a.	2.06 ± 0.41[c]

n.a.: not applicable.
[a] q_{Smean1} Value corresponding to the first addition of the tested substrate.
[b] Range of the obtained $Y_{O/S}$ values for CA.
[c] Mean ± confidence interval at a 95% confidence level.

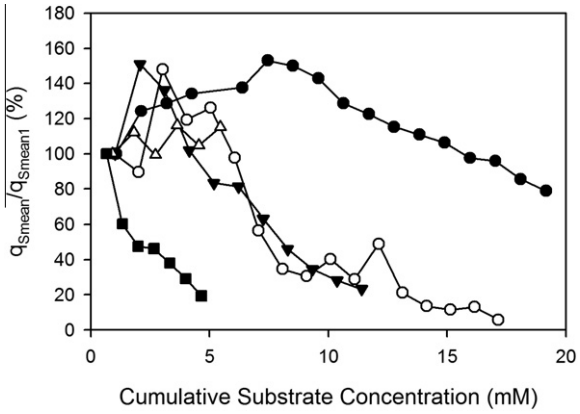

Fig. 4. Effect of the cumulative substrate concentration on the specific mean substrate consumption rate (q_{Smean}): phenol (filled circles), catechol (open circles), resorcinol (filled triangles), hydroquinone (open triangles), pyrogallol (filled squares). For each phenolic compound, results were expressed as a percentage of the value corresponding to the first pulse (q_{Smean1}).

where C_e is the DO concentration in the absence of an oxidizable substrate, and C is the instantaneous DO concentration. In order to compare experiments with different biomass concentrations, specific exogenous respiration rates (q_{Ex}) were calculated as the ratio between OUR_{Ex} and the biomass concentration in the respirometer. The biomass concentration was constant in each experiment. The ratio of substrate to biomass (g/gTSS) was 0.03 for PY, 0.04 for PH, RE, HY; and 0.07 for CA. Based on the OUR_{ex} values as a function of time, the oxygen consumed (OC) during the substrate oxidation was calculated as follows:

$$OC = \int_0^t OUR_{Ex} dt \quad (2)$$

The oxidation coefficient ($Y_{O/S}$), which represents the amount of oxygen consumed per unit of substrate oxidized, was calculated as the ratio OC_m/S_0, where OC_m is the maximum amount of oxygen consumed to degrade the added substrate, and S_0 is the initial substrate concentration. For all the tested compounds, abiotic control experiments (e.g., without biomass) were negative with regard to the oxygen consumption (data not shown).

2.4. Analytical procedures

Total suspended solids (TSS, mg/L) were used as a measure for the biomass concentration. Known sample volumes (8 mL in this work) were poured into pre-weighted centrifuge tubes, centrifuged and washed twice with distilled water, and placed at 105 °C for 24 h; TSS of each sample was calculated as the difference between final weight (dry sample + tube) and initial weight (tube alone) divided by the sample volume. Duplicate biomass measurements were performed to reduce experimental errors; average and maximum relative errors for TSS were 4% and 13%, respectively. Soluble COD was determined as follows: 3 mL of culture samples were centrifuged for 5 min at 13,000 rpm (Eppendorf 5415C); then COD of the supernatant was determined using a commercial test (Hach Cat. No. 21259). Total phenols (TPh) concentration of the supernatant was measured using the 4-aminoantipyrine method (Vazquez-Rodriguez et al., 2006).

3. Results and discussion

3.1. Biooxidation phenolic compounds by phenol-acclimated activated sludge

The specific exogenous respiration rate (q_{Ex}) of phenol-acclimated activated sludge during the biooxidation of several phenolic compounds was studied. Obtained results demonstrate that phenol-acclimated activated sludge were able to metabolize all the tested compounds. Fig. 1 shows that obtained profiles of q_{Ex} as a function of time corresponding to the addition of phenol (PH), resorcinol (RE), and hydroquinone (HY) were similar, suggesting that all these substrates were metabolized by the same enzyme, probably phenol 2-monooxygenase (PHMO). This enzyme catalyzes the oxidation of PH to CA by molecular oxygen (Enroth et al., 1998); however, other phenolic compounds, such as RE and HY, can also be oxidized to produce 1,2,4-benzenetriol (Maeda-Yorita and Massey, 1993; Eppink et al., 2000).

Among the various substrate inhibition models, the Haldane equation has been widely used to describe PH biodegradation kinetic of whole cells. According to this model, the critical substrate concentration ($S*$) can be defined as the substrate concentration (S) that produces the maximum specific oxygen consumption rate (q_{Exm}) (Christen et al., 2012). If the initial substrate concentration (S_0) is higher than $S*$, then initial q_{Ex} is lower than q_{Exm}. Because the substrate is metabolized, S decreases as a function of time and it tends to $S*$; in this case, an increase on q_{Ex} towards its maximum value as a function of time can be observed. Then, S becomes lower than $S*$ because the reaction continues. For this reason, q_{Ex} diminishes and eventually returns to zero due to the substrate depletion. Thus, respirometric profiles corresponding to PH, RE,

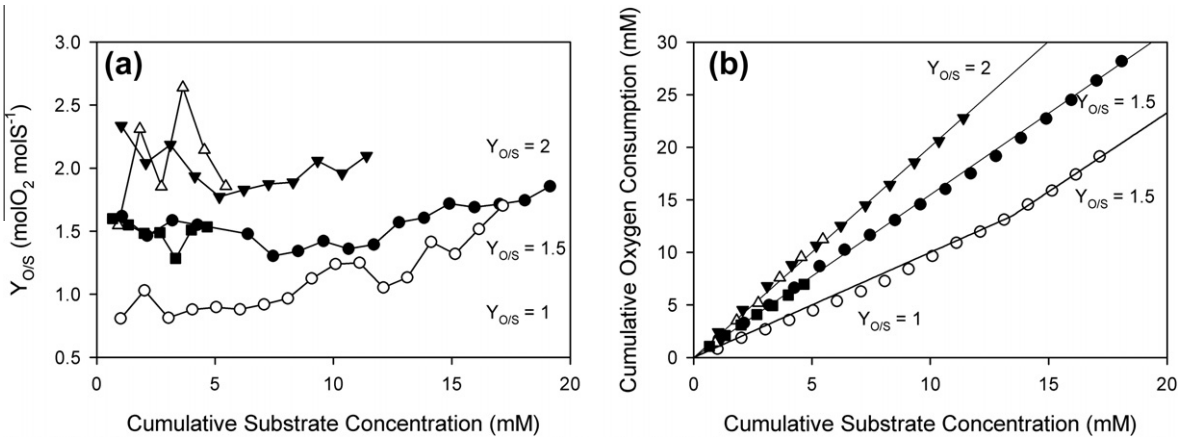

Fig. 5. (a) Oxidation coefficient ($Y_{O/S}$), and (b) cumulative oxygen consumption as a function of the cumulative substrate concentration: phenol (filled circles), catechol (open circles), resorcinol (filled triangles), hydroquinone (open triangles), pyrogallol (filled squares).

and HY suggest that critical concentrations of these compounds ($S*$) were lower than S_0.

Fig. 1 shows that obtained respirograms during the biodegradation of catechol (CA) and pyrogallol (PY) exhibited a more complex behavior than those corresponding to PH. When CA or PY were added to the respirometer, a sharp peak of respiration was observed. Then, respiration rate decreased gradually to a lower stable value; finally, when the substrate was depleted, q_{Ex} returns to zero. Mayer and Que (1984) observed a similar pattern with regard to the oxygen uptake rate during the oxidation of pyrogallol by catechol 1,2-dioxygenase (CA12DO). After the addition of pyrogallol, those authors observed a burst of oxygen consumption, which then decay into a new, nearly constant uptake rate. Additionally, those authors also demonstrated that the change in rate over the course of the reaction was due to the production of intermediates that were further oxidized. Other authors (Wang et al., 2006; Matera et al., 2010) also reported that CA12DO catalyze the oxidation of PY.

3.2. Changes on the specific respiration rate (q_{Ex}) during the biodegradation of phenolic compounds

The effect of consecutive additions of single compounds on the respiration rate of phenol-acclimated activated sludge are shown in Figs. 2 and 3. Maximum q_{Ex} (q_{Exm}) value corresponding to PH increased during the first two pulses; then, it became stable ranging between 2 and 3 $mmolO_2\ gTSS^{-1}\ h^{-1}$ up to the addition of the pulse #9. Finally, q_{Exm} decrease gradually; the lower q_{Exm} value was 1.4 $mmolO_2\ gTSS^{-1}\ h^{-1}$, corresponding to the pulse #20 of PH (Fig. 2a). With regard to RE, the addition of the first two pulses of RE produced an increase on q_{Exm} values; however, conversely to PH, a noticeably inhibition of the respiration rate was observed due to the consecutive additions of RE (Fig. 2b). q_{Exm} corresponding to last pulse (pulse #11) were about 30% of the overall maximum value (pulse #2). Fig. 2c shows that q_{Exm} values corresponding to HY were almost constant as a function of the added pulse. However, these respiration rates were about one tenth the values obtained with PH.

The trend of the respiration rate as a function of the number of added pulse of CA, or PY (Fig. 3) was similar to the observed with RE (Fig. 2); in both cases, consecutive additions of CA, or PY determined a gradually inhibition of q_{Exm} values. Fig. 3a shows that q_{Exm} corresponding to CA increased during the first three pulses up to about 6 $mmolO_2\ gTSS^{-1}\ h^{-1}$, and then, it decreased sharply throughout the pulses #4 to #17, indicating that CA was toxic against its own biodegradation. Respirograms corresponding to PY were similar to those obtained with CA; however, q_{Exm} values corresponding to PY were much lower (about five times lower) than those corresponding to CA. PY was even more toxic than RE; in this case, a decrease of about 80% of q_{Exm} value was obtained when the pulse #7 was added (Fig. 3b).

Taking into account the obtained q_{Ex} values during the first pulses (Figs. 2 and 3), the tested phenolic compounds can be ordered as follows: CA > PH >> PY RE > HY. This sequence agree with other authors that studied the oxygen consumption rate in the presence of phenolic compounds by intact cells, and enzyme (cell-free) preparations (Groseclose and Ribbons, 1981; Orupold et al., 2001; Ahuatzi-Chacón et al., 2004; Lepik and Tenno, 2011). The case of pyrogallol is controversial. Neujahr and Varga (1970) studied the oxygen consumption of an enzyme preparation from *Trichosporon cutaneum* grown on PH. Those authors found that the oxygen consumption rate in the presence of PY was 15% of the rate in the presence of CA, which is very close to the results obtained in the present work. Matera et al. (2010) demonstrated that the intradiol cleavage activity of CA12DO from *Rhodococcus opacus* with PY as the substrate was 15–25% the activity in the presence of CA. On the contrary, Groseclose and Ribbons (1981) reported the following sequence with regard to the oxygen consumption rate of several phenolic compounds by *Azotobacter vinelandii*: PY >> - CA > PH > RE. Wang et al. (2006) found that the cleavage activity of CA12DO from *Pseudomonas aeruginosa* with PY was 180% of the activity measured with CA was the substrate. These differences could reflect the effect of the substrate used to acclimate the activated sludge. For example, while in this work PH was used to feed the activated sludge reactor, Groseclose and Ribbons (1981) used RE. Besides, it must be considered that Groseclose and Ribbons (1981), and Wang et al. (2006) performed those assays at pH 7.5, while the experiments presented in this work were performed in phosphate buffer (15 mM) at pH 7. Abrash et al. (1989) demonstrate that PY can react with molecular oxygen in weakly alkaline conditions (e.g. pH = 7.5) to form purpurogalline, however, this reaction is negligible at pH = 7. Thus, in addition to the above mentioned difference on the feeding substrate, the extremely high activity reported by Groseclose and Ribbons (1981), and Wang et al. (2006) when PY was tested may also reflect the non-enzymatic oxidation of PY.

Maximum specific exogenous respiration rates (q_{Exm}) corresponding to the tested compounds in the present work were within the range reported by other authors. Yoong et al. (2000) reported q_{Exm} values ranged from 0.7 to 1.4 $mmolO_2\ gTSS^{-1}\ h^{-1}$ for activated sludge samples grown in a sequencing batch reactor with PH as the carbon source. Tallur et al. (2008) studied the oxidation of several phenolic compounds by *Micrococcus*; those authors found q_{Exm} values of 4.2, and 4.8 $mmolO_2\ gTSS^{-1}\ h^{-1}$ during the consumption of PH, and CA, respectively. Viggor et al. (2008) reported q_{Exm} values ranged from 4.5 to 22.8 $mmolO_2\ gTSS^{-1}\ h^{-1}$ during the PH degradation by different phenol-degrading pseudomonads. Pramparo et al. (2012) studied the aerobic biodegradation of CA, RE, and HY by activated sludge acclimated to *p*-nitrophenol. Those authors measured q_{Exm} values of about 0.3 $mmolO_2\ gTSS^{-1}$ - h^{-1} for CA, which were much lower than those obtained in the present work. This difference can be attributed to the substrate used to acclimatize the biomass, *p*-nitrophenol (Pramparo et al., 2012) or phenol (this study). The first step of the aerobic biodegradation of PH is its hydroxylation to CA (Ellis et al., 2006). However, according to Spain and Gibson (1991), biodegradation of *p*-nitrophenol begins with its conversion to HY but not to CA. For this reason, q_{Exm} values of *p*-nitrophenol acclimated sludge were much lower than those corresponding to the phenol acclimated sludge used in the present work.

3.3. Specific mean substrate consumption rate (q_{Smean})

Fig. 1 shows that respirometric profiles corresponding to the tested phenolic compounds were not comparable, particularly with regard to the maximum q_{Ex} value for each pulse. While CA and PY exhibited a high and sharp initial peak of the oxygen uptake rate, respirometric profiles corresponding to PH, RE, and HY suggested a Haldane-type dependence of q_{Ex} as a function of the substrate concentration. Due to this difference of the oxygen uptake kinetics, q_{Exm} values were not comparable. Moreover, q_{Exm} values are quite sensitive to the noise of the DO signal. For these reasons, to evaluate the toxic effect of the tested phenolic compounds to its own biodegradation, the specific mean substrate consumption rate (q_{Smean}) was calculated as follows. When a given oxidizable substrate is added to the respirometer, an increase of the OUR is observed; then, once the degradation of the substrate is accomplished, the OUR falls to a value close to the observed prior the pulse. The total degradation time (t_d) can be defined as the time interval between the increase and the decrease of the OUR. This approach was adopted by Buitron et al. (2005) as a control strategy to optimize the reaction time in an SBR based on DO profiles. If the added substrate concentration (S_0) is known, then the specific

mean substrate consumption rate (q_{Smean}) can be calculated as follows:

$$q_{Smean} = \frac{S_0}{t_d X} \quad (3)$$

where X is the biomass concentration in the open respirometer. While q_{Exm} depends on the shape (e.g., on the kinetics) of a respirometric profile, q_{Smean} is a more robust coefficient to measure the specific degradation activity because it only depends on the total degradation time. Moreover, in some cases obtained q_{Ex} profiles vary as a function of the added pulse, making very difficult the comparison of q_{Exm} between different phenolic compounds. Table 1 shows that with regard to the specific mean substrate consumption rates during the first pulse (q_{Smean1}), the tested phenolic compounds can be ordered as follows: CA > PH >> PY RE > HY, in accordance with the sequence previously obtained based on q_{Exm} values (Section 3.2).

Fig. 4 shows the effect of the cumulative substrate concentration (e.g., the product of the pulse number and its substrate concentration) on q_{Smean} corresponding to the tested phenolic compounds. For comparison purposes, in Fig. 4 results were expressed as a percentage of the value corresponding to the first pulse (q_{Smean1}). PY was the most toxic among the tested phenolic compounds; in this case, the IC_{50} (e.g., the cumulative substrate concentration necessary to reduce 50% the initial specific substrate consumption rate) was 2 mM (Fig. 4). Toxicity of CA was lower than the observed by PY, the IC_{50} corresponding to CA was about 7.5 mM.

Catechol 1,2-dioxygenase (CA12DO), and catechol 2,3-dioxygenase (CA23DO) catalyze the oxidative cleavage of PY and CA (Matera et al., 2010). Two mechanisms of inactivation of dioxygenases can be found in literature. Mayer and Que (1984) found that during the oxidative cleavage of PY by CA12DO, the enzyme was inactivated due to the formation of a pyrogallol-derived intermediate. Moreover, the inactivation degree was only a function of the amount of oxidized PY. Vaillancourt et al. (2002) proposed a general mechanism for O_2-dependent inactivation of extradiol dioxygenases by catecholic substrates: during the catalytic cycle the intermediate ternary complex (enzyme-substrate-dioxygen) dissociates releasing superoxide and causing the oxidation of the active site Fe(II) to Fe(III) (inactive form of the enzyme). In the present work, when PY was added to the respirometer these two mechanisms could be responsible for the inactivation the respiration activity. However, when CA was tested, PY was absent from the reaction mixture and only the O_2-dependent inactivation mechanism could exert inhibition; this difference could explain the lesser toxicity of CA in comparison with PY.

Fig. 4 shows that the toxicity of RE was similar to the obtained by CA; in this case, IC_{50} was about 8 mM. At first glance, this result seems quite surprising since RE was used by other authors as a model substrate during the study of the reaction mechanism of PHMO (Maeda-Yorita and Massey, 1993). However, it must be pointed out that the product of the hydroxylation of RE by PHMO is 1,2,4-benzenetriol (BTOL). Although BTOL could be oxidized by hydroxyquinol 1,2-dioxygenase to produce 3-hydroxy-cis,cis-muconate, this enzyme cannot be induced by PH, which was the substrate employed in the acclimation of activated sludge in the present work (Eppink et al., 1997). According to Kawanishi et al. (1989), the autoxidation of BTOL in the presence of Cu(II) produces hydroxyquinone which is further oxidized to yield an active species, which is the actual responsible for the toxicity of BTOL. Taking into account that trace amounts of Cu(II) were present during the assays performed in the present work (see Section 2.4), the inhibition of the degradation rate of RE as a function of the cumulative RE concentration (Fig. 4) could be due to the toxicity of BTOL or its autooxidation product. Moreover, during these assays soluble COD values were constant and close to the background value, indicating that all the above mentioned processes were intracellular or occurred at the cell surface.

Fig. 4 shows that changes on q_{Smean} corresponding to PH, and HY consumptions as a function of their cumulative concentrations were similar to the trend of q_{Ex} as a function of the number of pulse added (Fig. 2b, c) (Section 3.2). PH was less toxic in comparison with PY, CA, and RE; the lowest q_{Smean} corresponding to PH obtained in the present work was 70% of the initial value. Conversely to the other tested phenolic compounds, a slight increase of q_{Smean} as a function of the added HY was observed. Because PH was the sole carbon source during the acclimation of the activated sludge used in the present work, oxygenases of the phenol aerobic degradation pathway (e.g. PHMO) were actively expressed (Ahuatzi-Chacón et al., 2004; Mazzoli et al., 2007). Under these conditions, HY should be readily oxidized by PHMO to produce BTOL, which is a strong toxicant as it was commented previously. However, the increase of q_{Smean} (Fig. 4) suggested that the expression of enzymes of the HY aerobic pathway, such as hydroquinone 1,2-dioxygenase (HY12DO), were induced by the consecutive additions of HY. The oxidation of HY by HY12DO produces 4-hydroxymuconic semialdehyde, which finally enters to the TCA cycle without producing toxicity (Zhang et al., 2012).

3.4. Oxidation coefficient ($Y_{O/S}$) of the tested phenolic compounds

Based on the respirograms showed in Figs. 2 and 3, for each addition of substrate, the oxidation coefficient ($Y_{O/S}$) corresponding to the tested compounds were calculated as the ratio between the maximum oxygen consumed (OC_m) and the initial substrate concentration (S_0). Although this procedure is a common practice to evaluate a stoichiometric coefficient such as $Y_{O/S}$, Fig. 5a shows that results were very sensitive to experimental errors. Instead, $Y_{O/S}$ can also be calculated as the slope of the plot of the cumulative oxygen consumption as a function of the cumulative substrate concentration. Fig. 5b clearly demonstrates that $Y_{O/S}$ values corresponding to PH, PY, RE, and HY were constant as a function of the cumulative substrate concentration. Conversely, $Y_{O/S}$ corresponding to CA shifted from 1 to 1.5 $molO_2\ molCA^{-1}$ when the CA cumulative concentration was higher than 10 mM (Fig. 5b).

The oxidation coefficients ($Y_{O/S}$) corresponding the phenolic compounds tested in this work (Table 1) were within the range reported by other authors. Orupold et al. (2001) studied the oxidation of several phenolic compounds by activated sludge; those authors found $Y_{O/S}$ values of 2.6 ± 0.4, 1.5 ± 0.1, and 1.5 ± 0.1 $molO_2\ molS^{-1}$ for PH, CA, and RE, respectively. Divari et al. (2003) reported 1.6 $molO_2\ molS^{-1}$ during the PH oxidation by PHMO from *Acinetobacter radioresistens* S13. Lepik and Tenno (2011) studied the aerobic degradation of PH and RE by activated sludge acclimated to a wastewater of an oil-shale industry; those authors reported $Y_{O/S}$ values of 2.0, and 1.4 $molO_2\ molS^{-1}$ for PH, and RE, respectively. Unfortunately, no similar data were found in literature with regard to the aerobic biodegradation of PY by whole cells.

4. Conclusions

The aerobic degradation of PH, CA, RE, PY, and HY by phenol-acclimated activated sludge was studied using a respirometric technique. Initial biodegradation rate corresponding to CA and PH were higher than those corresponding to PY, RE, and HY. Additionally, PY exhibited the higher degree of toxicity to its degradation. While oxidation coefficients corresponding to PH, PY, RE, and HY were constant, an increase of this coefficient was observed during repeated additions of CA. Results presented in this work are useful in designing aeration devices for phenolic compounds removal facilities.

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