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ORIGINAL PAPER

Kinetic limitations during the simultaneous removal of *p*-cresol and sulfide in a denitrifying process

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Abstract The aim of this study was to evaluate the capacity of a denitrifying consortium to achieve the simultaneous removal of nitrate, sulfide and *p*-cresol and elucidate the rate-limiting steps in the mixotrophic process. Nitrite reduction appeared as the most evident rate-limiting step in the denitrifying respiratory process. The nitrite reduction rate achieved was up to 57 times lower than the nitrate reduction rate during the simultaneous removal of sulfide and *p*-cresol. Negligible accumulation of N_2O occurred in the denitrifying cultures corroborating that nitrite reduction was the main rate-limiting step of the respiratory process. A synergistic effect of nitrate and sulfide is proposed to explain the accumulation of nitrite. The study also points at the oxidation of S^0 as another rate-limiting step in the denitrifying process. Different respiratory rates were achieved with the distinct electron donors provided (*p*-cresol and sulfide). The oxidation rate of *p*-cresol (q_{CRES}) was generally higher (up to 2.6-fold in terms of reducing equivalents) than the sulfide oxidation rate (q_{S2-}), except for the experiments performed at 100 mg S^{2-} L^{-1} in which q_{S2-} was slightly ($\sim$1.4-fold in terms of reducing equivalents) higher than q_{CRES}. The present study provides kinetic information, which should be considered when designing and operating denitrifying reactors to treat industrial wastewaters containing large amounts of sulfurous, nitrogenous and phenolic contaminants such as those generated from petrochemical refineries.

Keywords Denitrification · Elemental sulfur · Kinetics · Phenolic contaminants · Mixotrophic · Sulfide · Wastewater

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Introduction

Wastewaters originating from the chemical and petrochemical sectors represent a great challenge for treatment to fulfill regulatory requirements. These industrial effluents contain large amounts of nitrogenous, sulfurous and aromatic pollutants [1–3], which demand suitable technologies to achieve their simultaneous removal. Denitrification has lately been proposed as a suitable treatment process to achieve the simultaneous removal of these contaminants [2–7]. This dissimilatory process involves four enzymatic steps in which nitrate is reduced to nitrite, nitric oxide (NO), nitrous oxide (N_2O) and nitrogen gas (N_2) [8]. Nitrate reduction to ammonia (DNRA) is another dissimilatory process that can also occur in denitrifying consortia leading to the production of ammonia [9, 10].

Denitrification can proceed under lithotrophic conditions using reduced sulfurous compounds, such as elemental sulfur (S^0), sulfide and thiosulfate, as an electron donor [2–7, 11, 12]. Denitrification can also be linked to the oxidation of a wide variety of organic compounds

including volatile fatty acids, alcohols and phenolic compounds [2, 4]. Therefore, denitrifying processes can potentially achieve the simultaneous removal of nitrogenous, sulfurous and aromatic pollutants from industrial effluents. Nitrate could be supplied to these denitrifying processes via nitrification treatment units strategically connected.

The capacity of different denitrifying reactors has recently been tested for the simultaneous removal of sulfurous, nitrogenous and organic contaminants showing heterogeneous results depending on several operational parameters, such as pH, temperature, N and S loading rate, hydraulic residence time (HRT), S/N ratio and C/N ratio, among others [13, 14]. Nevertheless, kinetic parameters available in the literature are not yet sufficient in order to know which are the rate-limiting steps during the application of denitrifying processes for the simultaneous removal of sulfide and organic pollutants, and the information is especially required as these contaminants coexist in many industrial effluents [1, 3, 7].

Several studies have described an inhibitory effect of sulfide on the last steps of denitrification consequently causing release of NO or N_2O [4, 15–17]. Dalsgaard and Bak [18] reported inhibition of nitrate reduction (from 37 to 71% compared to the control without sulfide) in a wide range of sulfide concentrations (46–152 μM). Several studies, in contrast, reported accumulation of only nitrite at different sulfide concentrations [2, 6, 11].

The aim of this research was to evaluate the capacity of a denitrifying consortium to achieve the simultaneous removal of sulfide and *p*-cresol and elucidate the rate-limiting steps in the mixotrophic process.

Materials and methods

Biomass stabilization in a denitrifying reactor

In order to avoid problems in results interpretation, the microbial consortium evaluated was firstly stabilized under defined denitrifying conditions. A 1.4-l upflow anaerobic sludge blanket (UASB) reactor was operated at 30°C and with a HRT of 2 days to obtain a consortium under denitrifying steady state conditions. The reactor was inoculated with 6 g volatile suspended solids (VSS) l^{-1} of denitrifying sludge previously fed with acetate as electron donor. The synthetic influent was divided into two parts (source of carbon and source of nitrogen) in order to preserve it and to avoid precipitation of metals. The two influent parts were initially pumped separately by a peristaltic pump and mixed just before entering the reactor. The composition of the carbon source medium was (g l^{-1}): *p*-cresol (0.32), acetate (0.31), KH_2PO_4 (1.2), K_2HPO_4 (3.2) and $MgCl_2{\cdot}6H_2O$ (0.4). Meanwhile, the composition of the nitrogen source medium was (g l^{-1}): $NaNO_3$ (1.7), Na_2SO_4 (1) and trace element solution (2 ml l^{-1}). The C/N ratio obtained during bioreactor operation was 1.13, which was used during mass balances. The trace element solution consisted of (g l^{-1}): $CaCl_2{\cdot}2H_2O$ (0.6), $Na_2MoO_4{\cdot}2H_2O$ (0.12), $FeCl_3{\cdot}6H_2O$ (0.03) and $CuSO_4{\cdot}5H_2O$ (0.02).

Nitrate, nitrite, acetate, *p*-cresol and its aromatic intermediates, *p*-hydroxy-benzyl alcohol, *p*-hydroxy-benzaldehyde and *p*-hydroxy-benzoate, were quantified in the denitrifying reactor. Biogas production and composition were also regularly monitored in the reactor.

Table 1 summarizes the reactor performance under steady-state conditions. Both *p*-cresol and acetate were totally removed, and the main carbonaceous product was bicarbonate with a yield (Y_{TIC}) of 0.66 ± 0.04 mg total inorganic carbon (TIC) [mg of total organic carbon (TOC) fed]$^{-1}$. Total inorganic carbon represents the sum of dissolved bicarbonate and the C–CO_2 measured in the headspace. Total organic carbon remaining in the effluent represents unidentified organic compounds. Nitrate removal efficiency was 99.5 ± 0.2%, and a high production yield of molecular nitrogen [Y_{N_2} = 0.88 ± 0.04 mg N_2 (mg of NO_3^-–N fed)$^{-1}$] was observed. Ammonia formation via DNRA was not detected in the denitrifying reactor.

Batch assays

Batch experiments were undertaken in serologic bottles of 160 ml. Sludge incubations contained 60 ml of basal medium and were inoculated with the previously stabilized denitrifying sludge (initial concentration of 1 g VSS l^{-1}).

Table 1 Performance of the UASB denitrifying reactor under steady state conditions

Parameter	Value [in mg (l day)$^{-1}$]
TOC_{in}	85.4 ± 1.2
TIC^a_{out}	56.7 ± 3.7
C-biomass	12.6 ± 1.9
TOC^b_{out}	7.2 ± 0.8
NO_3^-–N_{in}	75.5 ± 0.4
NO_3^-–N_{out}	0.4 ± 0.1
NO_2^-–N_{out}	1.0 ± 0.3
N_2 produced	65.8 ± 3.1
N-biomass	2.5 ± 0.4

Data represent mean values obtained from 63 days of steady-state denitrifying conditions ± standard deviation

TOC Total organic carbon, *TIC* total inorganic carbon

[a] The sum of dissolved TIC and the C–CO_2 measured in the headspace

[b] TOC_{out} constituted by unidentified compounds (no acetate and *p*-cresol detected)

The basal medium consisted of (g l^{-1}): KH_2PO_4 (0.6), K_2HPO_4 (1.6), $MgCl_2{\cdot}6H_2O$ (0.2) and trace element solution (2 ml l^{-1}) with the composition described above. The pH of the basal medium was controlled at 7.2 ± 0.1 by the phosphate buffer described above, which was corroborated by pH determinations during the incubation period. The culture controls (in the absence of sulfide) were supplied with stoichiometric concentrations of the electron donor and electron accepting substrate, resulting in a total C and N concentration of 44 mg *p*-cresol-C l^{-1} and 50 mg NO_3^-–N l^{-1} (C/N ratio of 0.88). For sulfide-amended cultures, the concentration of *p*-cresol remained constant as in the control, while the concentrations of sulfide and nitrate were increased to 25, 50 and 100 mg S^{2-} l^{-1}, and to 64, 85 and 120 mg NO_3^-–N L^{-1}, respectively, to stoichiometrically oxidize both electron donors. Sulfide was provided from a stock solution of $Na_2S{\cdot}9H_2O$. Serum bottles were sealed with rubber stoppers and aluminum crimps, and flushed with helium in order to saturate the headspace with this inert gas. All experimental treatments were carried out in duplicate and incubated at 30°C in a shaker at 200 rpm. Every incubation bottle was an independent experimental unit, which was sacrificed after sampling. Liquid and headspace samples were taken periodically to determinate substrates utilization and products formation.

Microbial performance was evaluated in terms of consumption efficiency [E, %, mg of C, S or N consumed (mg of C, S or N fed)$^{-1}$] × 100, yield [Y, mg of C, S or N produced (mg of C, S or N consumed)$^{-1}$], specific substrate consumption rate [q_S, mg of C, S or N consumed (g VSS day)$^{-1}$] and specific production rate [q_P, mg of C, S or N produced (g VSS day)$^{-1}$]. Specific consumption and production rates were determined on the maximum slope observed on linear regressions considering at least three sampling points. The coefficient of determination (R^2) was higher than 0.9 for all respiratory rates calculated.

Analytical methods

Total organic carbon and TIC were measured in a TOC-meter (Shimadzu Co. Model TOC-5000 A). Nitrate, nitrite, thiosulfate and sulfate concentrations were determined using a HPLC (Waters, Shelton USA) equipped with diode array detector by conductivity and with an anion column (IC-Pak A HC Waters 4.6 × 75 mm). The mobile phase was a borate–gluconate solution with the composition previously described [19] at 2 ml min^{-1}. *p*-Cresol and its aromatic intermediates were monitored by HPLC (Pekin-Elmer serie 200 UV) using a C18 reverse phase column and a UV detector at 280 nm. The mobile phase was an acetonitrile:water (70:30) mixture at 1.5 ml min^{-1}. VSSs were determined according to standard methods [20]. Acetate was measured by gas chromatography (GC) with a flame ionization detector. The temperatures of the oven, the injector and the detector were 120, 130 and 150°C, respectively. The gas composition in the headspace (CO_2, N_2 and N_2O) was determined by GC (Varian 3350) with a thermal conductivity detector. The temperatures of the column, the injector and the detector were 50, 100 and 110°C, respectively, with helium as the carrier gas at a constant flow rate of 16 ml min^{-1}. All liquid samples were filtered through a 0.45-μm nylon membrane before analysis. Due to the rapid oxidation of sulfide, samples were immediately titrated using the iodometric method described in standard methods to determine the sulfide concentration [20].

Results

Organotrophic culture control

The studied denitrifying culture achieved complete removal of both nitrate (Fig. 1) and *p*-cresol in a very short incubation period (5 h) in the absence of sulfide. The remaining TOC observed after this incubation period (Fig. 2) did not correspond to *p*-cresol, but to unidentified intermediates of the biodegradation pathway. The carbonaceous intermediates monitored, *p*-hydroxy-benzyl alcohol, *p*-hydroxy-benzaldehyde and *p*-hydroxy-benzoate, were not detected during the oxidation of *p*-cresol, and the extent of mineralization achieved (Y_{TIC}) was 0.74 (Table 2).

Albeit nitrate was very efficiently removed, nitrite reduction rate (q_{NO_2}) became only significant after complete reduction of nitrate (Fig. 1) and proceeded at a ~14-fold lower velocity compared to the nitrate reduction rate (q_{NO_3}, Table 3) causing accumulation of nitrite and poor denitrifying yield (Y_{N_2}, Table 2). N_2O accumulation was also observed in the organotrophic cultures, but accounted only for <5% of the nitrate initially supplied.

Mixotrophic cultures for the simultaneous removal of *p*-cresol and sulfide

Simultaneous removal of *p*-cresol and sulfide coupled to nitrate reduction occurred in mixotrophic cultures provided with both electron donors (Figs. 1, 2, 3) promoting higher q_{NO_3} compared to the organotrophic culture control (Table 3). The oxidation rate of *p*-cresol (q_{CRES}) was generally higher (up to 2.6-fold in terms of reducing equivalents) than that observed for sulfide (q_{S2-}), except for the cultures supplied with the maximum sulfide concentration tested (100 mg S^{2-} l^{-1}) in which q_{S2-} was

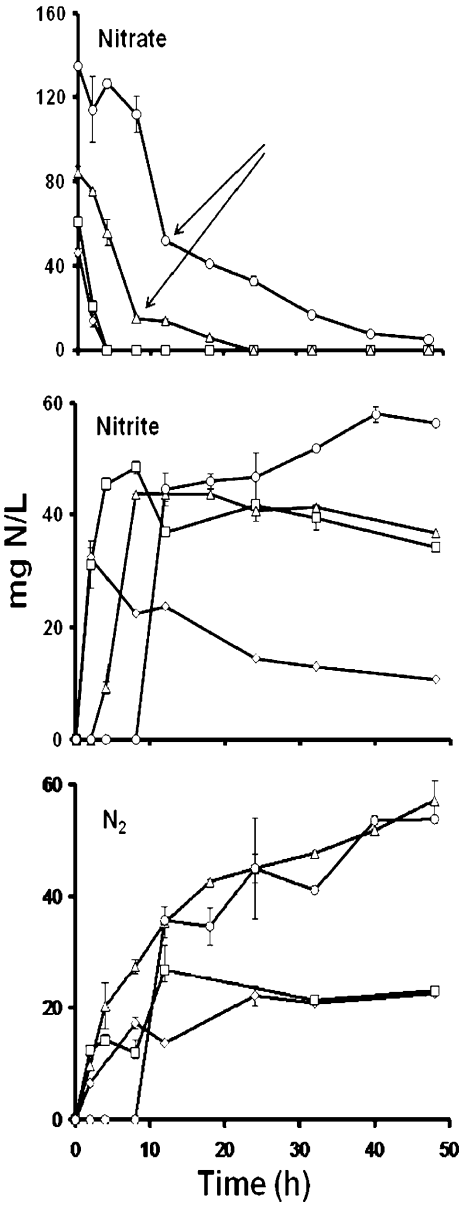

Fig. 1 Kinetic profiles of nitrogenous compounds monitored in denitrifying cultures at different concentrations of sulfide. *Open diamonds* organotrophic control without sulfide, *open squares* 20 mg S^{2-} l^{-1}, *open triangles* 50 mg S^{2-} l^{-1}, *open circles* 100 mg S^{2-} l^{-1}. *Arrows* indicate the time at which sulfide was completely removed from denitrifying cultures. Results represent average from duplicate determinations and *error bars* the standard deviation

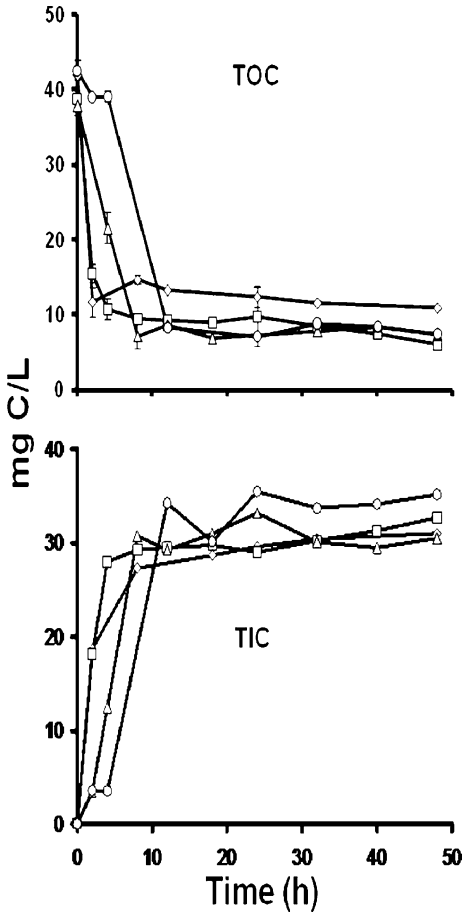

Fig. 2 Kinetic profiles of TOC and TIC in denitrifying cultures at different concentrations of sulfide. *Open diamonds* organotrophic control without sulfide, *open squares* 20 mg S^{2-} l^{-1}, *open triangles* 50 mg S^{2-} l^{-1}, *open circles* 100 mg S^{2-} l^{-1}. Results represent average from duplicate determinations and *error bars* the standard deviation

Table 2 Yields obtained in mixotrophic denitrifying cultures at different sulfide concentrations after 48 h of incubation

Sulfide concentration (mg l^{-1})	Y_{TIC}	Y_{N_2}	Y_{SO_4}
0	0.74 ± 0.04	0.48 ± 0.002	–
25	0.84 ± 0.04	0.38 ± 0.001	1.07 ± 0.001
50	0.8 ± 0.007	0.67 ± 0.040	0.91 ± 0.02
100	0.83 ± 0.03	0.41 ± 0.004	0.085 ± 0.01

slightly higher (~1.4-fold in terms of reducing equivalents) than q_{CRES} (Table 3).

Two clear respiratory phases were observed in the mixotrophic denitrifying cultures when high sulfide concentrations (50 and 100 mg S^{2-} l^{-1}) were supplied. Certainly, during the first incubation period (8–12 h), when both *p*-cresol and sulfide were available as electron donors, high q_{NO_3} were accomplished. Nevertheless, the q_{NO_3} values decreased up to ~9-fold, as compared with those observed during the first respiratory period, when *p*-cresol and sulfide were no longer available as electron donors (Figs. 1, 2, 3). Organic electron donors (derived from *p*-cresol) were not involved in the reduction of nitrate during the second respiratory phase observed as no TOC removal was evident during this incubation period (Fig. 2). Moreover, the sulfate production achieved during the first

Table 3 Maximum respiratory rates [in mg C, S or N (g VSS day)$^{-1}$] obtained in denitrifying cultures

Sulfide concentration (mg S l^{-1})	q_{NO_3}	q_{NO_2}	q_{CRES}	q_{S2-}
0	200 ± 8.0	14.4 ± 3.0	180 ± 4.0 (73 ± 1.6)[b]	–
25	344 ± 40	6.0 ± 1.0	288 ± 14 (117 ± 5.7)[b]	166 ± 17 (45 ± 4.3)[b]
50	333 ± 40 (52.7 ± 6.0)[a]	6.2 ± 1.0	300 ± 20 (121 ± 8.1)[b]	400 ± 7 (100 ± 1.8)[b]
100	308 ± 10 (33.3 ± 1.1)[a]	8.6 ± 1.5	111 ± 8.0 (45 ± 3.2)[b]	248 ± 7 (62 ± 1.7)[b]

Data represent average from duplicate determinations ± standard deviation

[a] Determined after complete removal of sulfide

[b] In milli-equivalents [g VSS day]$^{-1}$

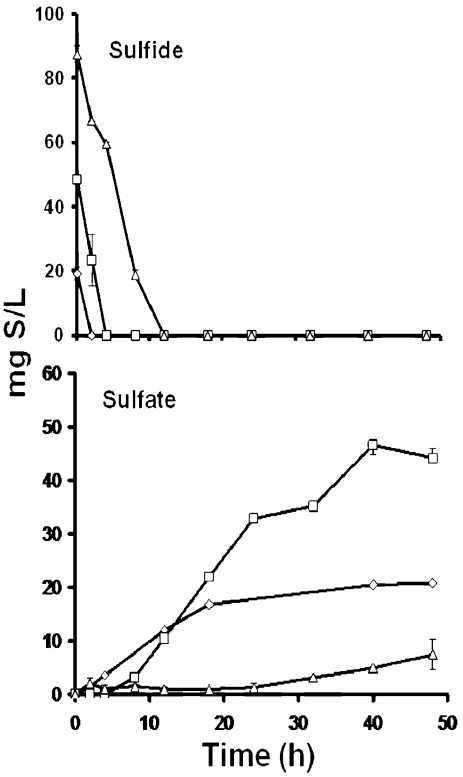

Fig. 3 Kinetic profiles of sulfurous compounds monitored in denitrifying cultures at different concentrations of sulfide. *Open diamonds* 20 mg S^{2-} l^{-1}, *open squares* 50 mg S^{2-} l^{-1}, *open triangles* 100 mg S^{2-} l^{-1}. Results represent average from duplicate determinations and *error bars* the standard deviation

incubation period did not account for the sulfide consumed (recovery < 20%, Fig. 3). Therefore, the results suggest that a sulfurous intermediate, derived from the oxidation of sulfide, was the main electron donor promoting the reduction of nitrate during the second respiratory phase observed. As thiosulfate was not detected in any denitrifying culture performed, it is suggested that S^0 was the main electron donor utilized during this incubation period. Although S^0 was not quantified, qualitative evidence was obtained by the formation of white particles in these incubations. Another indication that nitrate reduction was linked to the oxidation of S^0 during this incubation period was the concomitant production of sulfate observed (Fig. 3).

As observed in the organotrophic culture control, nitrite reduction was the rate-limiting step in the mixotrophic denitrifying cultures as the q_{NO_2} achieved was up to 57- and 8.5-fold lower than q_{NO_3} during the first and second incubation period, respectively (Table 3). Furthermore, negligible accumulation of N_2O occurred, corroborating that nitrite reduction was the rate-limiting step in the mixotrophic cultures performed.

The accumulation of nitrite caused Y_{N_2} values as low as 0.38 in the mixotrophic cultures, although there was not a clear and direct correlation between the concentration of sulfide supplied and the Y_{N_2} values obtained (Table 2). The extent of mineralization of *p*-cresol was not affected by any concentration of sulfide supplemented (Table 2). However, the conversion of sulfide to sulfate (Y_{SO_4}) decreased by raising the concentration of sulfide (Table 2). After 50 h of incubation, the Y_{SO_4} value was particularly low at the maximum concentration of sulfide evaluated, but contrasting with the high sulfide removal efficiency (100%) achieved during the same incubation period.

Discussion

The aim of this study was to evaluate the capacity of a denitrifying consortium to achieve the simultaneous removal of sulfide and *p*-cresol and elucidate the rate-limiting steps in the mixotrophic process. The results obtained clearly show the feasibility to simultaneously remove nitrate, sulfide and *p*-cresol under denitrifying conditions, although there are some bottlenecks in the respiratory process, which have been identified and should be considered when designing and operating denitrifying reactors for that purpose.

Nitrite reduction appeared as the most evident rate-limiting step in the denitrifying cultures studied. The experimental evidence found in this work have shown that

the q_{NO_2} achieved was up to 57-lower than q_{NO_3} during the simultaneous removal of sulfide and *p*-cresol. Moreover, negligible accumulation of N_2O occurred in the denitrifying cultures, corroborating that nitrite reduction was the main rate-limiting step of the respiratory process.

Several aspects may explain the accumulation of nitrite in the denitrifying cultures. Firstly, the q_{NO_2} values obtained in the presence of sulfide were up to 2.4-fold lower compared to that achieved in the organotrophic culture control in the absence of sulfide (Table 3). Therefore, an inhibitory effect of sulfide over nitrite reduction was palpable in mixotrophic incubations. This inhibitory effect may partly be explained by the precipitation of trace elements (e.g., iron and copper), which are essential for the activity of nitrite-reductases [21] caused by sulfide. The last observation was confirmed by a program of speciation (MINTEQ) utilized to identify precipitated compounds in our assays. Most iron species appeared precipitated even in the absence of sulfide, but there was a significant difference in copper species, which remain more soluble in the absence of sulfide. Manconi et al. [22] observed an increase on nitrite accumulation in a continuous reactor after precipitation of iron and copper. Cervantes et al. [8] also found accumulation of nitrite and N_2O caused by deficiencies in copper in a denitrifying culture. Thus, precipitation of trace metals, which are essential for denitrifying enzymes, might be one of the causes of the diminished q_{NO_2} induced by sulfide in our experiments. The last observation is also suggested by the fact that nitrite reduction rates remain lower in sulfide-amended cultures, compared to the controls in the absence of sulfide, even after sulfide was totally depleted.

Furthermore, nitrite reduction became only significant after most supplied nitrate had previously been removed from denitrifying cultures (Fig. 1). The last scenario was also observed by Schönharting et al. [16] in a denitrifying process exposed to sulfide. The authors attributed nitrite accumulation to kinetic aspects; namely, nitrate reduction proceeded faster than nitrite reduction, thus causing accumulation of nitrite in the cultures. Consequently, a synergistic effect of nitrate and sulfide may explain the accumulation of nitrite in the present study, which was corroborated by the accumulation of nitrite during the course of nitrate reduction in the organotrophic culture control in the absence of sulfide (Fig. 1).

On the other hand, for mixotrophic cultures, nitrite reduction was mainly promoted by the oxidation of a sulfurous intermediate derived from the oxidation of sulfide (presumably S^0) as nitrate reduction had previously depleted all sulfide, and no further TOC removal was observed during the course of nitrite reduction (Figs. 1, 2, 3). The oxidation of S^0 in denitrifying processes has previously been reported to proceed at very low rates [2, 4, 23], which potentially may also contribute to the accumulation of nitrite or N_2O in denitrifying reactors. It has been pointed out that mass transfer limitations might be responsible for the low oxidation rate of S^0 observed in denitrifying reactors [24] since its maximum aqueous solubility is 0.16 μM [25].

The low oxidation rate of S^0 affected the reduction of not only nitrite, but also nitrate when high concentrations (50 and 100 mg S^{2-} L^{-1}) of sulfide were supplied. Certainly, during the first incubation period (8–12 h) in mixotrophic cultures, when both *p*-cresol and sulfide were available as electron donors, high q_{NO_3} were accomplished (Fig. 1). Nevertheless, the q_{NO_3} values decreased up to ~9-fold, as compared with those observed during the first respiratory period (Table 3), when nitrate reduction was exclusively linked to the production of sulfate from the oxidation of S^0 (Figs. 1, 2, 3).

Although the present study and previous reports [2, 23, 24] point at the oxidation of S^0 as one of the rate-limiting steps in denitrifying processes, this drawback may be beneficial when the recovery of S^0 is one of the goals in treatment systems. In fact, partial oxidation of sulfide to S^0 in denitrifying reactors allows its recovery in a subsequent treatment step, either by filtration or by sedimentation [24, 26]. However, it is important to emphasize that operational strategies should be implemented in order to avoid the accumulation of S^0 in denitrifying reactors, which would promote the selective enrichment of bacteria capable of oxidizing sulfide to S^0. When the goal is the partial oxidation of sulfide to S^0, inversed fluidized bed reactors represent a suitable option. In these treatment systems, the produced S^0 may easily be separated from denitrifying biomass by the remarkable difference in density between S^0 and biofilm particles [27].

The present study also revealed differences in the respiratory rates observed with the distinct electron donors provided (*p*-cresol and sulfide). Indeed, although both energy sources could simultaneously be oxidized linked to nitrate reduction, the q_{CRES} obtained was generally higher (up to 2.6-fold in terms of reducing equivalents) than q_{S2-}, except for the experiments performed at 100 mg S^{2-} l^{-1} in which q_{S2-} was slightly (~1.4-fold in terms of reducing equivalents) higher than q_{CRES} (Table 3). Our results agree with those reported by Reyes-Avila et al. [2], who found that the denitrifying culture evaluated was able to simultaneously oxidize sulfide and acetate, although q_{S2-} was ~10-fold higher than the oxidation rate of acetate (Fig. 4). More recently, Beristain-Cardoso et al. [28] found a sequential respiratory profile in a denitrifying culture previously stabilized with sulfide as an electron donor. Batch incubations of this consortium revealed that the oxidation of phenol was only significant after complete conversion of sulfide to sulfate under denitrifying conditions. It is conceivable that the different respiratory profiles observed

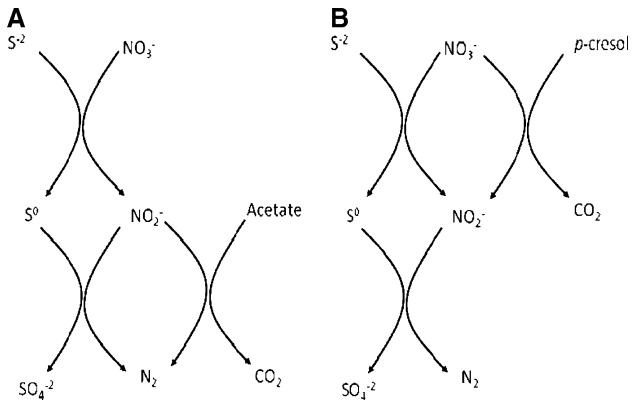

Fig. 4 Comparison of proposed mechanisms during the simultaneous removal of nitrate, sulfide and organic substrates in mixotrophic denitrifying processes (**a** from Reyes-Avila et al. [2], **b** present study)

among these studied may partly be explained by different phylogenetic profiles of the microbial communities evaluated.

The present study provides with kinetic information, which should be considered when designing and operating denitrifying reactors to treat industrial wastewaters containing large amounts of sulfurous, nitrogenous and phenolic contaminants, such as those generated from petrochemical refineries. For instance, the kinetic data obtained could be used to establish the nitrate, sulfide and COD loading rates in mixotrophic denitrifying systems, as well as the HRT and the biomass concentration required for achieving high removal efficiencies.

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