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Performance of a reactor containing denitrifying immobilized biomass in removing ethanol and aromatic hydrocarbons (BTEX) in a short operating period

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Abstract

A horizontal-flow anaerobic immobilized biomass reactor (HAIB) containing denitrifying biomass was evaluated with respect to its ability to remove, separately and in a short operating period (30 days), organic matter, nitrate, and the hydrocarbons benzene (41.4 mg L^{-1}), toluene (27.8 mg L^{-1}), ethylbenzene (31.1 mg L^{-1}), *o*-xylene (28.5 mg L^{-1}), *m*-xylene (28.4 mg L^{-1}) and *p*-xylene (32.1 mg L^{-1}). The purified culture, which was grown in the presence of the specific hydrocarbon, was used as the source of cells to be immobilized in the polyurethane foam. After 30 days of operation, the foam was removed and a new immobilized biomass was grown in the presence of another hydrocarbon. The average hydrocarbon removal efficiency attained was 97%. The organic matter, especially ethanol, was removed with an average efficiency of 83% at a mean influent concentration of 1185.0 mg L^{-1}. A concomitant removal of 97% of nitrate was observed for a mean influent concentration of 423.4 mg L^{-1}. The independent removal of each hydrocarbon demonstrated that these contaminants can be biodegraded separately, without the need for a compound to be the primary substrate for the degradation of another. This study proposes the application of the system for treatment of areas contaminated with these compounds, with substitution and formation of a biofilm in a 30-day period.

Keywords: BTEX; Ethanol; HAIB reactor; Denitrifying biofilm

1. Introduction

Many researches have focused on restoring the quality of waters to the potable levels required by environmental agencies, taking into account the development of technologies aimed at minimizing the costs and maximizing the efficiency of pollutant removal. In this context, different technologies have been applied to solve the problem of waste, subterranean or supply waters contaminated by compounds such as nitrate and aromatic hydrocarbons (benzene, toluene, ethylbenzene and xylenes, BTEX). One technological possibility is the use of horizontal-flow anaerobic immobilized biomass reactors (HAIB), which present an adequate performance and stability of the process due to the possibility of applying long cell retention times while operating with low hydraulic retention times [1]. In addition to providing favorable conditions for the development of biofilms, the use of the HAIB reactor is also technically, financially and environmentally viable. This system has proved to be a suitable tool for the treatment of toxic compounds, displaying high efficiency in the removal of pollutants, including BTEX [2–6].

In studies involving HAIB reactors used in the treatment of toxic pollutants, the system is usually operated over long periods until it attains a state of dynamic equilibrium, at which point it reaches its maximum removal efficiency, both of the pollutants and of the organic matter present in the medium. However, studying the efficiency of this system with short operating peri-

Abbreviations: BTEX, benzene, toluene, ethylbenzene and xylenes; COD, chemical oxygen demand; HAIB, horizontal-flow anaerobic immobilized biomass (reactor); HDT, hydraulic detection time; UASB, up-flow anaerobic sludge blanket reactor

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ods is crucial, since more immediate action is required in cases of environmental contamination by toxic compounds.

Gusmão et al. [6] found that the application of purified denitrifying biofilm is highly specialized for the removal of BTEX. In that study, the biofilm was kept on the reactor's support medium throughout the operating period (189 days) while the aromatic hydrocarbons were substituted in the feed substrate. Thus, the formation of the biofilm and adaptation to a given hydrocarbon could be important factors for the removal of another compound to occur. According to Alvarez and Vogel [7], individual compounds can stimulate the degradation of others through the induction of enzymes or can act as a primary substrate stimulating microbial growth and thereby favoring the cometabolism of another compound. On the other hand, the presence of a compound can exert an inhibitory effect due to toxicity, catabolic repression or competitive inhibition by enzymes.

Based on the above findings, the present study aimed to ascertain the removal efficiency of benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene and *p*-xylene, separately, with a biofilm grown and adapted for 30 days in a denitrifying horizontal-flow anaerobic immobilized biomass reactor operating with a hydraulic retention time of 12 h. The contribution of this study is therefore significant in that, once the microbial community had been established in a short period of operation, the system successfully removed ethanol, nitrate and BTEX, demonstrating the feasibility of its technological application.

2. Materials and methods

2.1. Purification and growth conditions

The source of inoculum was an up-flow anaerobic sludge blanket reactor (UASB) treating poultry slaughterhouse wastewater. The denitrifying culture was purified in a liquid anoxic medium as described by Dolfing et al. [8], separately from the reducing solution, and supplemented with sodium nitrate (350 mg L^{-1}—N–NO_3^-), ethylbenzene (10 mg L^{-1}) and ethanol 377 mg L^{-1} (82% purity). The inoculum was then purified by serial dilutions. The highest dilution of ethylbenzene amendment showing growth was again serially diluted. The culture was repeatedly cultivated by superficial streaking in Petri dishes incubated for 48 h at 30 °C ± 2 in a Gas Pack Jar (BBL™ Anaerobic Systems). One colony, which later proved to be a microbial consortium, was transferred to a fresh liquid medium containing a specific hydrocarbon.

2.2. Horizontal-flow anaerobic immobilized biomass (HAIB) reactor

A horizontal-flow anaerobic immobilized biomass (HAIB) reactor was used to evaluate the removal efficiency of benzene, toluene, ethylbenzene and *o*-xylene, *m*-xylene and *p*-xylene. Fig. 1 shows a schematic diagram of the HAIB reactor. The horizontal reactor was made of borosilicate glass with a 100 cm length, 5.0 cm diameter, a length/diameter ratio (*L*/*D*) of 20, a total volume of 1995 mL, and a liquid volume capacity of 800 mL. The reactor contained five intermediate sampling ports

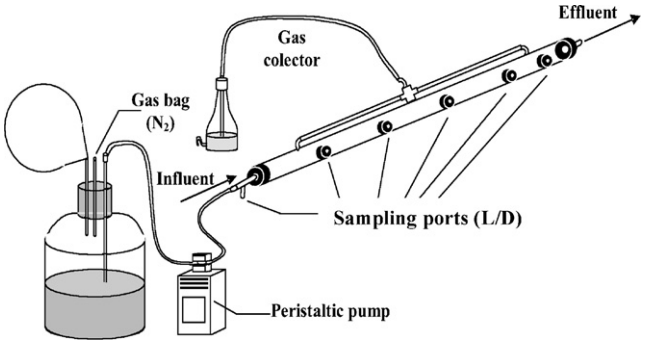

Fig. 1. Schematic representation of the HAIB reactor.

along its length (*L*/*D* of 4, 8, 12, 16, and 20). Cubic polyurethane foam particles (5 mm in size and 23 kg/m^3 in density) were used as the immobilization support for the microbial biomass, with a bed porosity of 40%. The reactor was installed in a temperature-controlled chamber (30 °C ± 2) and the influent was fed into it with a peristaltic pump. A microbial consortium, grown in the presence of the specific hydrocarbon and at the exponential growth phase, was used as the source of cells for immobilization in the polyurethane foam inside the continuous horizontal reactor. Immobilization of the biomass was promoted by influent feeding-circulation for 3 days, after which the HAIB reactor was fed with fresh substrate. The reactor was operated with a hydraulic retention time of 12 h for 30 consecutive days, with each of the hydrocarbons separately. After spatial characterization-profiling, the reactor was emptied and washed for 5 min with a chloric acid solution (10%, v/v) to eliminate any possible residue. This procedure was carried out prior to each feeding with another hydrocarbon, at which time the bed was packed with new polyurethane foam particles.

2.3. Feeding substrate

A feeding substrate was prepared using a microbial consortium medium [8] and amended with the specific hydrocarbon. Ethanol at final concentrations of 566 mg L^{-1} was used to enhance the dissolution of the hydrocarbons. The final values of ethanol concentrations were estimated theoretically using a chemical product of 82% purity. Final nitrate amendments were made based on Eqs. (1)–(5). Therefore, the equivalent redox potential for the electron acceptor was 5.8 times higher than the electron donor concentrations. Table 1 compares these different values.

A N_2 (100%) atmosphere was maintained in the substrate feeding flasks by means of a gas-balloon device:

$$C_6H_6 + 6NO_3^- + 6H^+ \rightarrow 6CO_2^- + 3N_2 + 6H_2O \quad (1)$$

$$C_7H_8 + 7.2NO_3^- + 7.2H^+ \rightarrow 7CO_2 + 3.6N_2 + 7.6H_2O \quad (2)$$

$$C_8H_{10} + 8.4NO_3^- + 8.4H^+ \rightarrow 8CO_2 + 4.2N_2 + 9.2H_2O \quad (3)$$

$$C_2H_6O + 2.4NO_3^- + 2.4H^+ \rightarrow 2CO_2 + 1.2N_2 + 4.2H_2O \quad (4)$$

Table 1
Operating regime used for the HAIB reactor feeding with benzene toluene, ethylbenzene and xylenes at HDT of 12 h

Reactor feeding characteristics	Operational time (days)	Concentration (mg L^{-1})		COD (mg L^{-1})		Removal (%)
		Influent	Effluent	Influent	Effluent	
Total organic matter	30	–	–	1322.3	142.8	89.0
Benzene		41.4	4.4	127.4[a]	13.5[a]	89.0
Ethanol		566.0	–	1194.9[a]	–	–
Acetic acid		[b]	62.3	–	66.5[a]	–
Nitrate (N–NO_3^-)		520.0	2.0	–	–	99.0
Unidentified excreted organic matter		–	–	–	62.8[a]	–
Total organic matter	30	–	–	1240.8	345.1	72.0
Toluene		27.8	0.3	87.0[a]	0.9[a]	99.0
Ethanol		566.0	–	1153.8[a]	–	–
Acetic acid		[b]	286.4	–	305.5[a]	–
Nitrate (N–NO_3^-)		451.4	6.1	–	–	97.0
Unidentified excreted organic matter		–	–	–	38.7[a]	–
Total organic matter	118	–	–	1205.7	38.5	97.0
Ethylbenzene		31.1	0.2	98.6[a]	0.6[a]	99.0
Ethanol		566.0	–	1106.4[a]	–	–
Acetic acid		[b]	[b]	–	–	–
Nitrate (N–NO_3^-)		547.9	15.5	–	–	97.0
Unidentified excreted organic matter		–	–	–	37.9[a]	–
Total organic matter	30	–	–	1258.5	163.2	87.0
o-Xylene		28.5	1.8	90.3[a]	5.7[a]	94.0
Ethanol		566.0	–	1168.2[a]	–	–
Acetic acid		[b]	[b]	–	–	–
Nitrate (N–NO_3^-)		516.7	11.5	–	–	98.0
Unidentified excreted organic matter		–	–	–	157.5[a]	–
Total organic matter	30	–	–	1619.3	537.9	68.0
m-Xylene		28.4	0.3	90.0[a]	0.9[a]	99.0
Ethanol		566.0	–	1529.3[a]	–	–
Acetic acid		15.2	502.9	16.2[a]	535.9[a]	–
Nitrate (N–NO_3^-)		417.5	4.3	–	–	99.0
Unidentified excreted organic matter		–	–	–	1.5[a]	–
Total organic matter	30	–	–	1648.5	208.3	87.0
p-Xylene		32.1	0.1	101.7[a]	0.3[a]	99.0
Ethanol		566.0	–	1546.8[a]	–	–
Acetic acid		[b]	[b]	–	–	–
Nitrate (N–NO_3^-)		510.0	28.3	–	–	95.0
Unidentified excreted organic matter		–	–	–	208.0[a]	–

[a] Theoretical values based on COD analysis.
[b] Below limit of detection method (5.0 mg L^{-1} for acetic acid).

$$2.5C_2H_6O + 2NO_3^- + 2H^+ \rightarrow 2.5C_2H_4O_2 + N_2 + 3.5H_2O \quad (5)$$

The reactor was evaluated using hydrocarbon concentrations of benzene 41.4 mg L^{-1}, toluene 27.8 mg L^{-1}, ethylbenzene 31.1 mg L^{-1}, *o*-xylene 28.5 mg L^{-1}, *m*-xylene 28.4 mg L^{-1}, and *p*-xylene 32.1 mg L^{-1}. The organic consumption was spatially characterized and profiled after 30 days along the length of the horizontal reactor, involving each aromatic hydrocarbon separately. The hydrocarbon removal was assessed throughout the reactor's length at the end of each operating regime.

To evaluate the system in its dynamic equilibrium state only in the condition of feeding with ethylbenzene, the reactor was operated for 118 days without replacing the support medium (polyurethane foam particles) immobilized with the biomass. During this phase, different concentrations of ethanol (377–566 mg L^{-1}), nitrate (350–690 mg L^{-1}) and ethylbenzene (13.1, 15.1 and 31.1 mg L^{-1}) were used in the feeding substrate.

2.4. Chemical and chromatographic analysis

Chemical oxygen demand (COD), alkalinity, pH, nitrate and nitrite analyses were carried out in line with the standard methods for the examination of water and wastewater [9]. Volatile acid concentrations were determined using a Gas Chromatograph HP 6890 (HP Innovax column—30 m × 0.25 mm with an internal diameter of 0.25 μm), as described by Moraes et al. [10]. Hydrocarbon concentrations were determined using a static headspace gas chromatographic method [2] on a 6890 HP gas chromatograph (HP-1 column—30 m × 0.53 mm with and internal diameter of 2.65 μm).

2.5. Microscopic analysis

Morphological characteristics of microorganisms immobilized in polyurethane foam matrices were monitored using phase contrast microscopy (Olympus BX60-FLA with software Image Pro-Plus), and also by scanning electron microscopy (Zeiss digital scanning microscope DSM-960). Samples for scanning electron microscopy were subjected to the technique described by Araújo et al. [11].

2.6. Molecular analysis

Biofilm samples, obtained at the end of the profiling-procedure (benzene 41.4 mg L^{-1}, toluene 27.8 mg L^{-1}, ethylbenzene 31.1 mg L^{-1}, *o*-xylene 28.5 mg L^{-1}, *m*-xylene 28.4 mg L^{-1}, and *p*-xylene 32.1 mg L^{-1}), were submitted to 16S rDNA amplification. The extraction of rDNA was accomplished following methodology described by Griffiths et al. [12]. Polymerase chain reaction (PCR) was performed using bacterial primers described by Nielsen et al. [13], targeting the universal bacterial 16S rDNA, which is a portion of about 424 bp. In *E. coli* refers to the forward primer 968f (5′-AAC.GCG.AAG.AAC.CTT.AC-3′) with GC-clamp and the reverse primer 1392r (5′-ACG GGC GGT GTG TAC-3′). PCR positive controls comprised of three genera of bacteria (*Pseudomonas* sp.; *E. coli* and *Desulfococcus* sp.) and a negative control using *Methanosarcina* sp). It was used a "Gene Amp. PCR System 2400" (Perkin-Elmer Cetus, Norwalk, CO) with amplification reactions of an initial denaturing step of 94 °C for 5 min followed by 35 cycles of denaturing temperature of 94 °C for 45 s, annealing at 38 °C for 45 s, extension at 72 °C for 1 min and final extension at 72 °C for 7 min.

PCR products were used for diversity assessments by denaturating gradient gel electroforese, the DGGE-profiling [13]. For this purpose a denaturing gradient of 40–60% (40% of acrylamide/bis; solution 50× TAE; 40 or 60% of formamide and urea) was used. Gel electrophoresis was carried out at a constant temperature of 65 °C, 75 V for 16 h. Gels were stained with ethidium bromide. DGGE-profiling documentation was accomplished in an Eagle Eye TM III (Stratagene) under excitation of 254 nm UV, using Eagle Sight software.

Biofilm sample, obtained at the end of the profiling-procedure (ethylbenzene 31.1 mg L^{-1}), was submitted to 16S rDNA amplification and sequencing. PCR-amplification targeted the universal eubacterial 16S rDNA portion of about 880 bp using forward primer 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and reverse primer 907r (5′-CCG TCA ATT CCT TTG AGT TT-3′) following the approach described by So and Young [14]. Positive controls comprised of three genera of bacteria (*Pseudomonas* sp.; *E. coli* and *Desulfococcus* sp.) and a negative control using *Methanosarcina* sp. PCR-samples were cloned into a plasmid pCR 2.1 TOPO-TA easy vector system (Invitrogen®), and transformed into *E. coli* DHα5. From each library randomly selected, clones were screened for positive inserts after digestion with the endo-nuclease *Hha*I. A total of 80 clones were sequenced in ABI 377 DNA Sequencer (Perkin-Elmer) using M13 primers (forward and reverse, separately). The resultant nucleotide sequences were assembled, checked for potential chimerical sequences and compared with the electronic database to identify the closest matchs. Sequences were aligned using the DNASTAR package (Lasergene Sequence Analysis Software) and sequences were checked against the NCBI-database.

3. Results and discussion

3.1. Removal of aromatic hydrocarbons

Studies involving the HAIB reactor applied in the removal of BTEX under denitrification conditions have shown this system to be highly efficient in the treatment of these compounds. Ribeiro [5] reported removal efficiencies of benzene (4.9 mg L^{-1}), toluene (7.2 mg L^{-1}), *m*-xylene (3.7 mg L^{-1}) and BTEX (15.9 mg L^{-1}) were 96.3, 95.7, 96.3 and 96.1%, respectively, after the reactor reached the steady state (137 days), for an initial average nitrate ($N–NO_3^-$) concentration of 403.0 mg L^{-1}. High BTEX removal efficiencies were also achieved by Nardi et al. [2], when the anaerobic reactor was fed with an ethanol solution and BTEX (approximately 5.0 mg L^{-1} of each compound). In the latter study, after 167 days of operation, the benzene, toluene and ethylbenzene concentrations in the effluent were lower than 0.1 mg L^{-1}, and no xylene isomers were detected at any time during the experimental period. However, the high removal efficiencies attained in those studies occurred only after the reactor had become stabilized after long periods of biofilm formation and adaptation to the toxic compounds.

In the present study, high BTEX, ethanol and nitrate removal efficiencies were achieved even with a short operating period (30 days). Fig. 2 illustrates the degradation dynamics of these compounds. The lowest removal efficiencies were attained under the feeding conditions of 41.4 mg L^{-1} benzene and 28.5 mg L^{-1} *o*-xylene (Table 1). Benzene is reportedly difficult to biodegrade in the absence of oxygen, while alkylbenzenes biodegrade readily under a variety of anaerobic conditions [15,16]. The removal efficiency of *o*-xylene was lower than that of its isomers *p*-xylene and *m*-xylene, and for toluene and ethylbenzene, which

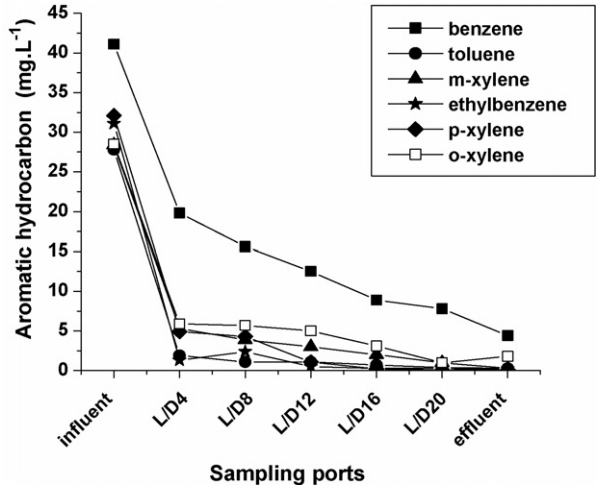

Fig. 2. Hydrocarbons concentration profiles carried out at the end of the operating regime of reactor fed with benzene, toluene, ethylbenzene and xylenes.

presented 99% removal efficiencies. Some reports suggest that the degradation of *o*-xylene occurs only in the presence of toluene [17–19]. The results we obtained in this study demonstrate that *o*-xylene was removed independently of the presence of toluene.

Chang et al. [20] conducted a study to characterize the substrate interaction of BTEX degradation with a mixed culture derived from a gasoline-contaminated aquifer in Korea. They found no interaction, stimulation, competitive inhibition, non-competitive inhibition, or cometabolism in the degradation of these compounds. Although no investigation into these metabolic aspects was conducted in this study, the independent removal of benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene, and *p*-xylene underpins the fact that they can be degraded separately, without requiring one of them to be a primary substrate for the degradation of another.

High hydrocarbon removal efficiencies have been achieved both with short (30 days) feeding conditions and with long operating periods (118 days with ethylbenzene). These results point to the high specificity of denitrifying biofilm in the removal of aromatic hydrocarbons, which was associated with the preadaptation of these cells when purified and grown in the presence of the toxic compound, thus allowing for the enzymatic induction and generation of more competent cells for the biodegradation of toxics.

The stimulation of biomass for the degradation of BTEX in underground waters contaminated by gasoline containing 24% ethanol was investigated in a study by Schneider et al. [21]. According to those authors, the negative effect of the presence of ethanol in gasoline lies in the fact that microorganisms degrade ethanol more intensively than the other organic compounds in gasoline, allowing the plume of hydrocarbons to increase continually. However, during their experiments, the researchers found that a high concentration of biomass remaining from the degradation of ethanol significantly accelerated the degradation process of the gasoline's other contaminants. Therefore, after 3 years of experiments, the presence of ethanol caused the reduction of benzene in the proximities of the source of the leak to be only 10%, but over the following 2 years, when the alcohol had biodegraded completely, this reduction increased to 99%. The authors concluded that the remaining biomass accelerated the reduction of benzene in the subsequent period, for the microorganisms were avid for substrates and the only ones available were those of the other contaminants. The results of this study corroborate the fact that ethanol acts as an accelerant of biodegradation in sites contaminated with BTEX.

3.2. Removal of organic matter

Fig. 3 shows the variations in the temporal concentrations of the monitored variables (COD, $N–NO_3^-$, $N–NO_2^-$, acetic acid, alkalinity and pH) which were recorded throughout the entire period (118 days) of operation with ethylbenzene. During this period, the reactor was fed with different concentrations of this toxic compound, which varied from 10 to 30 mg L^{-1}, with the spatial variations monitored for influent containing 13.1, 15.1 and 31.1 mg L^{-1} of ethylbenzene.

The reactor's performance was monitored constantly to assess the system's stability as a COD remover in the steady state. Overall, the reactor displayed a short start-up period (approximately 15 days), during which the effluent contained low concentrations of organic matter (Fig. 3a). Nitrate removal went hand-in-hand with organic matter removal and nitrite was detected as the intermediate compound (Fig. 3b and c). The spatial characterization-profiling of the volatile acids of all samples collected along the reactor's length, during the operating phase, suggests that the consumption of ethanol generated acetic acid, which was subsequently consumed (Fig. 4). The mean values of pH (8.8) and alkalinity (969.2 mg L^{-1} $CaCO_3^-$) (Fig. 3d and e) indicate that acidification of the effluent did not occur.

These results indicate that the reactor reached its dynamic equilibrium state, attaining high organic matter removal efficiencies which averaged 93%, with average nitrate removal of 90% for mean values of 956.3 and 390 mg L^{-1}, respectively, in the influent.

Organic matter removal efficiencies of around 76% were achieved in the reactor's short operating phases (30 days), when the reactor was fed with benzene (41.4 mg L^{-1}), toluene (27.8 mg L^{-1}), and *m*-xylene (28.4 mg L^{-1}). Fig. 5 and Table 1 show that, as a result of this operating regime, acetic acid concentrations increased in the effluent, diminishing the COD removal efficiency when compared with that achieved in the reactor's long operating phase when fed with ethylbenzene. In the latter condition, the mean removal efficiency of organic matter was 96%, with total consumption of the acetic acid generated in the metabolic process (Fig. 4). The increasing concentrations of acetic acid produced showed the absence of a proper steady-state phase, probably resulting from the short period of the biofilm's metabolic adaptation when operating for 30 days with the aforementioned feeding substrate. In the feeds with *o*-xylene and *p*-xylene, although the acetic acid was completely consumed along the reactor (Fig. 5c and e), the mean organic matter removal efficiency was 87% (Table 1), and the residual COD resulting from the excreted organic matter.

The highest rates of organic mater removal in the form of COD took place in the first portion of the horizontal reactor ($L/D = 4$). This portion of the reactor coincided with the system's highest values of biomass concentration (Fig. 5). The removal of nitrate was the direct result of organic matter consumption under all the feeding conditions (Fig. 5), presenting a mean removal efficiency of 97% (Table 1).

Gusmão et al. [6] study, in which the denitrifying HAIB reactor was fed with BTEX and operated for 189 consecutive days, the hydrocarbon removal efficiencies were 99% with an initial concentration of 26.5 mg L^{-1} benzene, 30.8 mg L^{-1} toluene, 32.1 mg L^{-1} *m*-xylene, 33.3 mg L^{-1} ethylbenzene and 26.5 mg L^{-1} BTEX. Nitrate (mean influent concentration of 449.0 mg L^{-1}) presented a mean removal efficiency of 93% and organic matter removal (mean influent concentration of 1184.0 mg L^{-1}) efficiencies were 87% on average. A comparison of the results obtained by Gusmão et al. [6] and those obtained in this study, in which the mean removal efficiencies of aromatic hydrocarbons, organic matter and nitrate were 97, 83 and 97%, respectively, indicates that the values of the parameters

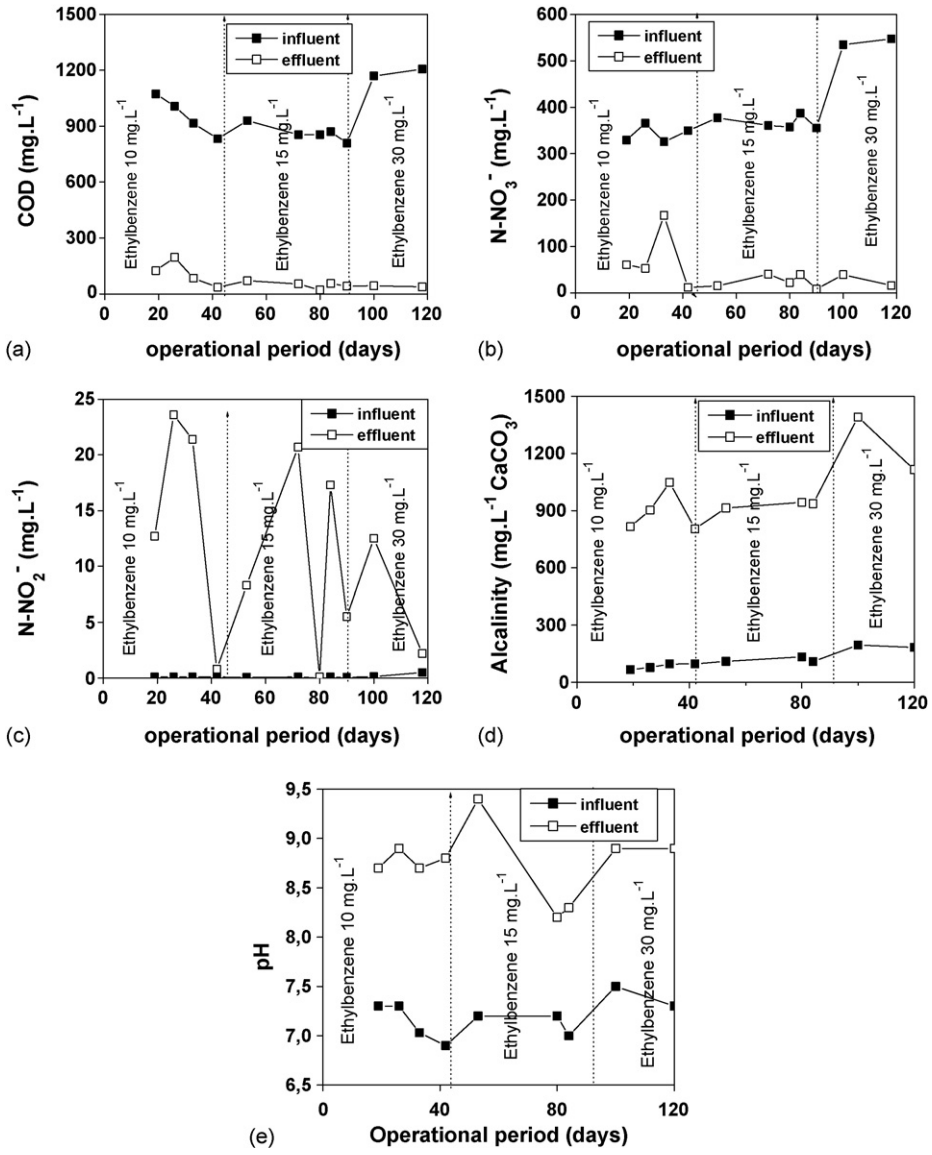

Fig. 3. Temporal variations of COD (a), $N–NO_3^-$ (b), $N–NO_2^-$ (c), alkalinity (d) and pH (e), obtained in the influent and effluent of reactor, during the operating regime with ethylbenzene.

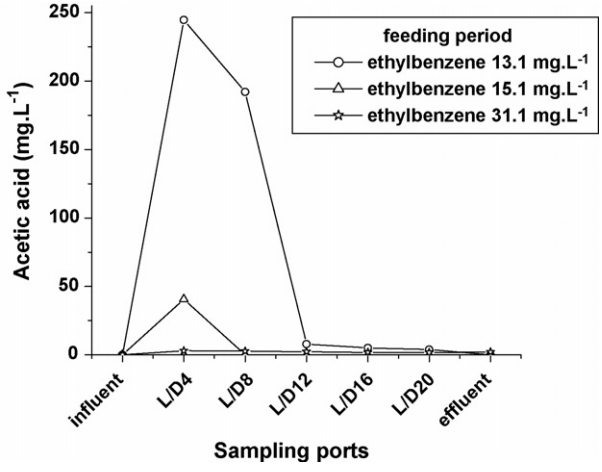

Fig. 4. Spatial characterization-profiling of volatile acids obtained during the operating regime phases with ethylbenzene amendments.

evaluated in the two studies were similar. These results highlight the strong potential of the anaerobic reactor immobilized with denitrifying biofilm operating in short times (30 days) to remove ethanol, nitrate and BTEX, without the need for long periods of biomass adaptation in order to reach high removal efficiencies.

3.3. Microscopic and molecular analysis

Microscopic characterization of the biofilm of ethylbenzene amendments found that after the adaptation period, rods were started to be predominant in the biofilm (Fig. 6a). Cell strings were frequently observed in all feeding conditions of the reactor, which may be associated to adverse conditions existing in the medium (Fig. 6b).

DGGE-profiling was used for assessing the microbial diversity of the biofilm samples obtained from all experiments. It was observed structural differences in the bacterial population

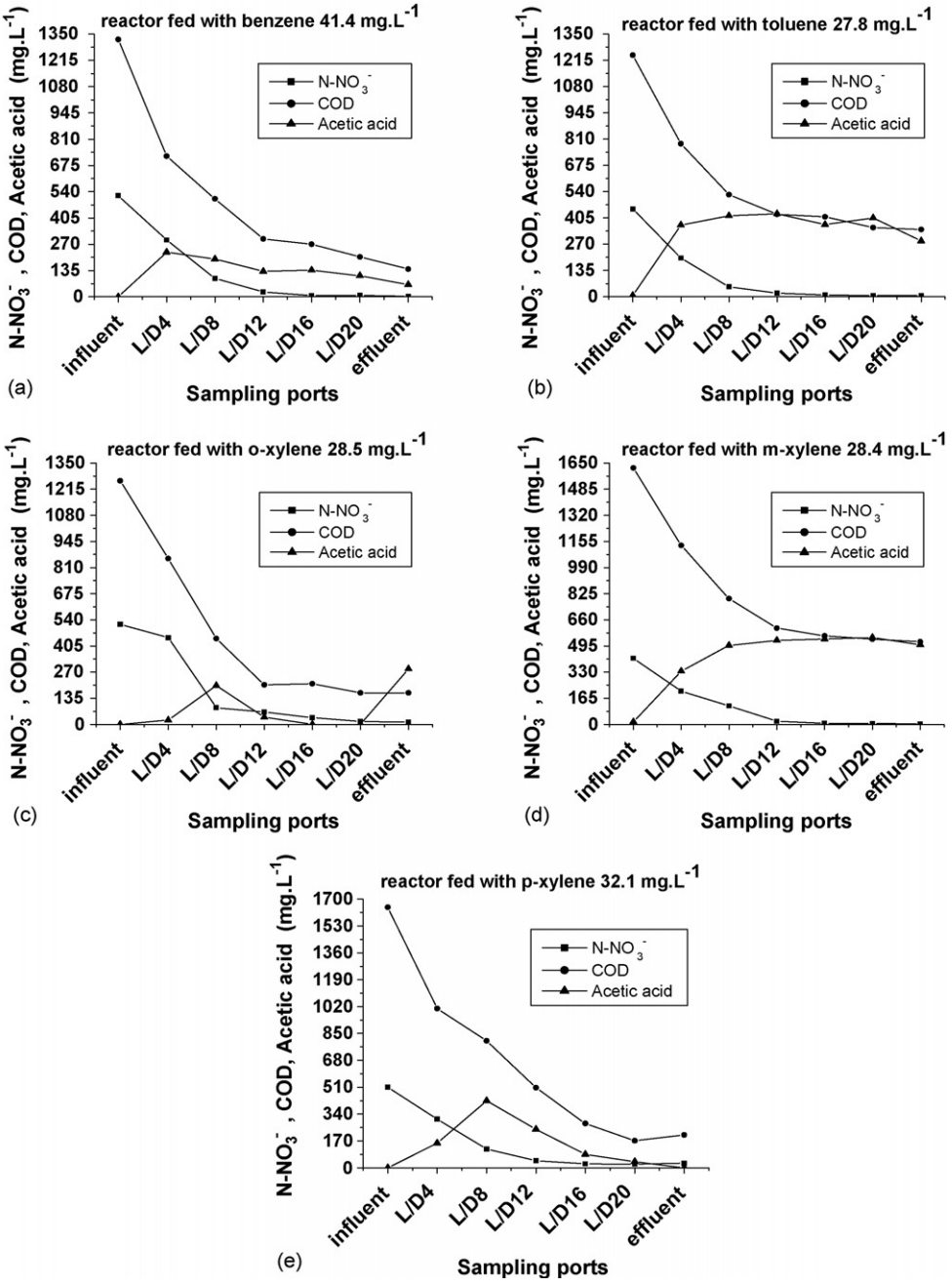

Fig. 5. Concentration profiles of organic matter (COD), acetic acid and N–NO_3^-. Results obtained at the end of the operating regime with benzene (a), toluene (b), *o*-xylene (c), *m*-xylene (d), and *p*-xylene (e).

as the result of different feeding conditions, but those were lower for the treatments with benzene and *m*-xylene (Fig. 7, lanes 1 and 5) and for the experiments with toluene and ethylbenzene (Fig. 7, lanes 2 and 3), respectively. A higher change in the DGGE band patterns were observed in the experiments using isomers of xylene (Fig. 7, lanes 4–6). The disappearance of some bands in the gel, which commonly is associated with significant decrease in population density or even extinction, might be considered to be the resultant of the direct effect of feeding conditions once all experiments were prepared in the similar manner. Different hydrocarbons were probably the main selective force affecting changes in the bacterial diversity. On the other hand, similarities were also observed between the DGGE band profiles. This suggested that the same bacterial strains, which were represented by bands in the similar position in the gel gradient, successfully colonized the biofilm in different feeding conditions. If it is also taken into account that each experiment was prepared separately having only in common the same source of inoculum and reactor's setup procedure; it is possible to suggest that the adopted approach is highly reproducible and efficiently robust in favoring the removal of benzene, toluene, ethylbenzene, *m*-xylene, *o*-xylene and *p*-xylene in the denitri-

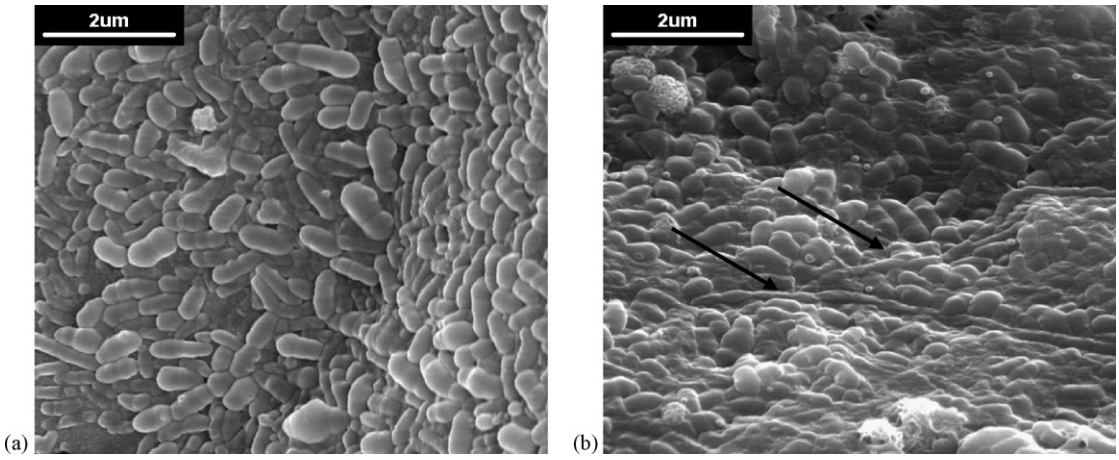

Fig. 6. Scanning electron microscopy (5000×) of microorganisms found in the biofilm of ethylbenzene 31.1 mg L^{-1} amendment: (a) rods and (b) cell strings observed at $L/D = 4$.

fying horizontal-flow anaerobic immobilized biomass reactor using as inoculum a previously adapted microbial community.

Sample sequencing obtained from the biofilm, after feeding it with ethylbenzene 31.1 mg L^{-1} found that randomly selected nucleotides sequences of about 880bp (27f and 907r primers of 16S rDNA) turned up to be almost exclusively related to the type specie of the genera *Paracoccus* (99% similarity with *Paracoccus versutus*). It was analyzed 80 clones and 77 matched with nucleotide sequence described for *Paracoccus* genera and another three clones matched with partial nucleotide sequences of *Variovorax* sp., *Pseudomonas stutzeri* and *Xanthomonas* sp.

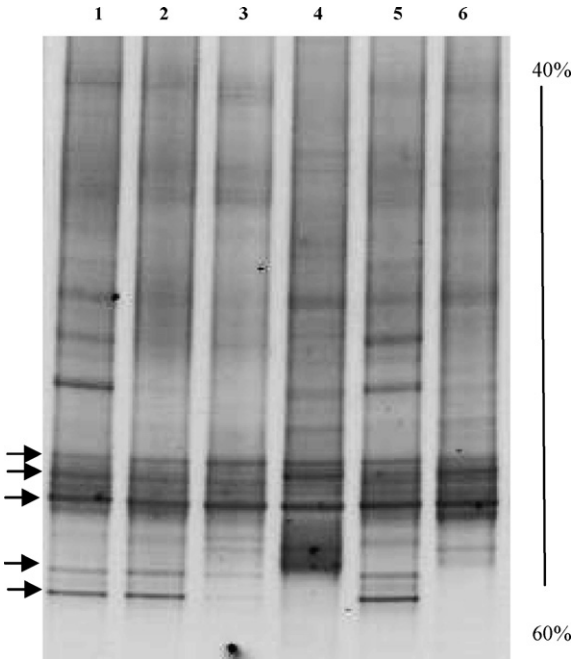

Fig. 7. DGGE-profiling of the reactor's biofilm samples obtained at the end of each different experiment. Lanes 1–6 show the band patters for the benzene (41.4 mg L^{-1}), toluene (27.8 mg L^{-1}), ethylbenzene (31.1 mg L^{-1}), *o*-xylene (28.5 mg L^{-1}), *m*-xylene (28.4 mg L^{-1}) and *p*-xylene (32.1 mg L^{-1}) amendments, respectively. The arrows point out to bands in the similar position in the gel gradient, displayed at the right side of the figure.

Few mutations were observed in some clones sequences during alignments, but in a global arrangement 77 clones matched with the described sequence of *Paracoccus versutus* (NCBI-AY014174). Therefore, the rDNA clone libraries showed that the reactor biofilm was almost exclusively colonized by one bacterial strain.

The species from the *Paracoccus* genus are probably important constituents of many residuary water treatment systems and are found in denitrified sand filters, activated sludge systems and some species have already been isolated of biological filters used in treatment of gases and contaminated soils [22]. The aromatic hydrocarbon degradation was also reported to *Pseudomonas* species [23]. Thus, the ability of different species of *Paracoccus* and *Pseudomonas* species to degrade unusual and potentially polluting compounds indicates their relevance in natural or contrived bioremediation systems.

4. Conclusions

We therefore conclude that benzene, toluene, ethylbenzene and the xylene isomers were removed independently, without the need for these substances to interact in order to degrade. Though operated in a short 30-day period, the reactor presented high removal efficiencies of both aromatic hydrocarbons and organic matter, especially ethanol and nitrate. The high removal efficiency of these substances was associated with the presence of a highly adapted biofilm and with the operating conditions imposed on the reactor.

DGGE-profiling and DNA-sequencing revealed a diversified and resourceful consortium composed of microorganisms from different phylotypes (*Paracoccus*, *Pseudomonas*) associated with pollutant degrading metabolic activities closed connected with this work.

The study of independent degradation of BTEX compounds in short reactor operating times suggests this system, immobilized with the biofilm described, and operated under the conditions imposed in the present study, can be usefully applied as a simple and low cost technology.

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