Chemical Engineering Journal 281 (2015) 272–280

Contents lists available at ScienceDirect

Chemical Engineering Journal

journal homepage: www.elsevier.com/locate/cej

Understanding the anaerobic BTEX removal in continuous-flow bioreactors for *ex situ* bioremediation purposes

Paulo Igor M. Firmino *, Raquel S. Farias, Amanda N. Barros, Patrícia M.C. Buarque, Elisa Rodríguez, Alexandre C. Lopes, André B. dos Santos

Department of Hydraulic and Environmental Engineering, Federal University of Ceará, Fortaleza, Ceará, Brazil

HIGHLIGHTS

- Good BTEX removal and reactor stability could still be reached at 24 h HRT.
- Effluent recirculation impact on BTEX removal depends on ethanol concentration.
- Shortage of ethanol had a positive effect on BTEX removal.
- Bacterial and archaeal richness changes did not match with the reactor operation.
- Dynamics and evenness seemed to be important to maintain the reactor stability.

ARTICLE INFO

Article history:
Received 19 December 2014
Received in revised form 20 June 2015
Accepted 25 June 2015
Available online 30 June 2015

Keywords:
BTEX
Anaerobic treatment
Contaminated water
Microbial community structure
UASB

ABSTRACT

This work aimed to understand the anaerobic BTEX removal in continuous-flow bioreactors for *ex situ* bioremediation purposes, evaluating the effect of some operational parameters on efficiency, stability and microbial community structure. The influence of the hydraulic retention time, effluent recirculation and co-substrate (ethanol) concentration was investigated on a mesophilic UASB bioreactor operated under methanogenic conditions. The changes on the bacterial and archaeal communities were evaluated in terms of diversity, evenness and richness. Good BTEX removal (~63%) and reactor stability could still be reached at a HRT of 24 h. The impact of the effluent recirculation on BTEX removal was not evident at high co-substrate (ethanol) concentrations, but it was significant when low concentrations were applied. The reduction of ethanol concentration had a positive impact on BTEX removal (from 80% to 86%), especially for benzene (from 51% to 62%). The optimal degree of evenness likely contributed to the relatively high stability of the system in terms of BTEX removal. Changes observed in bacterial and archaeal richness did not match with the functioning of the system. However, dynamics and evenness parameters seemed to be of importance in maintaining a stable reactor performance.

1. Introduction

Monoaromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylene isomers (BTEX), are important constituents of crude oil and its derivatives [1,2]. These compounds – which have a relatively high solubility and mobility in water – can account for up to approximately 18% (w/w) of a standard gasoline blend. Thus, from accidental leakages of underground fossil fuels storage tanks and pipelines, BTEX can contaminate extensively soils and aquifers, affecting drinking water sources [1,3].

In Brazil, ethanol is added to gasoline (20–25% v/v) according to its availability in the national market. Although this could mitigate harmful automotive emissions, it may aggravate the problem of aquifers contamination since ethanol may exert a cosolvent effect, increasing BTEX solubility in water. This fact would result in both a higher concentration of BTEX in water and the extension of the contamination [4]. In this context, the high toxicity and carcinogenic potential of BTEX compounds represent a serious environmental and public health problem, which needs to be addressed [2,3,5].

Among the different *in situ* or *ex situ* remediation technologies for BTEX-contaminated waters (e.g. advanced oxidation processes, adsorption and permeable reactive barriers) [6–8], biological processes (or bioremediation), especially the anaerobic ones, have

* Corresponding author at: Department of Hydraulic and Environmental Engineering, Campus do Pici, Bloco 713, Pici, CEP: 60455-900 Fortaleza, Ceará, Brazil. Tel./fax: +55 85 3366 9490.
E-mail address: igor@deha.ufc.br (P.I.M. Firmino).

http://dx.doi.org/10.1016/j.cej.2015.06.106
1385-8947/

drawn attention for being economical, efficient and environmentally friendly. In fact, *ex situ* bioremediation by anaerobic systems has been used successfully for the treatment of water contaminated with chemical or organic pollutants, including BTEX. However, just a small number of studies on BTEX removal in anaerobic systems have been reported in literature [9,10]. Thus, there is still a lack of information on how some operational parameters, such as hydraulic retention time, influent pollutant concentration, organic loading rate, effluent recirculation and others, can influence the biodegradation of these compounds [9].

For instance, literature reports that effluent recirculation impacts positively phenolics removal ability, compounds similar to BTEX in terms of aromaticity, since, besides improving mass transfer (contact between substrate and microorganisms), it favors influent dilution on the bottom of the reactor, thus maintaining the inhibitory compounds concentration within an appropriate range [11]. Concerning the presence of ethanol in waters contaminated with BTEX by gasoline tanks leakage, previous studies show that this compound is preferentially degraded over BTEX under different redox conditions (aerobic, nitrate-reducing, sulfate-reducing and methanogenic), hindering, therefore, aromatics degradation process [4,12].

Although much is known about the biochemistry of anaerobic BTEX biodegradation and the identity of the microorganisms involved in the process [2,13], currently, the way in which biodiversity and its components (evenness, richness, dynamics) influences the ecosystems function is still poorly understood. The investigation of potential links between environmental fluctuations and microbial community structure is one of the most challenging issues in natural and engineered environments [14]. Molecular fingerprinting techniques, e.g. DGGE, are commonly used to study structure–function relationships since it can be coupled to statistical analyses and several parameters calculations [15]. Therefore, increasing knowledge about structure–function relationships is likely the key to succeed in achieving an efficient and stable bioreactor performance for bioremediation purposes.

This work aimed to understand the anaerobic BTEX removal in continuous-flow bioreactors for *ex situ* bioremediation purposes, evaluating the effect of some operational parameters on efficiency, stability and microbial community structure. The influence of the hydraulic retention time, effluent recirculation and co-substrate (ethanol) concentration was investigated on a mesophilic up-flow anaerobic sludge blanket (UASB) bioreactor operated under methanogenic conditions. The changes on the bacterial and archaeal communities were evaluated in terms of diversity, evenness and richness.

2. Material and methods

2.1. Experimental set-up

The continuous-flow experiment was carried out in a lab-scale UASB reactor with a working volume of 3.3 L (Fig. S1). The reactor was inoculated with an anaerobic sludge (50 g VSS · L^{-1}) from a brewery mesophilic internal circulation (IC) reactor (Horizonte, Ceará, Brazil), whose specific methanogenic activity was 0.63 g COD · g VSS^{-1} · d^{-1}. In order to avoid the formation of preferential flow paths or short circuiting flows through the sludge blanket and facilitate the biogas release, avoiding the piston effect (sludge blanket rise due to entrapped biogas), a slow stirrer (5 rpm) was installed in the reactor [16].

The influent was stored at approximately 5 °C in a PVC container (total volume of 7 L) provided with a N_2 atmosphere (100%, White Martins, Brazil) from a Tedlar®gas sampling bag (Supelco, USA) in order to avoid BTEX volatilization inside the container and minimize the influent contact with the O_2 from the air. The reactor was fed by a peristaltic pump (Minipuls 3, Gilson, USA) through Tygon® Fuel and Lubricant tubing (Cole-Parmer, USA) – which is inert to the aromatic compounds tested – and operated at room temperature of approximately 27 °C. In some experimental periods, effluent was recirculated by a dosing pump (Concept Plus, ProMinent Dosiertechnik GmbH, Germany). The biogas produced was collected and measured by a previously calibrated gas meter (liquid displacement method).

2.2. Synthetic BTEX-contaminated water

The synthetic contaminated water consisted of an aqueous solution containing BTEX, i.e. benzene (99.5%, Dinâmica Química, Brazil), toluene (99.5%, Vetec, Brazil), ethylbenzene (99.0%, Sigma–Aldrich, USA), *o*-xylene (98.0%, Fluka, USA), *m*-xylene (99.0%, Sigma–Aldrich, USA) and *p*-xylene (99.0%, Sigma–Aldrich, USA), a co-substrate, basal medium (macro and micronutrients) and a buffer. The co-substrate was ethanol (99.8%, Dinâmica, Brazil), and the basal medium was prepared according to Firmino et al. [17]. To keep the pH around 7.0, the solution was buffered with sodium bicarbonate ($NaHCO_3$) in the proportion of 1 g $NaHCO_3$ to each 1 g COD. All chemicals were used as purchased without further purification.

2.3. Experimental procedure

The experiment with synthetic BTEX-contaminated water was divided in seven periods (Table 1), including the reactor start-up (acclimatization period) (period I), during which ethanol was the only carbon and energy source. After reaching steady operational conditions during the start-up period, the reactor was fed with BTEX at an average total concentration of approximately 18 mg · L^{-1} (~3 mg · L^{-1} of each compound) (period II). Subsequently, the hydraulic retention time (HRT) of the reactor was reduced from 48 to 36 (period III) and, then, to 24 h (stage IV) in order to assess the impact of this decrease on the reactor efficiency and stability.

In period V, HRT was re-established to 48 h, and an effluent recirculation flow rate of 0.7 L · h^{-1} was applied to the system to verify its effect on the mass transfer (substrate-microorganism contact) and, therefore, on anaerobic BTEX removal. Then, in period VI, in order to assess the effect of the co-substrate loading rate on anaerobic BTEX removal while the effluent was recirculated, ethanol concentration was decreased to approximately 0.11 g · L^{-1}. Finally, in period VII, the recirculation system was turned off in

Table 1
Operational parameters of the anaerobic methanogenic reactor over the experimental periods.

Period	I	II	III	IV	V	VI	VII
End of period (day)	49	201	251	282	308	343	363
HRT (h)	48	48	36	24	48	48	48
Total COD (g · L^{-1})	1.8	1.6	1.6	1.7	1.6	0.3	0.3
Ethanol (g · L^{-1})	0.85	0.72	0.76	0.77	0.76	0.11	0.10
BTEX (mg · L^{-1})	–	18.0	17.0	14.6	16.1	19.5	19.1
Benzene (mg · L^{-1})	–	3.4	2.9	2.6	2.6	3.0	2.8
Toluene (mg · L^{-1})	–	2.9	2.7	2.4	2.5	3.3	3.3
Ethylbenzene (mg · L^{-1})	–	3.0	3.0	2.5	2.8	3.5	3.5
o-Xylene (mg · L^{-1})	–	2.9	2.5	2.2	2.4	3.0	3.1
m,p-Xylenes[a] (mg · L^{-1})	–	5.9	5.9	5.0	5.7	6.8	6.5
Recirculation (L · h^{-1})	–	–	–	–	0.7	0.7	–

HRT, hydraulic retention time; COD, chemical oxygen demand.
[a] The isomers *meta*- and *para*-xylenes were quantified together due to the chromatographic method limitation.

order to confirm its impact on reactor performance at low co-substrate concentrations. The transition between the different experimental periods was carried out after verifying the effluent BTEX concentration stability (variation of up to 10%) in the last three analyzed points (equivalent to one week of operation).

2.4. Chemical and chromatographic analyses

COD and pH were determined according to Standard Methods for the Examination of Water and Wastewater [18], whereas volatile fatty acids (VFA) were determined by Kapp titrimetric method [19].

BTEX were determined by static headspace extraction (Triplus HS, Thermo Scientific, USA) followed by gas chromatography with photoionization detection (HS-GC-PID) (Trace GC Ultra, Thermo Scientific, USA) as described by Carneiro et al. [20]. All samples (15 mL) were previously diluted with ultrapure water (Milli-Q system, EMD Millipore, USA) directly into 20 mL headspace borosilicate glass vials (Supelco, USA), which were then sealed with PTFE/silicone septa and aluminum crimp seals (Supelco, USA).

Biogas characterization was carried out, in terms of air ($O_2 + N_2$), CO_2 and CH_4, by gas chromatography with thermal conductivity detection (GC-TCD) (GC-17A, Shimadzu Corporation, Japan) as described by Firmino et al. [21].

2.5. Microbial community analyses

To evaluate the structure (diversity, evenness and richness) of the bacterial and archaeal communities in the reactor, biomass samples were collected at the end of each experimental period from the bottom of the reactor and were immediately frozen at −20 °C until DNA extraction was performed. Genomic DNA was extracted according to Firmino et al. [21]. The universal bacterial primers 968-F-GC and 1401-R, and the archaeal primers A109(T)-F and 515-GC-R (IDT, USA) were used in the polymerase chain reaction (PCR). The PCR mixture (50 μL) contained 10 μL of reaction buffer (5X), 5 μL of $MgCl_2$ (25 mM), 0.25 μL of Taq polymerase ($5\ u \cdot \mu L^{-1}$) (Promega, USA), 1.0 or 1.5 μL of deoxynucleotide triphosphates (10 mM), l μL of the extracted DNA, 1.0 or 1.5 μL of PCR primers (10 μM) and Milli-Q water up to a final volume of 50 μL. The PCR thermocycling program used was previously described by Firmino et al. [21].

The Double Gradient DGGE technique, which provides an improved resolution of the bands [22], was used to analyze the PCR amplicons. DGGE was performed in a D-Code Universal Mutation Detection System (Bio Rad Laboratories, USA) using polyacrylamide gels with a urea/formamide denaturing gradient of 42–67% and 30–60% for bacterial and archaeal communities, respectively and superimposed with a porous gradient of acrylamide/bisacrylamide (6–10%). The electrophoresis conditions and gel staining were previously described by Firmino et al. [21].

The obtained DGGE patterns were processed using the Bionumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). After image normalization, bands were defined for each sample using the bands search algorithm of the program. Similarity values of the compared profiles were calculated from the densitometric curves of the scanned DGGE profiles by using the Pearson product-moment correlation coefficient [23] and were subsequently used to construct dendrograms by UPGMA clustering. Based on the DGGE data obtained, the ranged weighted richness (*Rr*), which indicates the richness and genetic diversity within a microbial community, the evenness/functional organization (*Fo*) [15] and the Shannon–Wiener diversity index (*H*) were calculated as described by Lebrero et al. [24].

2.6. Statistical methods

Statgraphics Centurion XV computer software was used for the statistical analysis of the data, being applied the Mann–Whitney Rank Sum and Kruskal–Wallis ANOVA on Ranks tests, non-parametric procedures which do not require a specific data distribution, to compare the performance of the reactor during the different experimental periods. The results of the tests were evaluated according to the *p*-value. If $p \leqslant 0.05$, the null hypothesis is rejected, i.e. the data groups are considered statistically different.

3. Results and discussion

3.1. Reactor start-up and adaptation to BTEX

During the reactor start-up (period I), when ethanol was the only carbon and energy source, a high average COD removal efficiency (∼96%) was achieved, and, consequently, the average methane yield was 0.41 L per g COD removed ($L \cdot g\ COD_{rem}^{-1}$) (Table 2). Then, after reaching operational stability, the reactor started to be fed with BTEX (period II), and an approximately 3.5% drop in the average COD removal efficiency was observed ($p < 0.01$). Nevertheless, there was no statistical difference between the methane yields of periods I and II ($p = 0.22$) (Table 2). Thus, apparently, although the aromatic hydrocarbons addition has caused a slight negative impact on the organic matter removal performance, it did not compromise the reactor operation.

For all BTEX compounds, with the exception of benzene (∼53%), higher removal efficiencies were obtained at the beginning of the period (>70% for toluene, >85% for *o*-xylene and >90% for the other compounds), which decreased over time (Fig. 1). It is likely that these higher efficiencies were achieved due to the initial adsorption of the compounds in the sludge blanket, which seemed to reach saturation after day 100 of operation (Fig. 1). In addition, at the end of the period, better removal efficiencies and effluent quality were obtained, especially for toluene (Fig. 1), suggesting a greater microbiota adaptation to the aromatic hydrocarbons.

In terms of average removal efficiency, the highest value was obtained for *m*- and *p*-xylenes and ethylbenzene (∼87%), followed, in decreasing order, by the compounds *o*-xylene, toluene and, finally, benzene (with only 53%) (Table 3). de Nardi et al. [25], using a mesophilic (30 °C) horizontal-flow anaerobic immobilized biomass (HAIB) reactor at a HRT of 11.4 h for the treatment of BTEX-contaminated waters (∼3 $mg \cdot L^{-1}$ for each compound) in the presence of ethanol, observed the same abovementioned pattern, i.e. the highest average efficiency was achieved for the isomers *m*- and *p*-xylenes (93%), whereas benzene was the most recalcitrant compound (82%). However, these authors obtained average removal efficiencies above 80% for all compounds.

In fact, it was already expected that the lowest efficiencies were achieved for benzene as several studies on the anaerobic BTEX degradation show that this compound is the most recalcitrant under such conditions [2,5,26]. This can be justified by the fact that benzene is thermodynamically very stable due to due to the symmetric π-electron system of the aromatic ring and the lack of potentially destabilizing or reactive substituents [27].

3.2. Effect of the HRT on BTEX removal

By reducing the reactor HRT from 48 to 36 (period III) and, then, to 24 h (period IV), no statistically significant differences were observed in the COD removal efficiency ($p = 0.28$) and the methane yield ($p = 0.52$) (Table 2). However, concerning BTEX removal, in period III (HRT = 36 h), the average removal efficiencies of all compounds decreased ($p \leqslant 0.01$), with the exception of toluene, for

Table 2
Operational performance of the anaerobic methanogenic reactor in terms of COD removal and methane production over the experimental periods.

Period		I	II	III	IV	V	VI	VII
HRT (h)		48	48	36	24	48	48	48
Ethanol ($g \cdot L^{-1}$)		0.85	0.72	0.76	0.77	0.76	0.11	0.10
Recirculation ($L \cdot h^{-1}$)		–	–	–	–	0.7	0.7	–
pH	Effluent	7.3 (0.2)	7.2 (0.2)	7.6 (0.6)	7.0 (0.6)	7.6 (0.2)	7.9 (0.2)	7.7 (0.1)
COD	Influent ($mg \cdot L^{-1}$)	1786 (147)	1570 (219)	1634 (141)	1662 (98)	1644 (255)	300 (50)	275 (37)
	Effluent ($mg \cdot L^{-1}$)	71 (24)	116 (57)	114 (60)	172 (110)	191 (105)	167 (96)	154 (37)
	Efficiency (%)	96.0 (1.4)	92.5 (4.2)	93.0 (3.9)	89.8 (6.1)	88.6 (5.8)	52.4 (13.5)	44.4 (8.9)
CH_4	($L \cdot d^{-1}$)	1.25 (0.16)	0.91 (0.10)	1.26 (0.19)	1.49 (0.12)	0.86 (0.06)	–	–
	($L \cdot g \cdot COD_{rem}^{-1}$)	0.41 (0.07)	0.37 (0.06)	0.40 (0.02)	0.37 (0.04)	0.46 (0.07)	–	–

HRT, hydraulic retention time; COD, chemical oxygen demand; COD_{rem}, chemical oxygen demand removed.
The standard deviation is shown in parentheses.

which a higher average value was observed ($p = 0.04$) (Table 3). Then, in period IV (HRT = 24 h), the removal efficiencies of benzene ($p = 0.31$) and toluene ($p = 0.62$) remained statistically similar to those obtained in period III (Fig. 1), whereas the efficiencies of the other compounds decreased moderately (~10%) ($p < 0.01$) (Table 3).

Although the influent BTEX concentrations were evidently lower in period IV ($p \leqslant 0.03$) (Table 3), which could have contributed to the reduction in the efficiency of ethylbenzene and xylenes, their effluent concentrations were significantly higher than those of the previous period ($p < 0.01$) (Fig. 1). Thus, in general, the HRT reduction seemed to affect adversely BTEX removal performance, most likely, as a result of the increase in the aromatics loading applied to the system (from 9.0 to 14.6 mg BTEX $\cdot L^{-1} \cdot d^{-1}$), which was not followed by a proportional cell growth of the BTEX-degrading microorganisms due to their specific kinetic properties [28].

Ramakrishnan and Gupta [29], during the treatment of synthetic wastewater containing phenolics ($752\ mg \cdot L^{-1}$), i.e. aromatic compounds whose anaerobic degradation is similar to that of BTEX, in a mesophilic (27 °C) hybrid anaerobic reactor, observed that, by reducing the HRT from 36 to 24 and, then, to 18 h, the removal efficiency of these aromatics decreased, respectively, from 99 to 96 and, finally, to 91.5%. However, after morphological analysis of the inoculum granules by scanning electron microscopy, the authors suggested that the decrease in the system bioactivity can be attributed to the intensification in toxicity to microbiota caused by the phenolics loading increase as a result of the HRT reduction.

In contrast, de Nardi et al. [25], using a mesophilic (25 °C) HAIB reactor to remove benzene ($14.5\ mg \cdot L^{-1}$), toluene ($12.5\ mg \cdot L^{-1}$) and *m*-xylene ($5.5\ mg \cdot L^{-1}$) from gasoline-contaminated water, observed no difference in their reactor BTX removal performance by reducing the HRT from 20 to 16 h. However, the removal efficiencies dropped sharply from 95% to 70% when the reactor started to be operated at a HRT of only 8 h [25].

Finally, it is important to mention that a good BTEX removal (~63%) and reactor stability could still be reached at a 24 h HRT (Table 3). Certainly, sometimes a reduction in the efficiency (from 77%, at 48 h, to 63%, at 24 h) is acceptable considering that the reactor volume would decrease by half.

3.3. Effect of the effluent recirculation and co-substrate concentration on BTEX removal

In period V, the HRT was re-established to 48 h, and an effluent recirculation flow rate of $0.7\ L \cdot h^{-1}$ (recirculation/feed ratio of approximately 10) was applied to the reactor. In comparison with period IV (HRT = 24 h), no significant differences were observed in the COD removal efficiency ($p = 0.40$), whereas the methane production was approximately 20% higher ($p = 0.02$) (Table 2). However, when comparing the reactor performance for the same HRT (periods II and V), there was a 3.9% drop in the average COD removal efficiency ($p = 0.04$). Nevertheless, statistical tests revealed no significant difference between the effluent quality ($p = 0.07$) and methane production ($p = 0.06$) of both periods (Table 2).

The removal efficiencies of all hydrocarbons have increased considerably compared to the previous period ($p < 0.01$) (Table 3). However, it is not possible to attribute the improved reactor performance exclusively to the effluent recirculation use since the pollutant loading applied in period V (HRT = 48 h) was 50% lower than in period IV (TDH = 24 h). Thus, in order to verify the impact of recirculation for the same HRT, the results were compared with those obtained at the end of period II. Unexpectedly, for all BTEX compounds, the statistical tests revealed no significant differences between the removal efficiencies ($0.07 \leqslant p \leqslant 0.94$) and the effluent concentrations ($0.05 \leqslant p \leqslant 0.52$) obtained in periods II and V (Fig. 1). Therefore, apparently, the recirculation did not affect the reactor BTEX removal performance.

Continuous-flow experiments with wastewater containing phenolic compounds shows that, normally, the effluent recirculation has a positive impact on the removal of these pollutants as, besides improving the mass transfer, it favors the dilution of the influent on the bottom of the reactor, thus maintaining the inhibitory compounds concentration within an appropriate range [11]. However, as we observed no changes in the aromatic hydrocarbons removal when recirculation was imposed, it is likely that neither mass transfer was a process-limiting factor nor pollutants concentrations were at inhibitory levels.

In period VI, the co-substrate loading was reduced (by ~7 times) in order to assess its impact on anaerobic BTEX removal since ethanol is preferentially degraded over these compounds under different redox conditions (aerobic, nitrate-reducing, sulfate-reducing and methanogenic), thus hindering the aromatic hydrocarbons degradation process [4,12]. The effluent quality in terms of COD remained similar to that of the previous period ($p = 0.71$). Consequently, due to the lower influent COD in this period ($p < 0.01$), lower removal efficiency values were achieved when compared to period V ($p < 0.01$) (Table 2). Additionally, it was not possible to register the amount of methane produced from a very low influent COD due to the limitation of the gas meter used.

As for BTEX removal, at the beginning of the period, an increase in the efficiency and, consequently, in the effluent quality (i.e. much lower effluent concentrations) of all compounds, noticeably for benzene, was observed (Fig. 1). However, over the period, the hydrocarbons effluent concentrations increased. Only for benzene, there was a gradual decrease in the efficiencies to values close to those obtained in the previous period (Fig. 1). Probably, this behavior could be the result of an adaptation of the microbial community to the new operational conditions (carbon source scarcity). Nevertheless, the removal efficiencies of all BTEX compounds in this period were considered statistically higher than those in the

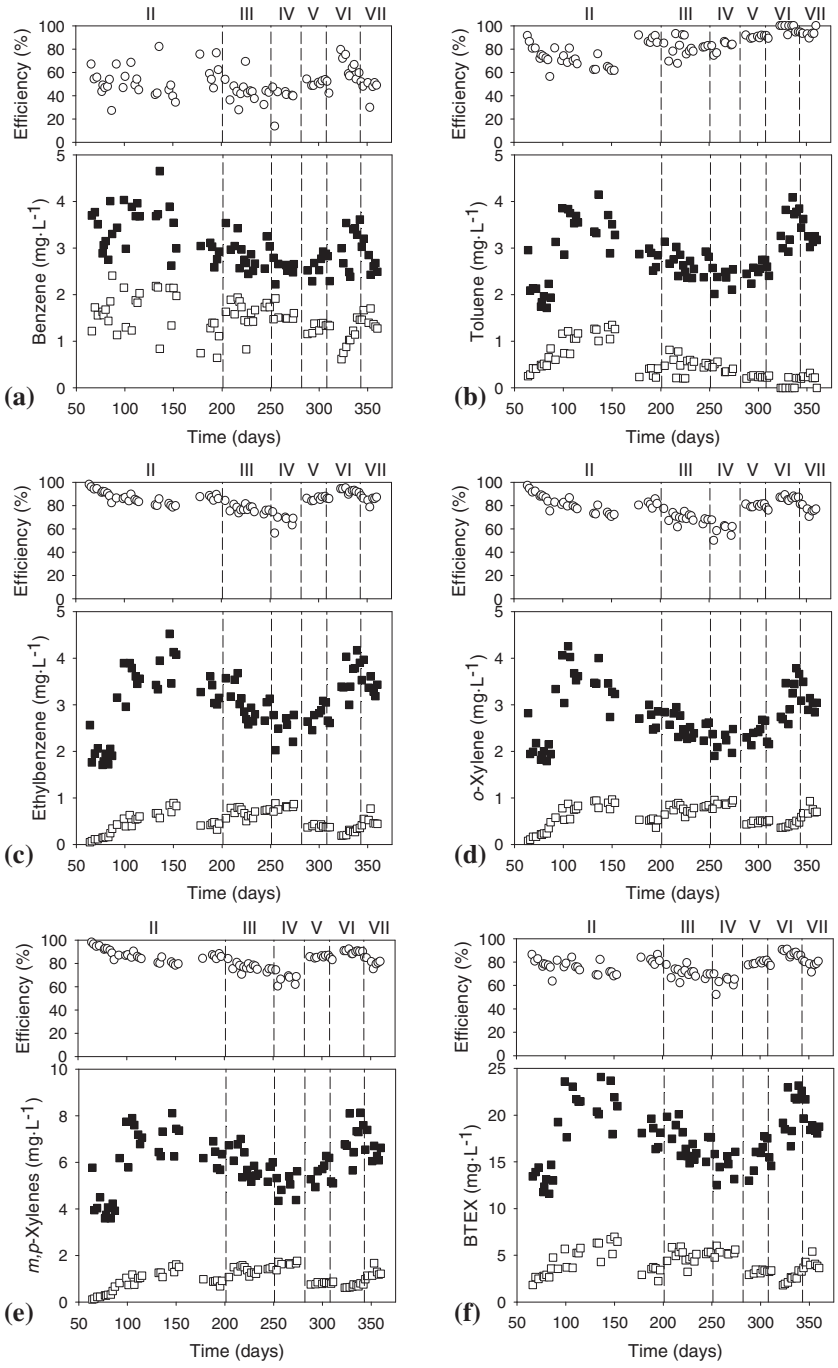

Fig. 1. Influent (■) and effluent (□) BTEX concentrations and their removal efficiencies (○). (a) benzene, (b) toluene, (c) ethylbenzene, (d) *o*-xylene, (e) *m*,*p*-xylenes, (f) total BTEX.

period V ($0.01 \leqslant p \leqslant 0.03$) (Table 3). With regard to the effluent quality, only for benzene ($p = 0.17$) and *o*-xylene ($p = 0.10$), it did not improve significantly (Table 3). Therefore, apparently, ethanol scarcity favored BTEX degradation, being in agreement with previous batch experiments, which showed that addition of preferential substrate, such as, for instance, acetate, hydrogen, methanol, glucose, aminoacids, fatty acids and yeast extract, completely inhibited toluene and xylenes degradation, under methanogenic and sulfate-reducing conditions, until such co-substrates were almost totally consumed [30].

It is interesting to remind that, even when high ethanol concentrations were used (periods II–V), BTEX removal occurred reasonably (40–90%). Thus, it is assumed that the reactor could contain microbial populations with specific affinity for BTEX, which were likely responsible for the initial attack on these hydrocarbons. Additionally, since there was an increase in the aromatic compounds removal efficiency after the co-substrate loading reduction, it is suggested that some microbial populations, responsible for the initial ethanol fermentation, were also able to start BTEX degradation, although the fermentation of these compounds to acetate and hydrogen is energetically less favorable than that of ethanol [31]. Hence, due to the scarcity of an easily degradable substrate, such microorganisms would have started to degrade the aromatic hydrocarbons, which could justify the better initial reactor performance.

Finally, in period VII, the recirculation system was turned off in order to confirm its impact on the reactor BTEX removal performance when low co-substrate concentrations were used.

Table 3
Operational performance of the anaerobic methanogenic reactor in terms of BTEX removal over the experimental periods.

Period		II	III	IV	V	VI	VII
HRT (h)		48	36	24	48	48	48
Ethanol ($g \cdot L^{-1}$)		0.72	0.76	0.77	0.76	0.11	0.10
Recirculation ($L \cdot h^{-1}$)		–	–	–	0.7	0.7	–
BTEX	Influent ($mg \cdot L^{-1}$)	18.0 (3.9)	17.0 (1.7)	14.6 (1.4)	16.2 (1.3)	19.5 (3.1)	19.1 (1.2)
	Effluent ($mg \cdot L^{-1}$)	4.2 (1.5)	4.9 (0.7)	5.3 (0.4)	3.2 (0.2)	2.7 (0.6)	4.1 (0.6)
	Efficiency (%)	77.1 (5.9)	70.8 (4.4)	63.2 (5.7)	80.0 (1.3)	85.9 (4.5)	78.4 (3.3)
B	Influent ($mg \cdot L^{-1}$)	3.4 (0.5)	2.9 (0.3)	2.6 (0.2)	2.7 (0.2)	3.0 (0.5)	2.8 (0.3)
	Effluent ($mg \cdot L^{-1}$)	1.6 (0.5)	1.6 (0.3)	1.6 (0.2)	1.3 (0.1)	1.1 (0.3)	1.5 (0.2)
	Efficiency (%)	53.0 (12.8)	43.5 (9.5)	38.3 (11.2)	51.2 (2.3)	61.8 (10.9)	46.9 (7.7)
T	Influent ($mg \cdot L^{-1}$)	2.9 (0.7)	2.7 (0.3)	2.4 (0.2)	2.6 (0.2)	3.3 (0.6)	3.3 (0.2)
	Effluent ($mg \cdot L^{-1}$)	0.7 (0.4)	0.5 (0.2)	0.4 (0.1)	0.2 (0.0)	0.1 (0.1)	0.2 (0.1)
	Efficiency (%)	74.9 (10.2)	81.2 (7.5)	81.8 (4.5)	90.5 (1.2)	96.5 (4.3)	93.7 (3.2)
E	Influent ($mg \cdot L^{-1}$)	3.0 (0.9)	3.0 (0.4)	2.5 (0.3)	2.8 (0.2)	3.5 (0.5)	3.5 (0.3)
	Effluent ($mg \cdot L^{-1}$)	0.4 (0.2)	0.7 (0.1)	0.8 (0.1)	0.4 (0.0)	0.3 (0.1)	0.5 (0.1)
	Efficiency (%)	86.7 (5.1)	77.2 (3.1)	67.3 (5.9)	85.9 (1.6)	91.3 (3.1)	85.2 (3.1)
o-X	Influent ($mg \cdot L^{-1}$)	2.9 (0.8)	2.5 (0.2)	2.2 (0.2)	2.4 (0.2)	3.0 (0.6)	3.1 (0.2)
	Effluent ($mg \cdot L^{-1}$)	0.6 (0.3)	0.8 (0.1)	0.9 (0.1)	0.5 (0.0)	0.4 (0.1)	0.7 (0.1)
	Efficiency (%)	81.8 (6.9)	69.5 (4.0)	59.4 (5.9)	80.1 (1.2)	84.8 (4.1)	76.7 (3.6)
m,p-X	Influent ($mg \cdot L^{-1}$)	6.0 (1.4)	5.9 (0.6)	5.0 (0.5)	5.7 (0.5)	6.8 (1.1)	6.5 (0.5)
	Effluent ($mg \cdot L^{-1}$)	0.8 (0.4)	1.4 (0.2)	1.6 (0.1)	0.8 (0.0)	0.7 (0.1)	1.2 (0.2)
	Efficiency (%)	86.8 (5.2)	76.6 (3.3)	66.9 (4.7)	85.7 (0.9)	88.9 (2.8)	81.1 (3.4)

HRT, hydraulic retention time; B, benzene; T, toluene; E, ethylbenzene; *o*-X, *orto*-xylene; *m,p*-X, *meta*- e *para*-xylenes.
The standard deviation is shown in parentheses.

Although the average COD removal efficiency decreased by 8% (Table 2), no significant differences were observed between the efficiency values ($p = 0.27$) and effluent COD ($p = 1.00$) of the periods VI and VII. Again, methane production was not registered due to the aforementioned reasons.

With the exception of toluene ($p = 0.19$), the removal efficiencies of all BTEX compounds decreased significantly ($p \leqslant 0.01$), thus compromising the effluent quality (Table 3). Hence, contrary to the observed in periods II and V (high ethanol concentration), apparently, the recirculation had a positive effect on BTEX removal for low co-substrate concentrations. Probably, the greater biogas production from high ethanol loadings would have been sufficient to guarantee favorable hydrodynamic conditions to an effective mass transfer, masking, therefore, the effect of recirculation on removal of these aromatic compounds.

3.4. Bacterial and archaeal community structure in the reactor

The effect of the operational conditions on the diversity, evenness and richness of the bacterial and archaeal communities was analyzed. Table 4 shows the diversity estimators range-weighted richness (*Rr*), Shannon diversity (*H*) and functional organization (*Fo*) of the bacterial and archaeal communities. The temporal dynamics of the communities was assessed by UPGMA clustering analysis based on the Pearson similarity coefficient (Fig. 2). These results are based on the DGGE profiles of the bacterial and archaeal communities (Fig. 3).

The bacterial community evenness was reflected by means of the *Fo* parameter (Table 3, Fig. S2), which can be considered as a measure for the degree of functional organization of the bacterial community, i.e. the higher the *Fo* value, the more specialized the bacterial community is [15]. According to the classification of Marzorati et al. [15], the reactor maintained a medium evenness value, i.e. between 34 and 41, indicating that most fitting species were dominant (a certain level of organization existed), but the majority of the species were present in decreasing lower amounts (functionally redundant species existed). This kind of communities can potentially better deal with changing environmental conditions since they have a pool of less dominant species, which can proliferate to replace the current ones under perturbations. In this context, the optimal degree of evenness of the bacterial communities likely contributed to the relatively high stability of the reactor in terms of BTEX removal, despite the variation observed in the influent BTEX concentrations and the operational conditions of the system. It is important to note that the degree of evenness of the bacterial communities in the inoculum sample (a medium *Fo* value of 38) could be the key to further maintain reactor stability since it has been reported that initial community evenness contributes to functional stability [32].

Clustering showed highly dynamic bacterial communities (Fig. 2a). This could have helped in maintaining functional stability of the reactor since a dynamic microbial community with a high initial evenness is considered of vital importance to guarantee functional stability in microbial communities [33]. A clear impact in the bacterial community of ethanol introduction in period I

Table 4
Shannon diversity index (*H*), ranged weighted richness (*Rr*) and functional organization (*Fo*) calculated from the DGGE patterns.

Parameters	Seed	Period I	Period II	Period III	Period IV	Period V	Period VI	Period VII
Bacteria								
Functional organization (*Fo*) (%)	38	41	36	35	35	36	40	34
Ranged weighted richness (*Rr*)	75	14	4	41	48	80	4	29
Shannon diversity (*H*)	3.1	2.1	1.7	3.0	2.9	3.0	1.9	2.8
Archaea								
Functional organization (*Fo*) (%)	43	38	36	37	37	36	37	38
Ranged weighted richness (*Rr*)	117	254	524	241	275	224	291	272
Shannon diversity (*H*)	2.8	3.2	3.6	3.2	3.2	3.2	3.3	3.3

Fo: Low *Fo*/high evenness (25%), Medium *Fo* and evenness (45%), High *Fo*/low evenness (85%) Marzorati et al. [15].
Rr: Low (<10), Medium (10–30), High (>30) Marzorati et al. [15].

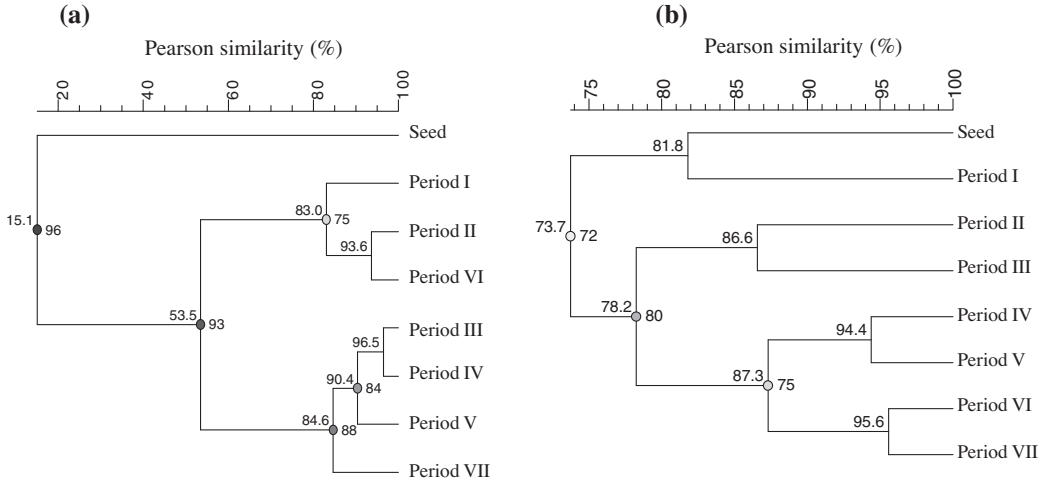

Fig. 2. Dendrograms based on the 16S rRNA DGGE profiles of the bacterial (a) and archaeal (b) communities. The labels indicate the experimental period at which each sample was collected.

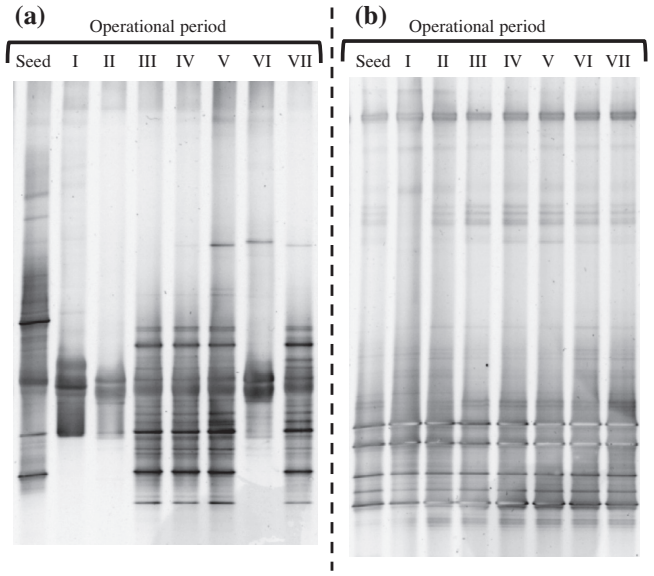

Fig. 3. Bacterial (a) and archaeal (b) DGGE profiles of 16S rRNA amplicons from the anaerobic reactor. Lane upper labels indicate the experimental period at which each sample was collected.

was observed. Pairwise comparison of the DGGE profiles revealed a Pearson similarity value of 15% between the inoculum and the rest of the samples (Fig. 2a), indicating that drastic changes occurred in response to ethanol introduction, in which the most fitted populations for the operational conditions imposed were able to emerge. Bacterial richness (*Rr*) and Shannon diversity (*H*) decreased from a high value of 75 (seed) to a medium value of 14 (period I) and from 3.1 (seed) to 2.1 (period I), respectively. This suggests the fitting of the bacterial community towards the restricted environmental conditions imposed (ethanol as sole carbon and energy source). The Shannon diversity index (*H*) takes into account the richness and the evenness of the species, and typically its value ranges from 1.5 (low species evenness and richness) to 3.5 (high species evenness and richness) [34]. Since the evenness remained almost constant during the whole experiment, it can be assumed that the variation in Shannon diversity was mostly affected by differences in bacterial richness rather than in community evenness.

In terms of bacterial dynamics, BTEX addition in period II did not cause a significant effect (83% similarity between the samples of period II and period I). However, *Rr* decreased from a value of 14 (period I) to a value of 4 (period II), and Shannon diversity also decreased from 2.1 (period I) to 1.7 (period II). It is likely that, in period II, bacterial communities continued to adapt to ethanol degradation (more specialized community), since BTEX added to the system represented only 3% of the total COD. An impact of decreasing the HRT from 48 (period II) to 36 h (period III) was observed, showing these samples a similarity value of 53.5 (Fig. 2a).

Increasing the organic loading rate (ethanol + BTEX) in the reactor (from 48 to 36 and, then, to 24 h) led to the recovery of *Rr* and Shannon diversity values (Table 4). However, the increase in species richness did not match with an improvement of reactor performance in terms of BTEX removal. The observed increase in richness can be explained by the increased availability of BTEX at the HRT of 36 and 48 h, as compared to the conditions maintained in periods I and II, where ethanol was the main carbon source available for microorganisms. In this context, it has been shown that the addition of species to the system is unlikely to have a substantial effect on the level of ecosystem functioning, if the new species perform an equivalent function than those already present in the system [35], which could explain the absence of a positive correlation within richness and BTEX reactor performance. Furthermore, the variations in the HRT could have a different effect on the different microorganisms inhabiting the system, based on their different kinetic properties, thus promoting the development of certain microorganisms over others. In fact, toluene removal efficiencies increased at periods III and IV, whereas the other BTEX compounds showed a decrease in their removal efficiencies.

In period V, when a recirculation flow was imposed to the system, *Rr* increased to a value of 80 (Table 4). Comparing this period V (with flow recirculation) with period II (without flow recirculation), BTEX removal efficiencies were statically similar in both periods (Table 3). Again, the increase in richness did not match with an improvement of reactor performance in terms of BTEX removal, likely due to the reasons explained above.

A significant impact of ethanol decrease on bacterial populations was observed (Fig. 2). The sample of period VI clustered together with samples of periods I and II (similarity of 83% between period I and periods II and VI, and similarity of 94% between periods II and VI). *Rr* and Shannon diversity decreased to values of 4 and 1.9, respectively, similar values to those

observed in periods I and II. This suggests a negative impact of ethanol decline on the microorganisms which were using this compound as carbon and energy source. However, the BTEX removal efficiency and the elimination capacity of the system improved in this period (85.9% and 27.8 mg · d^{-1}, respectively), indicating that ethanol exerted a negative effect on BTEX degradation. It is likely that some bacteria preferred ethanol as substrate for growth due to thermodynamic related advantages in periods with high ethanol concentration, but some of them could start to use BTEX under ethanol-limited conditions in period VI. In period VII (low ethanol concentration without recirculation), *Rr* and Shannon diversity increased (29 and 2.8, respectively), and BTEX removal efficiency decreased to 78.4%. The low ethanol and BTEX mass loadings applied in this period likely supported the selection of a highly diverse bacterial community since, as observed by some authors, a low mass loading supports the maintenance of a high bacterial diversity [36,37].

As well as for bacterial communities, the functional organization (*Fo*) of the archaeal communities presented a medium value (Table 4), which was maintained along the whole experiment, suggesting a strong capacity of the reactor to conserve functionality even under perturbed conditions [15]. In general, archaeal populations in the reactor displayed less temporal variability than bacterial communities (Fig. 2b). As compared to bacteria, ethanol addition (period I) did not cause a profound effect on archaeal populations (Fig. 2b) Nevertheless, clustering showed an effect of introducing BTEX into the bioreactor (similarity between periods I and II of 74%), increasing the *Rr* and Shannon diversity from values of 254 and 3.2, respectively (period I), to values of 524 to 3.6, respectively. An effect of decreasing the HRT from 36 (period III) to 24 h (period IV) was also observed, showing these samples a similarity value of 78% but maintaining similar *Rr* and Shannon diversity values (Table 4). Furthermore, an impact of decreasing ethanol concentration in the system (period VI) was observed (similarity of 87% between periods V and VI), whereas *Rr* and Shannon diversity were maintained (Table 4).

To what extent the diversity or the components of the diversity reflect the functioning and stability of an ecosystem is still a matter of discussion. It has been proposed that the ecosystem function is mostly influenced by components of the diversity, such as the evenness of the species distribution, the species composition, the positive species interaction (synergism, co-metabolism, syntrophy) and the dynamics of the communities [38]. For example, parameters such as the dynamic of the microbial community and a high initial evenness are considered of vital importance to guarantee functional stability [33]. In this work, the evenness parameter remained almost constant, and the operational parameters tested mainly affected bacterial richness, whose variations did not match with the functioning of the system in terms of BTEX removal. Hence, bacterial richness, as a component of the diversity, did not reflect the ecosystem function, whereas the reactor performance seemed to be mostly influenced by the high level of dynamics and the optimal evenness values of the community.

4. Conclusions

This work intended to understand a little more about the anaerobic BTEX removal in continuous-flow bioreactors for *ex situ* bioremediation purposes, evaluating the effect of some operational parameters on efficiency, stability and microbial community structure.

Good BTEX removal and reactor stability could still be reached at a HRT of 24 h. Concerning the impact of the effluent recirculation on BTEX removal, it was not evident at high co-substrate (ethanol) concentrations, but it was significant when low concentrations were applied. Finally, shortage of ethanol had a positive effect on BTEX removal, especially for benzene.

Regarding microbial community structure, the optimal degree of evenness likely contributed to the relatively high stability of the system in terms of BTEX removal. Although changes observed in bacterial and archaeal richness did not match with the functioning of the system, dynamics and evenness parameters seemed to be of importance in maintaining a stable reactor performance.

In conclusion, taking into account the efficiency and stability found, anaerobic treatment seems to be an interesting option for *ex situ* BTEX removal from contaminated waters, although some strategies to increase benzene removal must be investigated.

Acknowledgements

The authors would like to thank Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Process 481270/2010-8), Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES (Process PNPD 3022/2010) and Fundação Cearense de Apoio ao Desenvolvimento Científico e Tecnológico – Funcap (Process 078.01.00/09) for the research grants and financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2015.06.106.

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