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Applicability of Microaerobic Technology to Enhance BTEX Removal from Contaminated Waters

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Abstract As the addition of low concentrations of oxygen can favor the initial degradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) compounds, this work verified the applicability of the microaerobic technology to enhance BTEX removal in an anaerobic bioreactor supplemented with high and low co-substrate (ethanol) concentrations. Additionally, structural alterations on the bioreactor microbiota were assessed throughout the experiment. The bioreactor was fed with a synthetic BTEX-contaminated water (~ 3 mg L^{-1} of each compound) and operated at a hydraulic retention time of 48 h. The addition of low concentrations of oxygen (1.0 mL min^{-1} of atmospheric air at 27 °C and 1 atm) assured high removal efficiencies (> 80%) for all compounds under microaerobic conditions. In fact, the applicability of this technology showed to be viable to enhance BTEX removal from contaminated waters, especially concerning benzene (with a 30% removal increase), which is a very recalcitrant compound under anaerobic conditions. However, high concentrations of ethanol adversely affected BTEX removal, especially benzene, under anaerobic and microaerobic conditions. Finally, although bacterial community richness decreased at low concentrations of ethanol, in general, the bioreactor microbiota could deal with the different operational conditions and preserved its functionality during the whole experiment.

Keywords Monoaromatics · Contaminated water · Anaerobic treatment · Microaerobic treatment · Microaeration

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Introduction

Benzene, toluene, ethylbenzene, and xylenes (BTEX) are volatile monoaromatic hydrocarbons typically found in fossil fuels, such as diesel and gasoline. Consequently, these compounds are some of the most important soil and groundwater pollutants, being released into the environment from accidental fuel spills during transportation and leakages from underground storage tanks and pipelines [1].

In some countries, such as Brazil and USA, ethanol is used as a fuel additive in order to reduce both atmospheric pollution from fossil fuel combustion and petroleum import dependence [2, 3]. However, this alcohol increases BTEX solubility in water (cosolvency effect), which may aggravate the soil and groundwater contamination problem [4]. Moreover, ethanol hinders BTEX biodegradation since it is a preferential substrate over these hydrocarbons, which contributes to their persistence in the environment [2, 5].

Accordingly, as these aromatics are very toxic, being able to cause, for instance, neurological and respiratory damage, and even leukemia (specifically by benzene), many studies have focused on the development of effective (in situ and ex situ) bioremediation methods for BTEX-contaminated sites [6–9].

Although BTEX can be degraded under different redox conditions, anaerobic degradation is usually slower than aerobic one, particularly for benzene [6, 10, 11]. Nevertheless, aerobic systems have some drawbacks, such as significant sludge production and BTEX emission to atmosphere by volatilization and stripping during intense aeration, which also represents an important operational cost [7]. Therefore, anaerobic technology is attractive for being economical and environmentally friendly.

The limitation of anaerobic BTEX degradation can be overcome by adding low concentrations of oxygen into the system since it can favor the initial degradation of BTEX compounds. In fact, under microaerobic conditions, some microorganisms can use oxygen only to introduce hydroxyl groups into the aromatic ring (and not as the terminal electron acceptor) as in classical aerobic pathways, facilitating its subsequent cleavage by anaerobic metabolic pathways [12]. However, either batch or continuous-flow microaerobic BTEX degradation experiments found in literature have used only aerobic cultures adapted to low oxygen concentrations [13–15]. Thus, it is necessary to assess microaerobic processes in BTEX degradation, principally with anaerobic inocula operated under microaerobic conditions.

Hence, the main objective of this work was to verify the applicability of the microaerobic technology to enhance BTEX removal in an anaerobic bioreactor supplemented with high and low co-substrate (ethanol) concentrations. Additionally, structural alterations on the bioreactor microbiota were assessed throughout the experiment.

Material and Methods

Bioreactor

The experiment was performed in a lab-scale upflow anaerobic sludge blanket (UASB) bioreactor (working volume of 3.3 L), inoculated with a sludge (50 g VSS L^{-1}) from an anaerobic internal circulation (IC) bioreactor treating brewery wastewater (Horizonte, Ceará, Brazil) (Fig. 1). This experimental system was already adapted to BTEX compounds since it

was operated for more than 300 days with a synthetic BTEX-contaminated water in a previous study [16]. In the current investigation, it continued to be fed with the same contaminated water, whose composition and storage conditions are described elsewhere [16], and, in some experimental periods, microaerated by a peristaltic pump (Minipuls 3, Gilson, USA) at the effluent recirculation inlet (Fig. 1).

Experimental Procedure

The experiment was divided in four periods (Table 1). From period I to II, ethanol (co-substrate) concentration was decreased from 0.76 to 0.11 g L^{-1} in order to simulate a situation in which ethanol was already partially degraded in a gasoline-contaminated aquifer, i.e., at some distance from the contamination source (e.g., a leakage from an underground storage tank), and evaluate its influence on anaerobic BTEX removal. In fact, literature reports that ethanol concentration can be reduced from 1.0 to 0.1 g L^{-1} at a distance of approximately 30 m from the contamination source [17]. Then, still fed with an ethanol concentration as low as 0.12 g L^{-1} (period III), the bioreactor started to be operated under microaerobic conditions. A flow rate of 1.0 ml min^{-1} of atmospheric air at 27 °C and 1 atm (equivalent to 0.18 L O_2 L^{-1} feed) was introduced into the system at the effluent recirculation inlet. This flow rate was set based on Lopes [18] in order to ensure low dissolved oxygen (DO) concentrations in the liquid. In the aforementioned work, when a flow rate of 0.23 L O_2 L^{-1} feed (at 35 °C and 1 atm) was applied to microaerobic bioreactors to remove H_2S from a sulfate-rich wastewater, DO concentrations above 0.1 mg L^{-1} were not detected in the liquid. Additionally, doses of oxygen below 1.0 L O_2 L^{-1} feed are classified as microaeration and do not affect negatively anaerobic reactors [19]. Finally, in period IV, the microaeration impact on BTEX removal was assessed when the bioreactor was supplemented with a higher ethanol concentration (0.96 g L^{-1}). Each experimental period was finished after verifying a coefficient of variation less than 10% in the last three effluent BTEX concentration values.

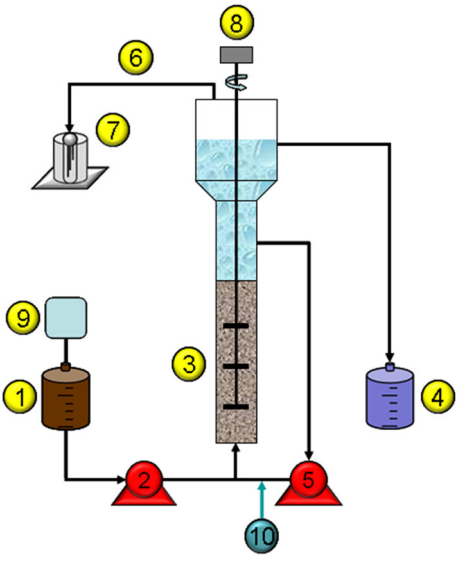

Fig. 1 Schematic of the experimental set-up used for BTEX removal under anaerobic and microaerobic conditions. 1, influent; 2, peristaltic pump (feeding); 3, UASB reactor; 4, effluent; 5, dosing pump (recirculation); 6, biogas; 7, gas meter; 8, stirrer (5 rpm); 9, Tedlar® bag (N_2); 10, microaeration (peristaltic pump)

Table 1 Bioreactor operational parameters

Period	I	II	III	IV
End of period (day)	22	59	81	112
HRT (h)	48	48	48	48
Total COD (g L^{-1})	1.6	0.3	0.3	2.1
OLR (kg m^{-3} day^{-1})	0.80	0.15	0.15	1.05
Ethanol (g L^{-1})	0.76	0.11	0.12	0.96
BTEX (mg L^{-1})	16.1	19.5	15.8	16.3
Benzene (mg L^{-1})	2.6	3.0	2.2	2.5
Toluene (mg L^{-1})	2.5	3.3	2.5	2.6
Ethylbenzene (mg L^{-1})	2.8	3.5	3.0	2.8
o-Xylene (mg L^{-1})	2.4	3.0	2.6	2.6
m,p-Xylenes[a] (mg L^{-1})	5.7	6.8	5.6	5.7
Recirculation (L h^{-1})	0.7	0.7	0.7	0.7
Microaeration[b] (mL min^{-1})	–	–	1.0	1.0
Dose of oxygen[b] (L O_2 L^{-1} feed)	–	–	0.18	0.18

HRT hydraulic retention time, *COD* chemical oxygen demand, *OLR* organic loading rate

[a] The isomers *meta*- and *para*-xylenes were quantified together due to the chromatographic method limitation

[b] At 1 atm and 27 °C

Analytical Methods

Chemical oxygen demand (COD) and pH were analyzed according to APHA [20]. BTEX were quantified 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) [21], whereas methane were determined by gas chromatography with thermal conductivity detection (GC-TCD) (GC-17A, Shimadzu Corporation, Japan) [22].

Bacterial and Archaeal Community Structure Analysis

The structure of the bacterial and archaeal communities was analyzed by double gradient-denaturing gradient gel electrophoresis (DG-DGGE) [23]. For this purpose, samples were collected at the end of each operational period (I–IV) and from the inoculum, and immediately preserved at − 20 °C. Genomic DNA was isolated from the samples with the Fast DNA Spin Kit for Soil (MP Biomedicals, LLC) according to the manufacturer instructions, while optimizing the time of lysis of the cells and the time necessary for the DNA binding to the silica matrix. After DNA isolation, the 16S rRNA gene of bacterial and archaeal communities was amplified by polymerase chain reaction (PCR) using the primers 968-F-GC and 1041-R for Bacteria, and the primers A109(T)-F and 515-R-GC for Archaea. PCR reaction conditions and thermocycling parameters were previously described in Firmino et al. [16] and Firmino et al. [22], respectively.

PCR amplicons were run in double-gradient-denaturing polyacrylamide gels with a porous gradient of acrylamide/bisacrylamide between 6 and 10% and with urea/formamide gradients ranging from 42 to 67% and from 30 to 60% for bacterial and archaeal communities, respectively. Electrophoresis was performed in a D-Code Universal Mutation Detection System (Bio Rad Laboratories, USA) using 0.5× TAE buffer at 60 °C. Bacterial samples were runned at 85 V for 16 h, while archaeal samples were runned at 65 V for 18 h. After gel staining for 1 h with SYBR Green I (Sigma-Aldrich, USA), gel images were

captured and then processed using the Bionumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

Ranged-weighted richness (*Rr*), Shannon diversity (*H*), evenness/functional organization (*Fo*) and temporal dynamics (UPGMA clustering) of the bacterial and archaeal communities were calculated form the DGGE profiles. *Rr* and evenness were calculated according to Marzorati et al. [24]. *Rr* reflects the number of different species/members within a microbial community, whereas *Fo*/evenness reflects the relative distribution of these members within the community. Shannon diversity was calculated according to Lebrero et al. [25] and considers both richness and evenness. Typical Shannon diversity values range between 1.5 (low evenness and richness) to 3.5 (high evenness and richness) [26]. For the construction of unweighted pair group method with arithmetic mean (UPGMA) dendrograms, indicating the similarity between samples and therefore the dynamics of the communities, the Pearson product-moment correlation coefficient was calculated from the DGGE profiles as previously described in Lebrero et al. [25].

Statistical Methods

The experimental data sets of the different periods were compared to each other by the Mann-Whitney rank sum test at a 95.0% confidence level [22].

Results and Discussion

BTEX Removal under Anaerobic Conditions

In period I, both high COD removal efficiency (~ 90%) and methane production (~ 0.45 L g COD_{rem}^{-1}) were obtained (Table 2). Concerning BTEX removal, all compounds presented average removal efficiencies above 80% (Table 3), except benzene, for which the lowest efficiencies (~ 50%) were achieved (Fig. 2). In fact, under anaerobic conditions, literature reports that benzene is the most recalcitrant BTEX compound [9, 10, 22] since its symmetric π-electron system and absence of reactive substituents gives it a very high thermodynamic stability [11].

Table 2 pH values, COD concentrations and removal efficiencies, and methane production

Period		I	II	III	IV
Ethanol (g L^{-1})		0.76	0.11	0.12	0.96
Microaeration (mL min^{-1})		–	–	1.0	1.0
pH	Effluent	7.6 (0.2)	7.9 (0.2)	7.8 (0.3)	7.7 (0.2)
COD	Influent (mg L^{-1})	1644 (255)	298 (52)	292 (26)	2054 (119)
	Effluent (mg L^{-1})	191 (105)	141 (45)	156 (44)	315 (32)
	Efficiency (%)	88.6 (5.8)	52.4 (13.5)	46.4 (15.0)	84.6 (1.8)
CH_4	(L g COD_{rem}^{-1})	0.461 (0.066)	0.385[a]	0.385[a]	0.282 (0.035)

COD chemical oxygen demand, COD_{rem} chemical oxygen demand removed

The standard deviation is shown in parentheses

[a] Theoretical value at 1 atm and 27 °C. The biogas production was not possible to be measured due to the gas meter limitation

Table 3 Monoaromatic hydrocarbons concentrations and removal efficiencies

Period		I	II	III	IV
Ethanol (g L^{-1})		0.76	0.11	0.12	0.96
Microaeration (mL min^{-1})		–	–	1.0	1.0
BTEX	Influent (μg L^{-1})	16,158 (1280)	19,537 (3109)	15,845 (757)	16,274 (1815)
	Effluent (μg L^{-1})	3227 (212)	2677 (576)	971 (320)	1928 (313)
	Efficiency (%)	80.0 (1.3)	85.9 (4.5)	93.8 (2.2)	88.2 (1.3)
B	Influent (μg L^{-1})	2658 (226)	2995 (488)	2188 (333)	2519 (451)
	Effluent (μg L^{-1})	1294 (102)	1122 (290)	159 (101)	472 (104)
	Efficiency (%)	51.2 (2.3)	61.8 (10.9)	92.5 (4.9)	81.2 (3.0)
T	Influent (μg L^{-1})	2545 (177)	3332 (559)	2536 (222)	2550 (345)
	Effluent (μg L^{-1})	240 (25)	103 (119)	53 (98)	263 (33)
	Efficiency (%)	90.5 (1.2)	96.5 (4.3)	97.9 (4.0)	89.6 (1.5)
E	Influent (μg L^{-1})	2817 (221)	3462 (532)	2953 (81)	2844 (318)
	Effluent (μg L^{-1})	395 (30)	290 (69)	48 (90)	233 (42)
	Efficiency (%)	85.9 (1.6)	91.3 (3.1)	98.3 (3.1)	91.8 (1.5)
o-X	Influent (μg L^{-1})	2438 (193)	2989 (566)	2578 (67)	2626 (350)
	Effluent (μg L^{-1})	484 (33)	437 (58)	244 (76)	341 (61)
	Efficiency (%)	80.1 (1.2)	84.8 (4.1)	90.5 (3.2)	87.0 (1.6)
m,p-X	Influent (μg L^{-1})	5700 (476)	6760 (1077)	5590 (298)	5734 (661)
	Effluent (μg L^{-1})	814 (39)	726 (83)	467 (75)	620 (96)
	Efficiency (%)	85.7 (0.9)	88.9 (2.8)	91.6 (1.5)	89.2 (1.4)

B benzene, *T* toluene, *E* ethylbenzene, *o-X orto*-xylene, *m,p*-X *meta*- and *para*-xylenes

The standard deviation is shown in parentheses

Under different redox conditions, ethanol is reported to be a preferential substrate over BTEX compounds, which hinders these monoaromatics degradation [4, 5]. Then, in period II, this co-substrate concentration was reduced to approximately 0.11 g L^{-1} to evaluate its influence on anaerobic BTEX removal. Although COD removal efficiency was lower than in period I, due to the lower influent co-substrate concentration, the effluent quality of period II was similar to that of the first period (Table 2). Moreover, since the influent COD was very low, the gas meter was not able to register the methane production. However, it was estimated a theoretical biogas production of only 95 mL day^{-1} at 1 atm and 27 °C.

Regarding BTEX removal, although there was an initial increase in both efficiency and effluent quality of all hydrocarbons, their effluent concentrations increased over period II, particularly for benzene, whose efficiencies decreased to values close to those of period I (Fig. 2). However, for all monoaromatics, the reactor was still statistically more efficient in the second period than in the first one (Table 3). Thus, the scarcity of a preferential substrate (ethanol) seemed to facilitate BTEX degradation, being corroborated by previous studies in which the anaerobic degradation of some monoaromatic hydrocarbons was inhibited until co-substrates (e.g., glucose, alcohols, and fatty acids) were almost completely consumed [27–29].

BTEX Removal under Microaerobic Conditions

In period III, under microaerobic conditions (0.18 L O_2 L^{-1} feed at 1 atm and 27 °C), COD removal efficiencies and effluent concentrations showed no statistical differences when compared to period II (Table 2). Therefore, microaeration seemed not to have affected significantly the organic matter removal. Additionally, as observed in period II, the gas meter was not able

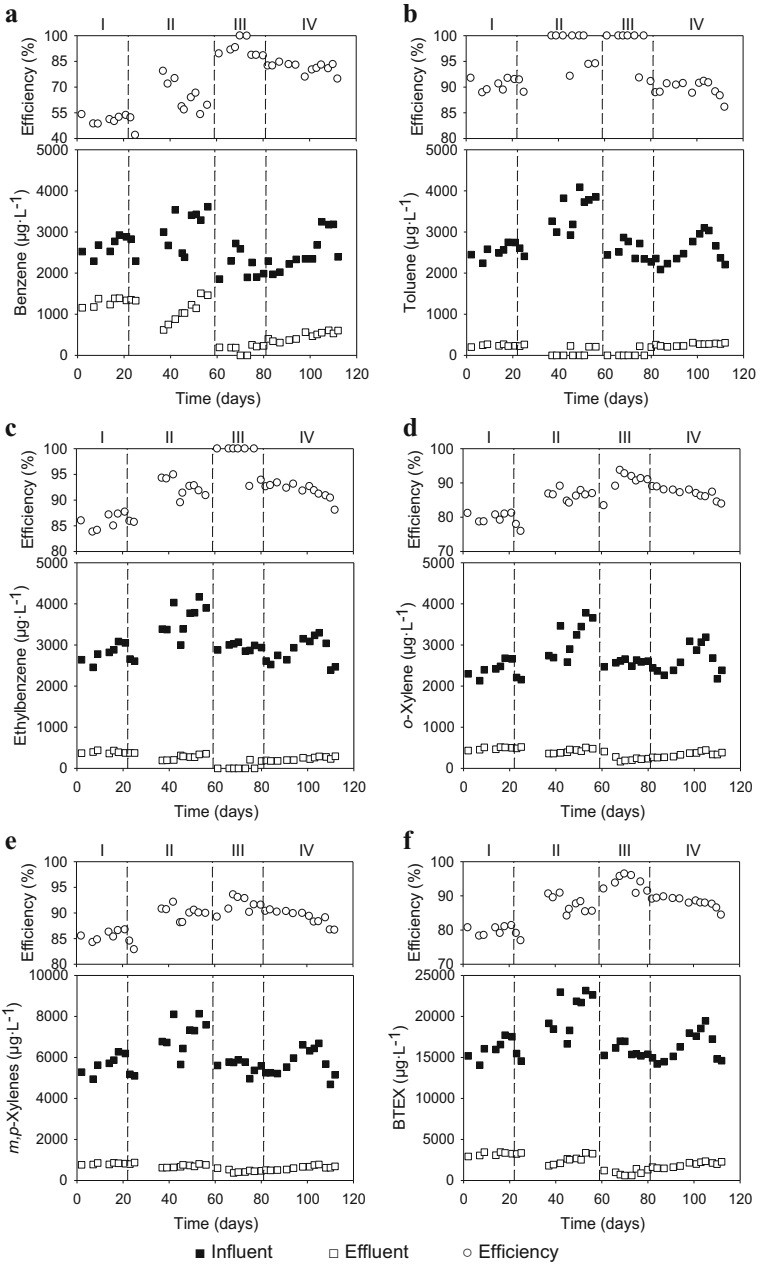

Fig. 2 Monoaromatic hydrocarbons influent (filled squares) and effluent (empty squares) concentrations, and their respective removal efficiencies (empty circles). Benzene (**a**), toluene (**b**), ethylbenzene (**c**), *o*-xylene (**d**), *m,p*-xylenes (**e**), and total BTEX (**f**)

to register the methane production since the influent COD was as low as 300 mg L^{-1} (organic loading rate (OLR) of 0.15 kg m^{-3} day^{-1}). However, it was estimated a theoretical biogas production of approximately 195 mL day^{-1} at 1 atm and 27 °C. Concerning BTEX removal,

except for toluene, the removal efficiencies of all compounds increased significantly (Fig. 2). Particularly for benzene, an almost 31% increase in their average removal efficiency was observed, which led to an average effluent concentration approximately sevenfold lower than that of period II (159 mg L^{-1}) (Table 3).

Under aerobic conditions, besides being the terminal electron acceptor, oxygen is used in the initial activation of aromatic compounds, in which mono- or dioxygenases enzymes insert it into the aromatic ring [9]. Hence, under these conditions, the biochemical activation of aromatic hydrocarbons consists in introducing one or more hydroxyl groups into the aromatic ring (monohydroxylation by mono-oxygenases or dihydroxylation by dioxygenases) in order to promote its cleavage [9, 12]. On the other hand, under microaerobic conditions, some microorganisms use oxygen only to introduce hydroxyl groups into the aromatic ring, as in the classic aerobic pathways, since its cleavage occurs through anaerobic metabolic pathways [12].

Yerushalmi et al. [15], in benzene degradation batch experiments with aerobic cultures adapted to low oxygen concentrations, observed that, under microaerobic conditions, the further oxidation of phenol—a less recalcitrant compound produced from the initial benzene hydroxylation by mono-oxygenases—to catechol, by aerobic pathways, only happens when adequate amounts of oxygen are available. Alternatively, these authors propose that, after the complete oxygen depletion, phenol might be degraded anaerobically to benzoate by the action of appropriate anaerobic microorganisms (Fig. 3). In fact, phenol is considered one of the possible key intermediates of the initial anaerobic benzene degradation [30].

Therefore, in the present study, it is likely that the addition of low oxygen concentrations has facilitated the initial activation of BTEX compounds, which is usually considered the rate-limiting step of the anaerobic degradation process, especially for benzene [9, 30]. Hence, probably, some microorganisms, using oxygenases, might have converted the aromatic hydrocarbons into less recalcitrant phenolic intermediates under anaerobic conditions, which affected positively the bioreactor removal performance.

Under methanogenic conditions, aromatic hydrocarbons are hardly degraded by a single microbial species. In fact, under such conditions, several studies report that the degradation of these compounds occurs through syntrophic interactions between fermentative bacteria and methanogenic archaea, in which the former microorganisms convert

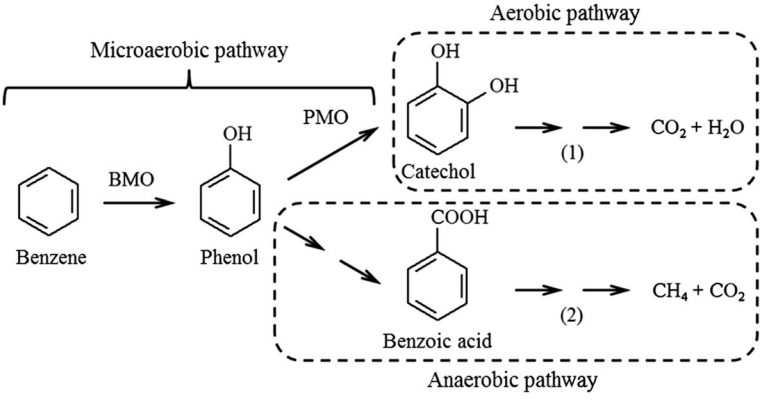

Fig. 3 Metabolic pathway of benzene degradation under microaerobic conditions proposed by Yerushalmi et al. [15]. BMO, benzene mono-oxygenase; PMO, phenol mono-oxygenase. The complete benzene mineralization depends on either the availability of adequate oxygen amount (1) or the presence of appropriate microorganisms (2)

the aromatic hydrocarbons into intermediates (e.g., acetate and hydrogen) which are consumed by the latter ones during methanogenesis [11, 31]. Actually, the fact that the microbiota is organized in granules (granular sludge) favors this syntrophy since the distance between the microbial groups is sufficiently small to assure interspecies intermediates transfer [32]. Additionally, most likely, the structural configuration of granular sludge, in which strictly anaerobic archaea in the granule core are protected from oxygen by probable facultative or microaerophillic fermentative bacteria in outer layers, seems to be a key feature to guarantee the applicability of microaeration to anaerobic bioreactor and enhance BTEX degradation.

In period IV, still under microaerobic conditions, when high ethanol concentrations were applied, COD removal efficiency increased significantly (Table 2). Concerning the average methane yield, there was a decrease from 0.461 (period I) to only 0.282 L g COD_{rem}^{-1} (Table 2). In addition, there was an increase in CO_2 concentration in the biogas (data not shown). Therefore, most likely, a fraction of the substrate might have been degraded aerobically. As for BTEX removal, lower average efficiencies were obtained when compared to period III, especially for benzene and toluene (Table 3). Moreover, a tendency for the effluent BTEX concentrations to increase over time was observed, reducing the removal efficiency (Fig. 2). Therefore, these results reinforce the hypothesis that the presence of high concentrations of an easily degradable substrate, such as ethanol, affects negatively the aromatic hydrocarbons removal, even under microaerobic conditions.

Regarding the microaeration impact at high co-substrate concentrations (periods I and IV), once more, no significant difference was found in toluene removal efficiency since, as mentioned above, it is considered a relatively less recalcitrant hydrocarbon under different redox conditions. On the other hand, the efficiencies of the other compounds increased significantly under microaerobic conditions (period IV) (Fig. 2), remarkably for benzene, with a 30% increase in the average efficiency (Table 3). Hence, although it was not possible to achieve the same BTEX removal performance of period III, in general, the addition of small amounts of oxygen (from atmospheric air) facilitated the aromatic compounds removal.

It is important to mention that the oxygen transfer, by injection of small bubbles of atmospheric air, to the liquid is not efficient, because, probably, the residence time of the bubbles in the system would not exceed 2 s. Therefore, the oxygen dissolution in the liquid, in microaerobic bioreactors, might mainly happen through the air present in the bioreactor headspace (gas-liquid interface); thus, the biogas residence time in this section is very important [18]. For the bioreactor used in the current study, whose headspace volume was 0.4 L, the biogas residence time for high ethanol loadings was 4.8 h, whereas, for low loadings, it could reach up to 2 days. Nevertheless, DO concentrations in liquid and oxidation reduction potential (ORP) kept below 0.1 mg L^{-1} (limit of detection) and − 200 mV, respectively, during both periods III and IV, being in accordance to literature [19].

Concerning BTEX removal by stripping, it is believed that this physical process was negligible since benzene, the least volatile compound [7], showed the highest increase in its removal efficiency values with the introduction of low amounts of oxygen (Table 3). Furthermore, it is important to mention that BTEX in biogas were sporadically analyzed by gas chromatography, and fractions of these aromatic hydrocarbons were found in the gaseous phase (data not shown). However, the method was not able to quantify these compounds in gaseous samples. Nevertheless, a remarkable reduction in the

chromatographic signal (peak areas) of all compounds, particularly for benzene, was observed under microaerobic conditions when compared to anaerobic ones, which contrasts the hypothesis of stripping by microaeration and indicates that, most likely, aromatic hydrocarbons were removed by a biological process rather than a purely physical process.

Finally, even for the high efficiencies achieved under microaerobic conditions, it is important to mention that a post-treatment is required to remove residual concentrations of COD and some BTEX compounds, such as benzene, in order to meet the quality standards established by environmental legislations. Nevertheless, the use of microaerobic technology for BTEX removal seems to be an advance, which could also be applied to other recalcitrant compounds, such as polycyclic aromatic hydrocarbons (PAH), phenols, dyes, and micropollutants (e.g., hormones, pharmaceuticals, and personal care products).

Bacterial and Archaeal Community Structure in the Bioreactor

The effect of ethanol concentration and of the application of microaerobic conditions on bacterial and archaeal communities can be observed in their corresponding DGGE profiles (Fig. 4). The ecological parameters *Rr* (richness), *Fo* (functional organization) [24], and *H* (Shannon diversity) of the communities were calculated from the DGGE profiles (Table 4) in order to evaluate microbial community changes. Similarly, the dynamics of the communities were evaluated from the DGGE profiles by calculating Pearson similarity coefficients, which were used to construct UPGMA dendrograms reflecting microbial similarities within samples.

Ethanol decrease in period II caused the major impact in the bacterial communities (Fig. 4). *Rr* and *H* in this sample decreased from 80 (period I) to 4 (period II) and from 3.0 (period I) to 1.9 (period II), respectively, likely due to the decrease in substrate availability. However, less

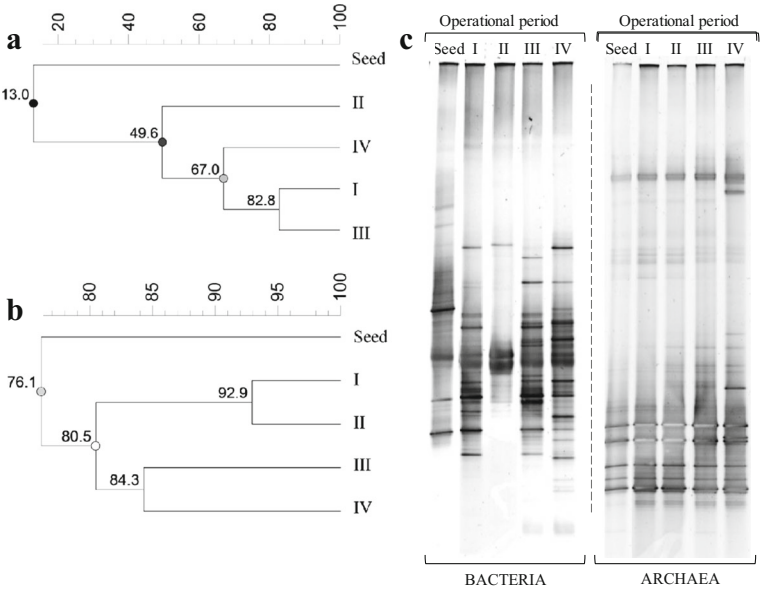

Fig. 4 Bacterial (**a**) and archaeal (**b**) communities dendograms, and microbial DGGE profiles (**c**). The labels specify the experimental periods at which the sludge samples were collected

Table 4 Microbial structure parameters

Parameters	Seed	Period I	Period II	Period III	Period IV
Bacteria					
Fo (%)	38	36	40	33	37
Rr	75	80	4	81	120
H	3.1	3.0	1.9	3.3	3.3
Archaea					
Fo (%)	43	36	37	34	31
Rr	117	224	291	273	326
H	2.8	3.2	3.3	3.3	3.4

Fo (functional organization): low *Fo*/high evenness (25%), medium *Fo* and evenness (30–70%), high *Fo*/low evenness (70%) [24]

Rr (ranged weighted richness): low (< 10), medium (10–30), high (> 30) [24]

H (Shannon diversity index): 1.5 to 3.5 (low and high species evenness and richness, respectively) [26]

variation in *Fo* were observed (Table 4). In fact, this parameter remained constant throughout all experimental periods, showing intermediate values ranging from 33 to 40%, which indicate a medium functional organization of the communities and medium evenness. This kind of communities have been described as balanced populations with the ability to deal with changing environmental conditions due to most of the community consists of species present in low amounts which can substitute the dominant species (in terms of functionality) in face of environmental variations) [24]. UPGMA clustering also reflected the impact of ethanol decrease on bacterial communities. Sample in period II clustered separately from the rest of the samples (periods I, III, and IV) showing a low similarity of 49.6% (Fig. 4). These results suggest that co-substrate decrease mainly affected bacterial richness rather than community evenness, which remained at values which could assure the functional stability of the reactor. In fact, as mentioned above, from periods I to II, effluent quality remained similar in terms of COD (mg L^{-1}) (Table 2), and, for all monoaromatics, the reactor was still statistically more efficient in period II than in period I (Table 3), indicating that some bacteria previously consuming ethanol due to thermodynamics advantages could have started to use monoaromatics for growth. In this work, evenness, and consequently, the presence of functionally redundant species on the community (species which can perform the same function), instead of richness or diversity, seemed to be a key ecological parameter to maintain functionally as previously observed by other authors [33–35].

In period III, when microaerobic conditions at low ethanol concentrations were imposed, *Rr* and *H* recovered values similar than those observed in period I (from 4 to 81 and from 1.9 to 3.3, respectively), and *Fo*/evenness remained at medium values (from 40 to 33) (Table 4). Accordingly, sample belonging to this period clustered together with sample corresponding to period I with a high Pearson similarity (82.8%) (Fig. 4), thus returning bacterial community to be similar to that prevailing during period I. The increase in diversity/richness due to oxygen introduction can be explained by the introduction of an electron acceptor in the system, which lead to the emergence of new bacterial species. During this period, an increase in BTEX removal efficiencies (except for toluene) was observed, which, in this case, matched with an increase in bacterial richness and the maintenance of *Fo*.

Rr, *H*, and evenness maintained similar values to those observed in period III when ethanol was restored to high concentrations (0.96 g L^{-1}) (Table 4). A slight increase in *Rr* (from 81 in period III to 120 in period IV) was observed likely due to the higher

availability of ethanol, which lead to the diversification of bacterial species. The availability of ethanol at high concentrations under microaerobic conditions lead to moderate changes in bacterial communities, showing sample corresponding to period IV a Pearson similarity value of 67% with samples belonging to periods I and III (Fig. 4). Despite richness and evenness almost not changing from period III to period IV, COD removal efficiencies increased significantly (Table 2), whereas monoaromatics removal efficiencies decreased (especially for toluene and benzene) (Table 3).

As compared to bacterial populations, archaeal communities were less affected by the variations in ethanol concentration and oxygen availability (Fig. 4). In general, high Pearson similarity values were observed between all samples, indicating slight changes in archaeal communities due to operational conditions. However, two different clusters corresponding to anaerobic and microaerobic conditions were observed (Pearson similarity = 80.5%), indicating changes in archaeal populations for their adaptation to the oxygen presence. Likely, the granular sludge properties (less oxygen-sensitive bacteria dominate in the outer zones of the granule which protecting archaea from oxygen) [36], and the low retention time of oxygen in the blanket sludge contributed to maintain archaeal populations. Despite experimental measurements of biogas production at low ethanol concentrations were not possible due to technical limitations, calculated theoretical biogas generation and the lack of damage of archaeal populations due to the operational conditions suggest that the methanogenesis process continued to occur even at a low ethanol concentrations. In fact, *Rr* and *H* values of archaeal communities were high, and evenness presented medium values (Table 4) during all periods, reflecting these results healthy and flexible archaeal populations able to adapt to changes [24, 37].

In this work, ethanol concentration variations and microaerobic conditions mainly affected bacterial richness, whose values did not always link to COD and BTEX removal performance. On the contrary, bacterial evenness remained constant at medium values, suggesting the importance of a good functional organization of the communities for the performance of the bioreactor.

Conclusions

The addition of low concentrations of oxygen (from atmospheric air) assured high removal efficiencies (> 80%) for all compounds under microaerobic conditions. High concentrations of ethanol adversely affected BTEX removal, especially benzene, under anaerobic and microaerobic conditions.

Although bacterial community richness decreased at low concentrations of ethanol, in general, the bioreactor microbiota could deal with the different operational conditions and preserved its functionality during the whole experiment.

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Compliance with Ethical Standards

Disclosures The authors indicate no potential conflicts of interest.

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