Effect of the co-substrate ethanol and intermediates (phenol, benzoate, and toluene) on anaerobic benzene removal

Efeito do cossubustrato etanol e intermediários (fenol, benzoato e tolueno) na remoção anaeróbia de benzeno

João Paulo da Silva Siqueira[1] , Robério Mires de Freitas[2] ,
Paulo Igor Milen Firmino[3] , André Bezerra dos Santos[3]*

ABSTRACT

Benzene represents an environmental and health risk because it has high solubility and mobility in water and also has carcinogenic properties. It is considered one of the most recalcitrant compounds among the aromatic hydrocarbons in anaerobic environments. Despite the investigations already conducted, the effect of important intermediates using mixed cultures as anaerobic sludge is still quite controversial. From this perspective, the objective of this research was to evaluate the influence of ethanol as a co-substrate and the addition of anaerobic benzene degradation by-products (i.e., phenol, benzoate, and toluene). The experiments were carried out in borosilicate glass bottles (115 mL) with a reaction volume of 70 mL (basal medium + microorganisms) inoculated with acclimatized anaerobic sludge (2 g VSS·L^{-1}). Benzene removal was accelerated when ethanol was used as a co-substrate, with the consumption rate increasing from 0.0445 mg·L^{-1}·day^{-1} (without ethanol) to 0.0563 mg·L^{-1}·day^{-1} (with ethanol), suggesting the participation of cometabolic processes. Benzene was removed with the subsequent formation of phenol and benzoate, consumed later, with no accumulation of by-products in the medium. Finally, intermediate addition negatively influenced benzene removal, with benzoate showing the greatest interference (-55% reduction), as it is a key intermediate in the anaerobic benzene removal.

Keywords: anaerobic digestion; benzene; benzoate; phenol; intermediates.

RESUMO

O benzeno representa um risco ambiental e para a saúde, porque possui alta solubilidade e mobilidade na água e também propriedades cancerígenas. É considerado um dos compostos mais recalcitrantes entre os hidrocarbonetos aromáticos em ambientes anaeróbios. Apesar das investigações já realizadas, o efeito de importantes intermediários utilizando culturas mistas como lodo anaeróbio ainda é bastante controverso. Nessa perspectiva, o objetivo desta pesquisa foi avaliar a influência do etanol como cossubstrato e da adição de subprodutos da degradação anaeróbia do benzeno (isto é, fenol, benzoato e tolueno). Os experimentos foram realizados em frascos de vidro borossilicato (115 mL) com volume de reação de 70 mL (meio basal + microrganismos) inoculados com lodo anaeróbio aclimatado (2 g VSS·L-1). A remoção do benzeno foi acelerada quando o etanol foi utilizado como cossubstrato, com a taxa de consumo aumentando de 0,0445 (sem etanol) para 0,0563 mg·L·dia-1 (com etanol), sugerindo a participação de processos cometabólicos. O benzeno foi removido com posterior formação de fenol e benzoato, consumidos posteriormente, sem acúmulo de subprodutos no meio. Finalmente, a adição de intermediário influenciou negativamente a remoção de benzeno, com o benzoato apresentando a maior interferência (-55% de redução), por ser um intermediário-chave na remoção anaeróbia de benzeno.

Palavras-chave: digestão anaeróbia; benzeno, benzoato, fenol, intermediários.

INTRODUCTION

Benzene is an aromatic hydrocarbon related to the extraction of crude oil and products derived from oil processing and is one of the compounds with major impacts on the environment (KUMAR *et al.*, 2022; LIU; HUANG; LI, 2020; YU *et al.*, 2022). Benzene represents an environmental and health risk as it has high solubility and mobility in water (SONWANI *et al.*, 2021) and also has carcinogenic properties (DEHGHANI *et al.*, 2022; MEDEIROS *et al.*, 2022).

[1]Instituto Federal de Educação, Ciência e Tecnologia do Piauí – Corrente (PI), Brazil.
[2]Universidade Federal do Ceará, Instituto de Ciências do Mar – Fortaleza (CE), Brazil.
[3]Universidade Federal do Ceará, Departamento de Engenharia Hidráulica e Ambiental – Fortaleza (CE), Brazil.
*Corresponding author: andre23@ufc.br

Although benzene is considered one of the most recalcitrant compounds among the aromatic hydrocarbons in anaerobic environments, some studies have shown promising results, indicating that anaerobic microorganisms under methanogenic conditions (MASUMOTO *et al.*, 2012; SIQUEIRA *et al.*, 2018; WU *et al.*, 2022) and linked to the reduction of different electron acceptors, such as sulfate, nitrate, Fe(III), and Mn(IV) (FIRMINO *et al.*, 2015; MÜLLER *et al.*, 2021; SU *et al.*, 2022; ULRICH; BELLER; EDWARDS, 2005; VAN LEEUWEN *et al.*, 2022) could remove this aromatic compound.

The greatest adversity of the anaerobic benzene biodegradation is related to the high chemical stability of the aromatic ring (LADINO-ORJUELA *et al.*, 2016; ZHANG *et al.*, 2021). Hence, its removal was considered unrealizable sometimes (CHAKRABORTY; COATES, 2004). Benzene stability can be decreased in the presence of molecular oxygen, in which monoxygenases have a very active role.

Studies indicate that anaerobic microorganisms present in cultures enriched with specific microorganisms (ULRICH; BELLER; EDWARDS, 2005; TOTH *et al.*, 2021), acclimated microbial consortia, and operational conditions adjustments (e.g., microaeration, electron acceptor change, and redox mediators) make the removal of benzene and other hydrocarbons possible, leading to less toxic by-products or full mineralization (FIRMINO *et al.*, 2018; SIQUEIRA *et al.*, 2018; SU *et al.*, 2022).

The addition of ethanol to fermentation processes is another interesting strategy that can also stimulate the biodegradation of hydrocarbons (SIQUEIRA *et al.*, 2018). Indeed, the addition of ethanol as a co-substrate during anaerobic manipulation of organic substrates strengthens the metabolism of mixed microbial cultures, with this syntrophic/cooperative pathway persisting stably even after the end of ethanol supplementation (AULENTA *et al.*, 2021).

The anaerobic benzene degradation pathway includes benzene activation and channeling to intermediates and is often initiated by one of the four following reactions:

- addition of fumarate through the activity of benzylsuccinate synthesis;
- methylation by the transfer of a methyl group before fumarate addition;
- carboxylation, which is the conversion of the chemical compound to benzoate, observed in benzene metabolism;
- hydroxylation, which is the hydroxyl group addition to the aromatic ring (KUNAPULI *et al.*, 2008; LADINO-ORJUELA *et al.*, 2016; WANG *et al.*, 2022).

Despite the investigations, the effect of intermediates using mixed cultures as anaerobic sludge is still quite controversial. From this perspective, the objective of this study was to evaluate the influence of using ethanol as a co-substrate on the anaerobic degradation of benzene and, subsequently, the impact of adding some likely intermediate compounds (e.g., phenol, benzoate, and toluene) on this process.

MATERIALS AND METHODS

Experimental procedure

This study was divided into two experiments as described in Table 1. The effect of ethanol as a co-substrate was initially investigated on the anaerobic benzene removal, also monitoring the formation of known intermediates (e.g., phenol, benzoate, and toluene). In the second experiment, phenol, benzoate, and toluene were added individually with the co-substrate ethanol to observe the effect of the initial intermediate presence on the removal of benzene, intermediate, and formation of other by-products.

All experiments were performed in batches (in duplicate), with a reaction volume of 70 mL of basal medium and inoculum, in 115-mL borosilicate glass bottles, and an initial inoculum concentration of 2 g VSS·L^{-1}. Then, to establish anaerobic conditions inside the bottles, they were sealed with Viton® septa and aluminum seals, and their headspace was purged with nitrogen gas for 1 min. Subsequently, the assays were maintained under mechanical agitation (150 rpm) for 20 min at 30°C to guarantee the total consumption of dissolved oxygen remaining in the basal medium. Finally, with a glass syringe, benzene (~4.2 mg·L^{-1}) and, when necessary, the co-substrate ethanol (0.5 g·L^{-1}) and the intermediates phenol, benzoate, and toluene (tested individually at concentrations close to that of benzene) were added to the reaction medium (Table 1). The compound concentrations were set according to previous continuous-flow experiments (FIRMINO *et al.*, 2015; 2018; SIQUEIRA *et al.*, 2018; SIQUEIRA; FIRMINO; DOS SANTOS, 2023). The batch assays were carried out and monitored for 42 days when a steady benzene removal efficiency was observed. After this period, the compounds were reinjected, maintaining the initial characteristics of each experiment, and evaluated for the same period.

Chemical control consisted of benzene and ethanol added in the same basal medium and bottles described above and monitored over time to see if any abiotic removal of benzene and formation of intermediates occurred.

The degradation kinetics of benzene and intermediates was evaluated according to the second-order model (Equation 1), as it resulted in the best fit with the experimental data:

$$1/C = 1/C_0 + kt, \tag{1}$$

Where:

C: the reagent concentration (mg·L^{-1}) for any time t;

Table 1 – Experiment planning and compound concentration.

Experiment	Identification	Description	Benzene (mg·L^{-1})	Ethanol (mg·L^{-1})	Intermediate* (mg·L^{-1})	
1	B	Benzene	3.99 (0.10)	–	–	
1	BE	Benzene + ethanol	3.99 (0.06)	0.5	–	
2	BEPh	Benzene + ethanol + phenol	4.36 (0.14)	0.5	Phenol	4.72 (0.22)
2	BEBz	Benzene + ethanol + benzoate	4.84 (0.47)	0.5	Benzoate	5.63 (0.90)
2	BETo	Benzene + ethanol + toluene	4.28 (0.22)	0.5	Toluene	4.69 (0.21)

*Mean values considering duplicates and reinjections.

C_0: the reagent concentration (mg·L^{-1}) at time $t = 0$;

k: the kinetic constant (mg·L^{-1}·day^{-1});

t: the experiment time (days).

Inoculum and basal medium

An acclimatized microbial consortium was used, which was collected from a microaerobic reactor on a laboratory scale, and treated water contaminated with benzene (~4.2 mg·L^{-1}) and ethanol (0.5 g·L^{-1}) for 130 days. The seed sludge was collected from a UASB reactor treating domestic sewage. The basal medium (macro- and micronutrients) was prepared according to Firmino *et al.* (2010) and consisted of (mg/L) NH$_4$Cl (280), K$_2$HPO$_4$ (250), MgSO$_4$·7H$_2$O (100), CaCl$_2$·2H$_2$O (10), and 1 mL·L^{-1} of trace elements containing (mg·L^{-1}) H$_3$BO$_3$ (50), FeCl$_2$·4H$_2$O (2000), ZnCl$_2$ (50), MnCl$_2$·4H$_2$O (500), CuCl$_2$·2H$_2$O (38), (NH$_4$)$_6$Mo7O$_{24}$·4H$_2$O (50), AlCl$_3$·6H$_2$O (90), CoCl$_2$·6H$_2$O (2000), NiCl$_2$·6H$_2$O (92), Na$_2$SeO$_3$·5H$_2$O (162), EDTA (1000), and HCl 36% (1). To keep the pH around 7.0, the medium was buffered with sodium bicarbonate (NaHCO$_3$) in the proportion of 1 g NaHCO$_3$ to each 1 g COD ethanol.

Chemical and chromatographic analyses

Benzene and toluene were determined by static headspace extraction (Triplus HS, Thermo Scientific, USA) followed by gas chromatography with flame ionization detection (HS-GC-FID, headspace-gas chromatography flame ionization detection) (Trace GC Ultra, Thermo Scientific, USA) according to Carneiro *et al.* (2014). All samples (10 mL) were previously diluted with ultrapure water (Milli-Q system, EMD Millipore, USA) directly into headspace borosilicate glass vials (20 mL) (Supelco, USA), which were then sealed with PTFE/silicone septa and aluminum seals (Supelco, USA).

Phenol and benzoate determination was performed by liquid chromatography (Shimadzu Corporation, Japan) according to Siqueira, Firmino and dos Santos (2023). Before analysis, samples were filtered through Millipore HA membranes (0.45-µm pore).

Statistical analyses

The normality and homogeneity of the benzene kinetic data and the added intermediate compounds were evaluated using the Shapiro-Wilk and Fligner-Killeen tests, respectively, considering a significance value of 0.05 for both tests. Confirming the normality of the data, which showed a normal distribution (results greater than 0.05 in the normality and homogeneity tests are considered parametric data), statistically significant differences at $p < 0.05$ with analysis of variance (ANOVA) were verified. Data showing differences were identified using the Tukey's test. All tests were performed using the free software R in version 4.3.0.

RESULTS AND DISCUSSION

Effect of the co-substrate ethanol on anaerobic benzene removal

The effect of the co-substrate ethanol was assessed on the anaerobic benzene removal. It is noteworthy that a methanogenic sludge acclimated to benzene and ethanol was used as inoculum, simulating a biological *ex situ* treatment for contaminated groundwater. A comparison between benzene degradation kinetics in the presence of ethanol and intermediates is shown in Table 2.

In the chemical controls, the removal of benzene was negligible and there was no intermediate formation, indicating a biotic process. In this regard, the benzene consumption rate was approximately 0.0445 ± 0.0030 mg·L^{-1}·day^{-1}, while the rate was approximately 0.0563 ± 0.0054 mg·L^{-1}·day^{-1} when ethanol was the co-substrate (experiment BE), which was statistically different and showed the importance of cometabolic processes on benzene removal. Ethanol can be fermented into volatile fatty acids (e.g., acetic, propionic, and butyric acids).

Benzene removal over time and the formation and consumption of possible anaerobic benzene intermediates are shown in Figure 1. The consumption of benzene occurred right at the experiment beginning, starting the stabilization approximately on the 10th day and ending at 42 days of monitoring, with an efficiency of approximately 90%. Regarding the possible intermediates on anaerobic benzene removal, phenol and benzoate were identified in all conditions, while toluene was never detected.

Benzene degradation under methanogenic conditions can be very slow unless the consortium is stable and acclimatized (MASUMOTO *et al.*, 2012). They observed complete benzene and its intermediates (e.g., phenol, toluene, and benzoate) removal and a methane-rich biogas formation.

Antagonistic and synergistic effects have been reported during the degradation of mixture of hydrocarbons and ethanol. Field studies involving hydrocarbons in gasoline and ethanol indicate that alcohol can hinder hydrocarbon biodegradation. For example, benzene, toluene, ethylbenzene, and xylenes (BTEX) were removed initially consuming the oxygen available in the environment (CORSEUIL *et al.*, 1998, 2011; MA *et al.*, 2015; MA; RIXEY; ALVAREZ, 2015; RAMA *et al.*, 2019). More recently, studies have shown that different concentrations of ethanol influence the co-solvency, dissolution, and BTEX concentrations in groundwater and that higher ethanol concentrations in the medium with gasoline (85%) led to faster BTEX removal rates. There was concomitant biodegradation of ethanol and BTEX in relation to lower concentrations of the mixture of ethanol with gasoline (24%), thus opposing the preferential ethanol biodegradation that was often observed (RAMA *et al.*, 2019).

Table 2 – Consumption rates of benzene and the intermediate compounds.

Identification	Description	Benzene kinetics* (mg·L^{-1}·day^{-1})	Intermediate kinetics* (mg·L^{-1}·day^{-1})
B	Benzene	0.0445 (0.0030)[a]	—
BE	Benzene + ethanol	0.0563 (0.0054)[b]	—
BEPh	Benzene + ethanol + phenol	0.0459 (0.0088)[ab]	0.0565 (0.0056)[a]
BEBz	Benzene + ethanol + benzoate	0.0254 (0.0022)[c]	0.0519 (0.0050)[a]
BETo	Benzene + ethanol + toluene	0.0456 (0.0027)[a]	0.0406 (0.0012)[b]

Different letters in each column indicate statistically significant differences by the Tukey's test at $p < 0.05$; *Mean values considering duplicates and reinjections.
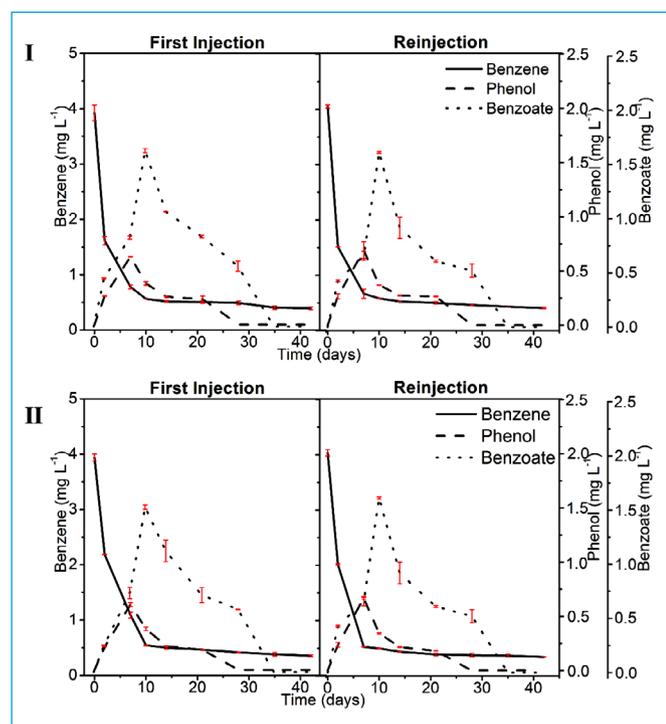
Figure 1 – Benzene degradation and formation of by-products from (I) experiments B and (II) BE.

Effect of intermediates on the anaerobic benzene removal

The effect of intermediates on the anaerobic benzene removal was assessed. In this regard, phenol (BEPh), benzoate (BEBz), and toluene (BETo) were tested individually in the presence of ethanol as the co-substrate (Table 1). As previously reported, phenol and benzoate were detected in the experiments in which only benzene was used, which can already be defined as intermediate compounds in benzene degradation. However, benzoate can also be a key intermediate, which, when added to the system, causes an imbalance, being preferentially consumed compared with benzene and delaying the microbial benzene removal rate.

In general, benzene removal showed a reduction when the intermediates were present. However, when benzoate was added to the medium, the benzene removal rate was reduced by approximately 55%, i.e., from 0.0563 ± 0.0054 to 0.0254 ± 0.0022 mg·L^{-1}·day^{-1}. In the presence of phenol and toluene, benzene degradation rates were 0.0459 ± 0.0088 and 0.0456 ± 0.0027 mg·L^{-1}·day^{-1} (Table 2). Despite being considered an intermediate compound for benzene degradation, phenol did not interfere as much as benzoate upon benzene removal rate, as both kinetics were very close and statistically similar (Table 2). The same trend was verified for toluene (Table 2).

Benzene aromatic ring cleavage in the absence of oxygen can be performed mainly by three enzymatic steps:

- hydroxylation: benzene is converted to phenol;
- methylation: a methyl group is incorporated into benzene and forms toluene;
- carboxylation: benzene is converted directly to benzoate (ABURTO-MEDINA; BALL, 2015; LADINO-ORJUELA *et al.*, 2016; WANG *et al.*, 2022).

The aromatic cleavage of benzene can occur in more than one way, especially when a microbial consortium is used (ULRICH; BELLER; EDWARDS, 2005). The by-products generated from benzene biotransformation, i.e., phenol and toluene, can be subsequently transformed into benzoate (MASUMOTO *et al.*, 2012).

The phenol, benzoate, and toluene removal rates in the presence of benzene are shown in Table 2. The phenol and benzoate showed the removal rates very close to that of benzene (Table 2) with no statistically significant differences ($p > 0.05$), and phenol achieved the highest values. Benzoate, which is the compound most negatively interfered with benzene removal, showed average kinetic values of 0.0519 ± 0.0050 mg·L^{-1}·day^{-1}. Toluene was the compound with the lowest removal rates (Table 2).

Toluene is considered an intermediate of benzene degradation when microorganisms use methylation as a pathway, where a methyl group is initially added to the aromatic ring, forming toluene (KUNAPULI *et al.*, 2008; LADINO-ORJUELA *et al.*, 2016; ULRICH; BELLER; EDWARDS, 2005). However, when monitoring possible intermediates formed from benzene degradation, toluene formation is not observed (Figure 1).

In general, the microorganisms previously acclimatized to benzene were also acclimatized to phenol and benzoate because they possibly participated in the benzene degradation pathway. On the contrary, toluene is not a compound to which the microorganisms present in the inoculum used in this study were acclimatized. Thus, it had a lower mean value in the degradation rate among the intermediates. It is worth emphasizing that, despite toluene having the lowest degradation rate, its presence did not interfere with benzene removal, as found for benzoate.

Formation of intermediates in experiments operated with simultaneous addition of benzene and intermediates added individually

Figure 2 illustrates the benzene and intermediates behavior over time. Toluene was never detected as an intermediate of benzene removal. When benzene and phenol were initially added (BEPh), benzoate was identified shortly after the experiment began, with a peak of up to 1.09 mg·L^{-1} on the 10th day of monitoring and exhausted from the medium on the 28th day, both in the first and second injections.

In the experiments in which benzene and benzoate were initially added (BEBz), the presence of phenol was observed in all samples, maintaining a pattern with an average concentration of 0.48 mg·L^{-1}, a different behavior from the initial experiments (B and BE) where only benzene was present. Benzoate is probably a compound that is easier to be biologically consumed compared with phenol, and, despite the biomass acclimatization, there may have been a shift in the reaction equilibrium caused by benzoate presence.

When benzene and toluene were initially added (BETo), a stable phenol concentration was found between the 2nd day and the 21st day of monitoring, not being detected in the medium after this period. Benzoate, on the contrary, behaved in a standard way, with a peak detected on the 10th day of monitoring and total consumption on the 28th day.

Intermediates monitoring results corroborate the research hypotheses. Benzene removal likely occurs in up to two metabolic routes, i.e., hydroxylation and carboxylation, due to phenol and benzoate formation. As toluene was not observed in the monitoring, methylation is very unlikely to have occurred.
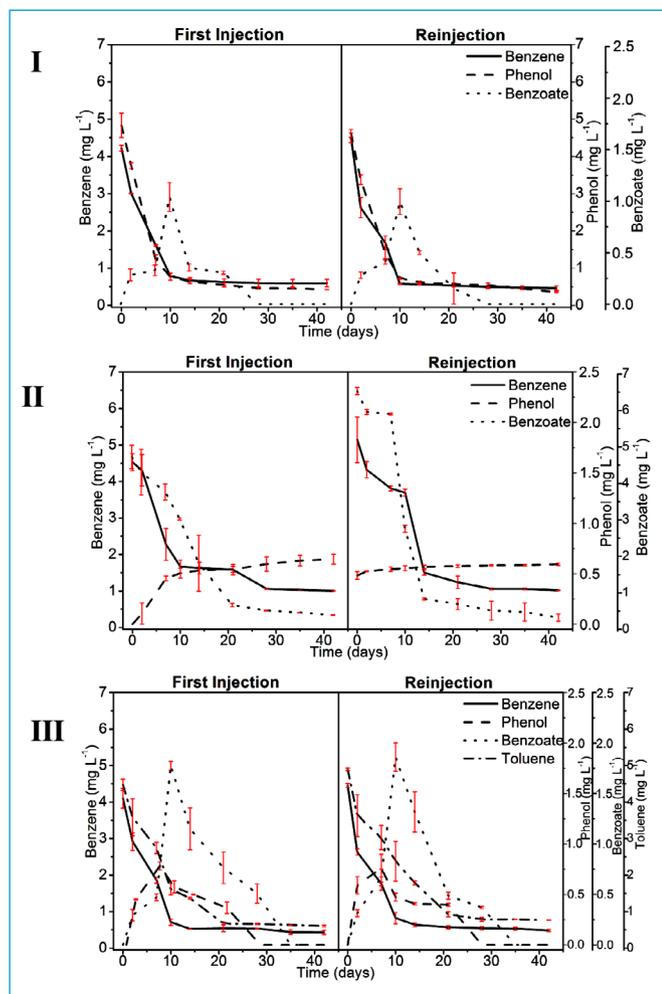
Figure 2 – Benzene degradation and intermediate formation: (I) BEPh, (II) BEBz, and (III) BETo.

When using sulfate-reducing microorganisms to obtain indications of the initial activation mechanism, Abu Laban *et al.* (2009) tested the use of the substrate, performed cometabolism tests and tracking of by-products (i.e., phenol, toluene, and benzoate), and stated that the hydroxylation of benzene to phenol was a possible route of benzene degradation, which was later converted to 4-hydroxybenzoate. However, in tests with an extra addition of phenol, a decrease in the degradation of benzene was verified. Therefore, phenol was considered by the authors as a key intermediate.

Two intermediates were also detected, i.e., phenol and benzoate, the latter of which presented a similar behavior to phenol as in the above-mentioned study, negatively influencing benzene degradation when added simultaneously. For this investigation, benzoate was considered a key intermediate. Hence, the activation process of the aromatic ring of benzene by hydroxylation might not occur in isolation but could occur simultaneously with the carboxylation process due to the diversity of the microbial consortium used, as well as the benzoate being the intermediate that most interfered benzene removal and showed, among the intermediates, better degradation kinetics.

CONCLUSION

Benzene removal kinetics were faster in the presence of ethanol, and benzene removal occurred with the subsequent formation of phenol and benzoate, which were consumed later, without accumulation of by-products in the medium.

Finally, the addition of intermediates had a negative influence on benzene removal, with benzoate being the intermediate that showed the greatest interference. In addition, the presence of benzoate caused a moderate accumulation of phenol, another by-product of benzene degradation, reinforcing the role of benzoate as a key intermediate in the anaerobic removal of benzene in this investigation.

Benzene could possibly be converted into phenol and later into benzoate, in general, considering the peak times of each by-product generated and their consumption in the first and second injections.

Research has indicated the way of benzene degradation by hydroxylation, observing a marked phenol formation from marked benzene consumption (ABURTO *et al.*, 2009; ULRICH; BELLER; EDWARDS, 2005; VOGEL; GRBIC-GALIC, 1986; ZHANG *et al.*, 2013).

AUTHORS' CONTRIBUTION

Siqueira, J.P.S.: Data curation, Formal Analysis, Investigation, Methodology, Writing - original draft. Freitas, R.M.: Data curation, Formal Analysis. Firmino, P.I.M.: Conceptualization, Funding Acquisition, Project administration, Supervision, Writing - review & editing. dos Santos, A.B.: Funding Acquisition, Project administration, Supervision, Writing - review & editing.

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