Chemosphere 173 (2017) 49–60

Contents lists available at ScienceDirect

Chemosphere

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

Metabolical shifts towards alternative BTEX biodegradation intermediates induced by perfluorinated compounds in firefighting foams

Renato Nallin Montagnolli [a], Paulo Renato Matos Lopes [b], Jaqueline Matos Cruz [a], Marina Turini Claro [a], Gabriela Mercuri Quiterio [a], Ederio Dino Bidoia [a, *]

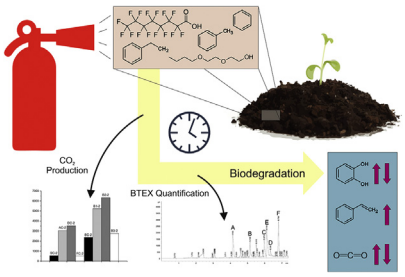
[a] Departamento de Bioquímica e Microbiologia, Instituto de Biociências, UNESP – São Paulo State University, Avenida 24 A, 1515 – Bela Vista, 13506-900, Rio Claro, SP, Brazil

[b] Faculdade de Ciências Agrárias e Tecnológicas, UNESP – São Paulo State University, Rodovia Comandante João Ribeiro de Barros (SP 294), Km 651, 17900-000, Dracena, SP, Brazil

HIGHLIGHTS

- Firefighting foams can disturb soil microbiota activity.
- Perfluorinated substances shifted metabolism in BTEX co-contamination.
- Differential catechol and styrene production rates were found.
- Inhibition and stimulation effects due to AFFF compounds were observed.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 October 2016
Received in revised form
27 December 2016
Accepted 28 December 2016
Available online 30 December 2016

Handling Editor: I. Cousins

Keywords:
Gas-chomatrography
Respirometry
Catechol
Styrene
AFFF

ABSTRACT

The type and concentration of perfluorinated compounds (PFCs) can induce different types of enzymes and promote alternate patterns of BTEX transformation. However, it is not known how the presence of active fluorocarbon-degrading microbial populations affects the transformation of BTEX. In addition to chemical analysis at the molecular level, our research approached the aqueous film forming fire-fighting foams (AFFF) and BTEX co-contamination at a large-scale with respirometers to quantify the total microbial metabolism of soil via CO_2 output levels. The intended outcome of this research was to obtain and characterize shifts in BTEX degradation at a set realistic environmental condition while measuring byproducts and CO_2 production. Both methodologies complimentarily provided an in-depth knowledge of the environmental behavior of fire-fighting foams. The biodegradation was monitored using headspace sampling and two types of gas chromatography: thermal conductivity detector and flame ionization detector. Headspace samples were periodically withdrawn for BTEX biodegradation and CO_2 production analysis. Our research suggests the discovery of an altered metabolic pathway in aromatic hydrocarbons biodegradation that is directly affected by fluorinated substances. The fluorinated compounds affected the BTEX biodegradation kinetics, as PFCs may contribute to a shift in styrene and catechol concentrations in co-contamination scenarios. A faster production of styrene and catechol was detected. Catechol is also rapidly consumed, thus undergoing further metabolic stages earlier under the presence of PFCs. The release of AFFF compounds not only changes byproducts output but also drastically disturbs the soil microbiota according to the highly variable CO_2 yields. Therefore, we observed a high

* Corresponding author.
E-mail address: ederio@rc.unesp.br (E.D. Bidoia).

http://dx.doi.org/10.1016/j.chemosphere.2016.12.144

sensitivity of microbial consortia due to PFCs in the AFFF formulation, therefore shifting their BTEX degradation routes in terms of intermediate products concentration.

1. Introduction

Perfluorinated compounds (PFCs) are important constituents in aqueous film forming foams (AFFFs), as they are responsible for the surface tension reduction that promotes spreadability and facilitates rapid fire suppression. AFFFs have been used to extinguish fuel fires in the petrochemical industry, in emergency situations and at fire-training sites. Due to the repeated nature of historical fire-fighting activities, PFCs and other AFFF components along with flammable fuels and solvents (e.g. gasoline, diesel and jet fuel) have been continually released into subsurface environments, creating mixed waste contamination in soil and groundwater (Moody and Field, 2000). Newly identified PFASs, along with high concentrations of PFOS and PFOA (Vecitis et al., 2009), have indeed been detected at military sites that are also contaminated with significant quantities of petroleum hydrocarbons. This poses considerable challenges for the remediation of contaminated groundwater. These strategies should, however, be studied within the context of hydrocarbon co-remediation as PFC-impacted sites are often accompanied by petroleum hydrocarbon contamination.

Aromatic hydrocarbons such as mixtures of benzene, toluene, ethylbenzene and xylene (BTEX) can effectively be biodegraded by diverse aerobic microbial communities (Deeb and Alvarez-Cohen, 1999). There are also great chances of BTEX to be found as co-contaminant in petrochemical fires. The BTEX makes up to 20% of the formulation of gasoline, thus resulting in major exposition to humans, whether in chronic or accidental release into the environment (Silva et al., 2009). Understanding the relationship between hydrocarbon-degrading and PFC-transforming soil and groundwater microorganisms is a key consideration in designing remediation systems that target both defluorination and hydrocarbon mineralization at AFFF-impacted sites. The ability of a diverse array of microorganisms to aerobically degrade petroleum hydrocarbons, specifically BTEX, is known and has been well established in the literature (Margesin and Schinner, 2001). It is hypothesized that under the appropriate environmental conditions the compounds can be co-remediated, yielding maximum defluorination and complete hydrocarbon mineralization. However, it is not known how the presence of active PFCs-degrading microbial populations affects the transformation of BTEX.

The type and concentration of PFC substrate can induce different types of enzymes to promote alternate patterns of BTEX transformation. Various substrates are known to induce certain enzyme systems and promote changes in enzyme expression correlates to a difference in quantified transformation products (El-Naas et al., 2014). The robust properties of BTEX activated oxygenases can be affected by co-metabolic PFC substrates. In other words, BTEX transformation pathways can be driven by such compounds. Thus, the knowledge on AFFF effects and its environmental fate allow a better approach in bioremediation processes that can reduce the time and resources expenses associated with hydrocarbon fires.

Current research emphasizes the biotransformation of compounds containing fluorine in their chemical structure. Much of the current experimental efforts were designed to demonstrate and promote the transformation of the PFC persistent substances (Harding-Marjanovic et al., 2015; Backe and Field, 2013; Dasu et al., 2013). However, fire-fighting foams contain many additional substances to achieve proper foaming capabilities and its functional properties. AFFFs are complex mixtures in their retail formulations, whose main components are a solvent (typically a glycol ether), fluorinated surfactants (amphoteric anionic partially fluorinated or perfluorinated), and surfactants based on hydrocarbons (Alm and Stern, 1992; Kissa, 1994). Surfactants in fluorine-containing mixtures, for example, contribute to the AFFF performance as sealants that prevent re-ignition of fuel and solvents.

Moreover, AFFF contains diethylene glycol butyl ether, also known as DBGE. This compound, which acts as co-solvent and anti-freezing of fire foaming agent, is responsible for 12%–15% of the total formulation of AFFF. The DBGE may also serve as a substrate and carbon source for microbial communities. Still, the interference of DGBE during the biodegradation of BTEX have not yet been studied and its impact on microcosms must be considered. Unlike PFCs, the DGBE has no fluoride in its structure and it is easily consumed by the microbiota. Therefore, it is safe to assume that DGBE presence may promote major changes in other substrate uptake ratios that can ultimately affect the biodegradation process.

BTEX degradation has been shown to be dependent upon many environmental factors, including nutrient availability and substrate interactions (Leahy and Olsen, 2006). However, it is unknown how this confluence of factors may affect the conditions that facilitate optimal BTEX biodegradation pathways. In addition to chemical analysis at the molecular level, our research approached the AFFF + BTEX co-contamination at a macroscopic scale with respirometers to quantify the total microbial metabolism of soil via CO_2 output levels. A greater focus was given to presence of DGBE and PFCs role in optimizing BTEX mineralization. The objective of this research was not to define a complete metabolic map of BTEX degradation, but to detect the effects of the individual components of full AFFF formulations in biodegradation. Thus, the intended outcome of this research was to obtain and characterize shifts in BTEX degradation at a set realistic environmental condition while measuring byproducts and CO_2 production.

2. Materials and methods

The biodegradation of AFFF and BTEX compounds in environmental samples aimed both the analysis of individual metabolites and large scale CO_2 production. The different methodologies complimentarily provided an in-depth knowledge of the environmental behavior of fire-fighting foams. The biodegradation was monitored using *headspace* sampling and two types of gas chromatography (GC): thermal conductivity detector (TCD) and flame ionization detector (FID). Headspace samples were periodically withdrawn for BTEX biodegradation and CO_2 production analysis. Thus we could demonstrate BTEX and DGBE biodegradation when mixed in a soil matrix to infer any mutual interference caused by the AFFF co-contamination.

2.1. Soil and AFFF samples

Soil samples were collected from the Replan Petrobras oil refinery in Paulinia, Brazil (22°43′24.2 ″S 47°08′00.3″ W). A large scale application of AFFF occurred at this refinery site on January 8, 1993 in one of the largest fires to ever strike Brazilian oil refineries.

While almost 4,000,000 L of diesel and gasoline burnt, circa 37,000 L of AFFF was spent and released into the soil (Figueredo and Sabadini, 2003). Soil samples were collected in July 2015 in an area close to the stock tanks refinery fuel. The soil was removed with PFC-free instruments at a 0.4 m depth. The samples were then placed in fluoride-free containers and stored in freezer (−80 °C).

There are no records on the AFFF brand used at the 1993 incident, however, it is known that most of the AFFF imported to Brazil at that time was either Ansul or 3 M branded. At that time, Brazil did not produce its own fire foam type AFFF, however in this experiment, we used the Sintex AFFF produced in Brazil to replicate the most common formulations of AFFF are based in fluortelomer thioamide sulfonated compounds (FtTAoS).

2.2. BTEX biodegradation

2.2.1. Experimental setup

The assays were assembled and conducted in 160 ml glass bottles. The assays were assembled in sterile environment according to Table 1 quantities. The biodegradation process in each test containing BTEX and AFFF was observed for 20 days, with the following components:

- 50 ml of minimum saline media, composed of 85.0 mg.L^{-1} KH_2PO_4, 218.0 mg.L^{-1} K_2HPO_4, 334.0 mg.L^{-1} $Na_2HPO_4.2H_2O$, 5.0 mg.L^{-1} NH_4Cl, 36.4 mg.L^{-1} $CaCl_2.2H_2O$, 22.5 mg.L^{-1} $MgSO_4.7H_2O$ and 0.25 mg.L^{-1} $FeCl_3.6H_2O$. Trace elements were also added to the media as 13.0 mg.L^{-1} $NiSO_4.6H_2O$, 0.65 mg.L^{-1} $(NH_4)_6Mo7O_2.4H_2O$, 105.0 mg.L^{-1} $Na_2SiO_3.5H_2O$, 0.17 mg.L^{-1} $MnSO_4$, 0.14 mg.L^{-1} $SnCl_2.2H_2O$ and 17.3 mg.L^{-1} Na_2SeO_3. The buffer $NaHCO_3$ was used at the approximate concentration of 2.7 ± 0.5 g.L^{-1} to achieve pH 7.2.

- 50.0 μL of AFFF. The portion containing perfluorinated substances is known by the brand name Lodyne (composed of a FtTAoS homologues mixture). Together with DGBE, these substances make up most of the Sintex AFFF 6% formulation. The concentration of DGBE in the samples constituted over 80% of the total organic carbon. DGBE and Lodyne were each inserted individually to some assays, instead of the full AFFF formulation.

- 9.0 μL BTEX mixture, with 25.0 mg.L^{-1} of each compound. The isomer o-xylene was used among the xylenes, since it is the most commonly occurring isomer. Given the volatility of the BTEX compounds, the final concentration took into account Henry's Law calculations. The mixture of BTEX was stabilized for 24 h before being injected into the vials; - 5.0 g of soil sample from Replan, Brazil; - 300.0 μg of B12 cofactor as an enhancement for microbial growth.

The vials were sealed with fluoride-free rubber caps, which would also prevent the volatilization of BTEX and the adsorption of volatile compounds over time. Additionally, soil-free medium controls (MC-1) also assessed the potential impacts of media components onto fluorinated surfactants. A set of sterile controls (NC-1) was prepared by exposing the soil to three consecutive autoclave cycles followed by overnight freezing at −20 °C, and then amending the medium with AFFF and BTEX. These controls were also established to verify any losses of the compounds due to sampling procedures or inadequate sealing. All experiments (Table 1) were run in triplicates in separate microcosms and shaken at 100 rpm in a 29 ± 0.1 °C incubator for 20 days. The headspace was sampled periodically with sterile syringes and immediately analyzed. The autoclaved control (NC-1) was supplemented with antibiotic solution to ensure the elimination of microbial activity.

2.2.2. Headspace analysis by GC-FID

The biodegradation of BTEX was monitored by GC-FID. The concentration of BTEX and its biodegradation intermediates was regularly monitored though 100 μL sample injections in a GS-GasPro chromatographic column (30 m × 0:32 mm; Agilent Technologies, Inc., Santa Clara, USA) taken from the headspace within the 160 ml bottles. Chromatography was performed on Agilent 7890A GC-FID equipment.

The parameters used were modified from the method 113–4332 Agilent Technologies to decrease the running time from 20 min for 5 min per sample as well as improve signal response. The initial temperature was 110 °C for 50 s, followed by a gradual increase of 110 °C–205 °C at 20 °C.minute^{-1} and then 205 °C–260 °C at 50 °C per minute. Finally, temperature was held at 260 °C for 3 min. The carrier gas helium was set at the flow rate of 39.7 cm s^{-1} and heated to 110 °C at the injection chamber. The injector temperature was set to *split* mode. FID detector temperature was set to 250 °C. The BTEX chromatogram showed sufficient resolution to quantify the consumption of BTEX with initial signal intensity greater than 2500 pA.

The gas samples have not undergone any type of preparation and were injected as they were removed from the flask. The withdrawal of the gas sample was taken by a glass syringe equipped with gas valves directly through the rubber stoppers to prevent opening the serum bottles risk any interfere with the biodegradation process.

2.3. CO_2 output

2.3.1. Experimental setup

Respirometric assays applied to bioremediation studies offers otherwise unobtainable environmentally analogue CO_2 production data compared to other approaches (Montagnolli et al., 2009, 2014; Lopes and Bidoia, 2009; Graves et al., 1991; Bartha and Pramer, 1965). We determined the CO_2 concentration in a simulated micro-atmosphere within respirometer flasks aiming to quantify the parameters of biodegradation over time and the different microbial adaptation stages until metabolism ceased. Such datasets were directly related to biodegradability and the expected development of this biomass (Fiuza and Vila, 2004) from the AFFF and BTEX substrates. The experimental design used BTEX and AFFF concentrations proportional to the GC-FID experiments (Table 2).

According to Table 2, many controls without BTEX were also setup. All experiments were performed in triplicate, with separate microcosms without shaking in an incubator at 30 °C for 105 days.

Table 1
BTEX biodegradation assay contents.

Assay ID and description	Media (50 mL)	Soil (5 g)	DGBE (700 μL)	Lodyne (50 μL)	AFFF (110 μL)	BTEX (10 μL)
MC-1 (Media control)	X					X
NC-1 (Sterile control)	X	X[a]			X	X
SC-1 (Soil control)	X	X				X
B1-1 (with AFFF)	X	X			X	X
B2-1 (DGBE only)	X	X	X			X
B3-1 (Lodyne only)	X	X		X		X

[a] Autoclaved soil.

Table 2
Respirometry assay contents.

Assay ID and description	Soil (5 g)	DGBE (700 μL)	Lodyne (50 μL)	AFFF (110 μL)	BTEX (10 μL)
SC-2 (Soil control)	X				
AC-2 (AFFF control)	X			X	
DC-2 (DGBE control)	X	X			
FC-2 (Lodyne control)	X		X		
BC-2 (BTEX control)	X				X
B1-2 (with AFFF + BTEX)	X			X	X
B2-2 (DGBE only + BTEX)	X	X			X
B3-2 (Lodyne only + BTEX)	X		X		X

Even though shaking would promote better aeration, we chose to replicate an environmental sample where the soil matrix is kept static instead of continuously stirred. It is important to state that the chosen concentrations and conditions ideally corresponded to four criteria: (i) resemblance to actual data of environmental contamination; (ii) low toxicity; (iii) response of the microbial community with difference statistically significant between tests; and (iv) within the measuring range of the technique. Samples were regularly collected from the headspace with syringes.

We carefully planned the concentration of aromatics to be added to each culture media. If the amount of BTEX was too high, it could present a toxic effect on living cells. The substrate inhibition and its critical concentration widely varies between microorganisms and microbial consortia. The 100.0 $mg.L^{-1}$ BTEX concentration was a safe option that took into account both previous literature reports and the pilot experiments. Li et al. (2006) demonstrated, for example, inhibition from benzene is expected for concentrations higher than 100 $mg.L^{-1}$ in *Planococcus* sp. Hamed et al. (2003) reported that *Pseudomonas putida* cannot completely metabolize benzene and toluene at concentrations exceeding 380 $mg.L^{-1}$ and 420 $mg.L^{-1}$, respectively. High initial concentrations of BTEX can inhibit microbial activity due to complex interactions at the molecular level with enzyme mechanisms and (Mathur and Majumder, 2010), so they were well-defined in our experimental design for maximum biodegradation. The choice of 25.0 $mg.L^{-1}$ per component was also defined taking into account the experiments from Dou et al. (2007), for BTEX metabolism under anaerobic nitrate and sulfate reduction conditions.

2.3.2. Headspace analysis by GC-TCD

Oxygen and carbon dioxide was measured by injecting 250 μL of microcosm headspace into a gas chromatograph (GC-8A TCD Shimadzu) with thermal conductivity detector. GC-TCD was operated with helium as carrier gas at a flow rate of 46.7 mL $min^{-1.}$ Samples were injected at a 280 °C chamber, while the oven operated isothermally at 60 °C. The detector temperature was 250 °C. HP-Plot Q capillary column 30 m × 530 mm × 40 mm was used in the system. The produced CO_2 volumes were normalized to standard temperature and pressure conditions (273 K and 101.3 kPa). The headspace was sampled with syringes using the same BTEX removal protocol, where 250 μL was injected instead of 100 μL. The oxygen in the headspace was maintained between 15 and 25% (v/v) by periodically amending pure oxygen into the microcosms using 0.2 μM gas-tight syringe and then depressurizing the bottle for several seconds with a sterile needle inserted into the valve plug.

The lack of microbial activity in autoclaved controls was confirmed by the lack of consumption of organic carbon or oxygen over the days of the experiment. All microcosms were equilibrated with AFFF and BTEX 24 h before collecting the first sample in 3 days. In order to calibrate the equipment though each measurement day, we used two standard gas mixtures with 65, 25 and 10% of CO_2; and 25, 55 and 20% of O_2 proportions.

2.3.3. Chemical oxygen demand (COD) of DBGE

The DGBE portion of retail AFFF formulation are important from the biodegradation point of view. Large amounts of DGBE can enter and accumulate in the environment, but the environmental risks of this release are not clear (Krystyna et al., 2012). Some microorganisms have the ability to use ethers as the sole source of carbon and energy. These microorganisms were examined by exclusively enrichment assays, isolation and identification of microbial followed by evaluation of its performance degradation and metabolic products. However, knowledge of the inhibitory effect on the development of micro-organisms in relation to the rest of AFFF formulation has not yet been elucidated. Therefore, we also wanted to investigate the impact of DGBE on microbial substrate intake to provide bioremediation real bioremediation scenarios.

We tested the chemical oxygen demand (COD) from our respirometric microbiota before and after biodegradation. Although not observed in this research, some studies suggest toxicity of DGBE to some cell cultures (Johnson et al., 2005). Thus, COD tests were made to test adaptability of the microbial communities to DBGE and check any potential interference solely caused by DGBE. The contents of Table 2 assays were diluted with 100 mL of water to obtain a suspension of micro-organisms adapted to DBGE and had their COD measured using the dichromate method (Jirka and Carter, 1975).

3. Results and discussion

3.1. Detection of BTEX biodegradation and by-products

The biodegradation of the individual BTEX components throughout 20 days is shown in Fig. 1. The difference between datasets from B1-1, B2-1 and B3-1 assays were considered statistically significant. These assays were also different from controls (MC-1, NC-1 and SC-1). On the other hand, control assays did not statistically differ when compared to each other, except for the soil control (SC-1). A detailed statistical analysis of the data can be found in the Supporting Information, Tables S1 to S4.

When no DGBE was present, either pure or as part of the AFFF formulation, we observed that the biodegradation of each of BTEX components of the mixture occurred much faster in comparison to other assays, especially in Fig. 1c. In average, B1-1 and B3-1 consumption ceased 6.8 days earlier than the B2-1 assays. This is due to the preferential consumption of DGBE over BTEX by the microbial community, whose metabolic pathways prioritize DGBE as substrate, thereby decreasing the degradation of BTEX components. Thus, when there is no DGBE as an agent of competitive inhibition, BTEX consumption is much higher. In other words, specific metabolic pathways are activated when BTEX is available as sole source of carbon.

The perfluorinated compounds in B3-1 also have an active role in BTEX metabolism. By comparing the B1-1, B2-1 and B3-1 with the soil control (SC), where no PFCs were added, we observed much

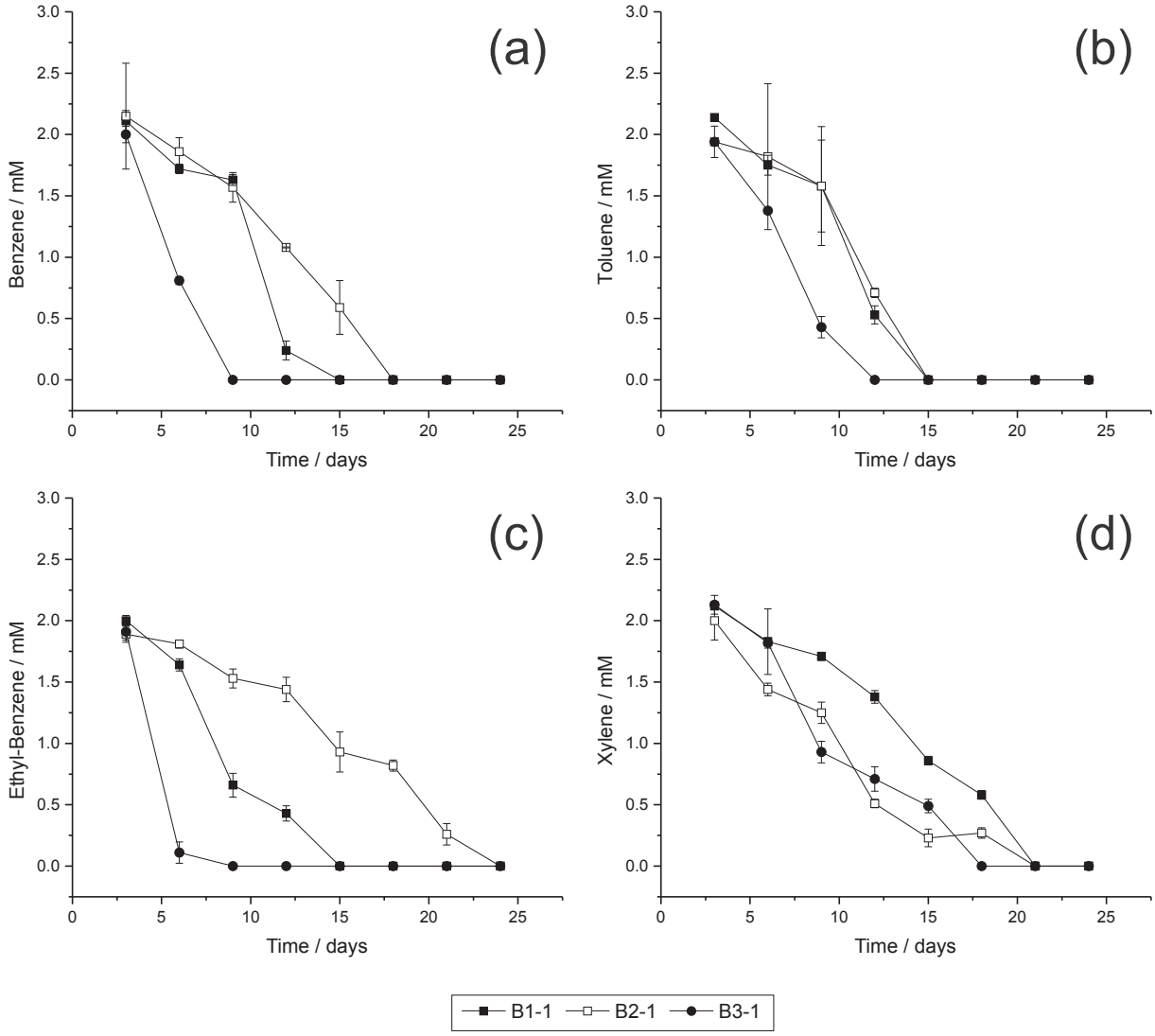


Fig. 1. Benzene (a), toluene (b), ethylbenzene (c) and xylene concentration in the biodegradation assays B1-1 (AFFF), B2-1 (DGBE only) and B3-1 (Lodyne only).

lower degradation rates. Therefore, the fluorinated compounds in media affected the overall BTEX biodegradation kinetics (Fig. 2).

Even the lowest BTEX consumption rates found in B2-1 assays surpassed SC-1 substrate consumption. Fig. 2 also shows that autoclaved soil (NC-1) slowly allowed the reestablishment of the microbiota, showing a minimal benzene degradation towards 24 days. The autoclaved assays were conceived to verify the sterility our experiments as the headspace sampling procedure were made with sterile syringes and needles. We noticed from previous pilot assays that extending the experiment to intervals greater than 30 days using the same sampling and analysis protocols could cause small traces of contamination due to the rubber cap wearing. Further contamination would compromise the technical accuracy of the experiments and our conclusions on biodegradation. For this reason, the final data point was performed at the 24-day period.

The soilless assay (MC) presented very low to none microbial activity that caused the biodegradation of aromatic hydrocarbons. Thus, the outcome of these controls demonstrated that no major leaks or adsorption of volatiles occurred throughout the experiment.

Although Fig. 2 only shows the comparison of control tests in benzene, the same substrate consumption profile occurred in vials containing toluene, ethylbenzene and xylene. The biodegradation profile for toluene, ethyl-benzene and xylene control assays is provided in Fig. S1 of the Supporting Information. BTEX biodegradation of the mixture occurred at similar proportions for each component. Except for ethyl-benzene, no other assay showed a higher or lower biodegradation rate of a particular element. In other words, the benzene was not degraded at a higher rate than toluene nor o-xylene was degraded at a greater rate than benzene. The biodegradation rate of all BTEX components, except ethylbenzene, was equal.

Ethyl benzene benefited the most by the addition of perfluorinated substances in its culture medium, especially in B3-1 assays. We also found an expressive increase in biodegradation of benzene and toluene in assays where fluorinated compounds were added (B1-1 and B3-1), although at lower rates than ethylbenzene. Triggered by such differential biodegradation profile between assays, our research group performed an in-depth analysis of the chromatogram to search for intermediates of aromatic hydrocarbon biodegradation. We found two new peaks during the biodegradation process that could be related to intermediates formation. A series of stock solutions containing known BTEX aerobic biodegradation intermediates (dihydrobenzenediol, catechol, muconate,

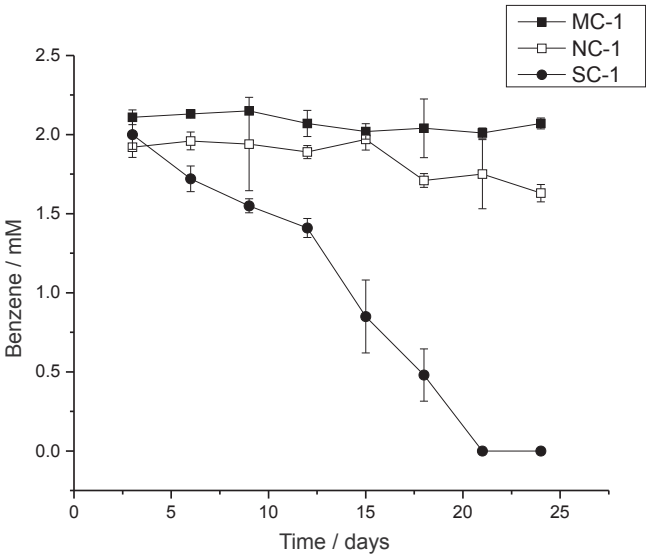


Fig. 2. Benzene concentration in MC (media control), NC (autoclaved control) and SC (soil control).

formate, methylbenzoate, benzaldehyde, benzoate, vanillin, styrene, dihydroxyethylbenzene and acetophenone) were tested with our chromatographic method to determine the retention time associated to the substances from the new peaks and their concentration through time. The peaks were found to be associated with styrene and catechol formation as shown in Fig. 3.

According to Fig. 3 insert, catechol is an expected intermediate of many aromatic compounds present in oil derivatives, including benzene and toluene. In general, the BTEX biodegradation process can be exemplified based on previous studies by Mazzeo et al. (2010). According to the authors, *Pseudomonas putida* is able to break the BTEX components using a metabolic pathway based on direct oxidation of aromatic rings by mono-oxygenases or di-oxygenases to form catechol. This, in turn, is subsequently broken into 2,3-dioxygenase and metabolites generated in the second stage are consumed in the Krebs cycle. Therefore, we can reasonably infer that an equivalent microbial pathway took place within our assays.

Styrene is a byproduct of ethyl benzene biodegradation; however, this is only one of several possible pathways for the degradation of that compound. Furthermore, styrene (vinyl benzene), is commonly detected along with BTEX compounds in contaminated groundwater. Although the individual BTEX compounds are widely used as solvents and in manufacturing (Swoboda-Colberg, 1995), legacy studies about gasoline leaks from underground storage tanks and distribution pipelines points BTEX as the primary contributor of BTEX contamination in ground water. We further expanded this investigation by demonstrating that perfluorinated compounds may contribute to a shift in styrene concentrations in co-contamination scenarios. The formation of these compounds on SC-1, B1-1, B2-1 and B3-1 assays is shown in Fig. 4. This specific change in styrene production profile was not found in SC-1 assays without fluorinated compounds.

According to Fig. 4, the production kinetics is noticeably different in B3-1 assays for both compounds, in which a larger and faster production of styrene and catechol was detected. Catechol is also rapidly consumed in B3-1, whereas no consumption was observed in B1 and B2. Therefore, we observed that the catechol, a transient by-product of the metabolic pathway of benzene and toluene (Mason and Geary, 1990), undergoes further metabolic stages earlier under the presence of perfluorinated compounds in a contamination scenario, instead of the full AFFF formulation. Still, the presence of AFFF and DGBE contribute to the development of this compound, although no production/consumption spike occurred in B1-1 and B2-1.

The determination of enzymatic mechanisms is beyond the scope of this research; however, our results confirm the previous reports on BTEX bioprocesses and slightly changes the expected biodegradation pattern with PFCs. Microbial adaptation to BTEX was extensively studied, as brilliantly complied and discussed in El-Naas et al. (2014) studies. Current knowledge on BTEX suggests that the adaptive history of a microbial community affects the default compounds biodegradation pathways and co-contaminants may yield improved performance of aromatic compounds removal from the environment. Yeom et al. (1997) demonstrated, for example, that even microbes adapted to only one of aromatic compounds such as *Alcaligenes xylosoxidans* may switch their enzymatic mechanisms towards other compounds, acquiring the ability to degrade toluene, m-xylene and ethyl benzene faster than control assays though unknown pathways. In summary, we found that PFCs can trigger a new metabolic pathway that causes an unpredictable biodegradation pattern in BTEX co-contamination scenarios.

3.2. Detection of CO_2 production during biodegradation

The CO_2 production during 105 days of biodegradation was calculated and plotted as a function of time using the response signal measured by GC-FID. The weekly CO_2 production is shown in Fig. 5.

The control assays (Fig. 5a) and assays containing BTEX and perfluorinated compounds (Fig. 5b) were plotted separately to simplify data visualization. As expected, control assays yielded the lowest weekly CO_2 production average (57.43 ± 2.7 μmol CO_2.week^{-1}). This is due to the low amount of organic matter in the SC-2 soil matrix also responsible for the low basal respiration of the indigenous microbiota. The other control assays (AC-2, DC-2, FC-2 and BC-2) revealed the influence of individual components in the biodegradation process.

Different profiles of weekly CO_2 production were observed for each substrate. The dataset ANOVA considered the difference between each treatment group significant at $p < 0.05$. In general, a spike pattern of increased and declined CO_2 peaks was observed across 105 days of biodegradation. We observed a larger average weekly CO_2 production in B1-2, B2-2 and B3-2 assays. Whenever fluorinated compounds were present on their own (such as in B3-2 assays), a quick burst of microbial activity occurred over the first 40 days of monitoring, however metabolism abruptly dropped below BC-2, NC-2 and DC-2 levels afterwards. In fact, the average B3-2 values (142.21 ± 7.0 μmol CO_2.week^{-1}) are even lower than the ones observed in CB (147.15 ± 5.7 μmol CO_2.week^{-1}). Therefore, fluorocarbons alter the long-term metabolism soil microorganisms. This is further evidenced by the low basal activity of soil samples contaminated exclusively with Lodyne in FC assays average (31.60 ± 3.7 μmol CO_2.week^{-1}). The decrease in microbial activity is most likely related to the toxicity of PFCs. In other words, the presence of PFCs decreased long-term CO_2 output from substrate consumption by the soil microbiota.

In a previous work from our research group, Montagnolli et al. (2015) found a similar spiked CO_2 output trend in gasoline compounds. In that case, a CO_2 spike was followed by a rapid growth inhibition due to the toxic effect of gasoline degradation by-products, in addition to volatilization of compounds to be used as substrate. Therefore, even though there is still enough substrate to be biodegraded within respirometric flasks, the total consumption of organic matter present in the assays is not possible due to

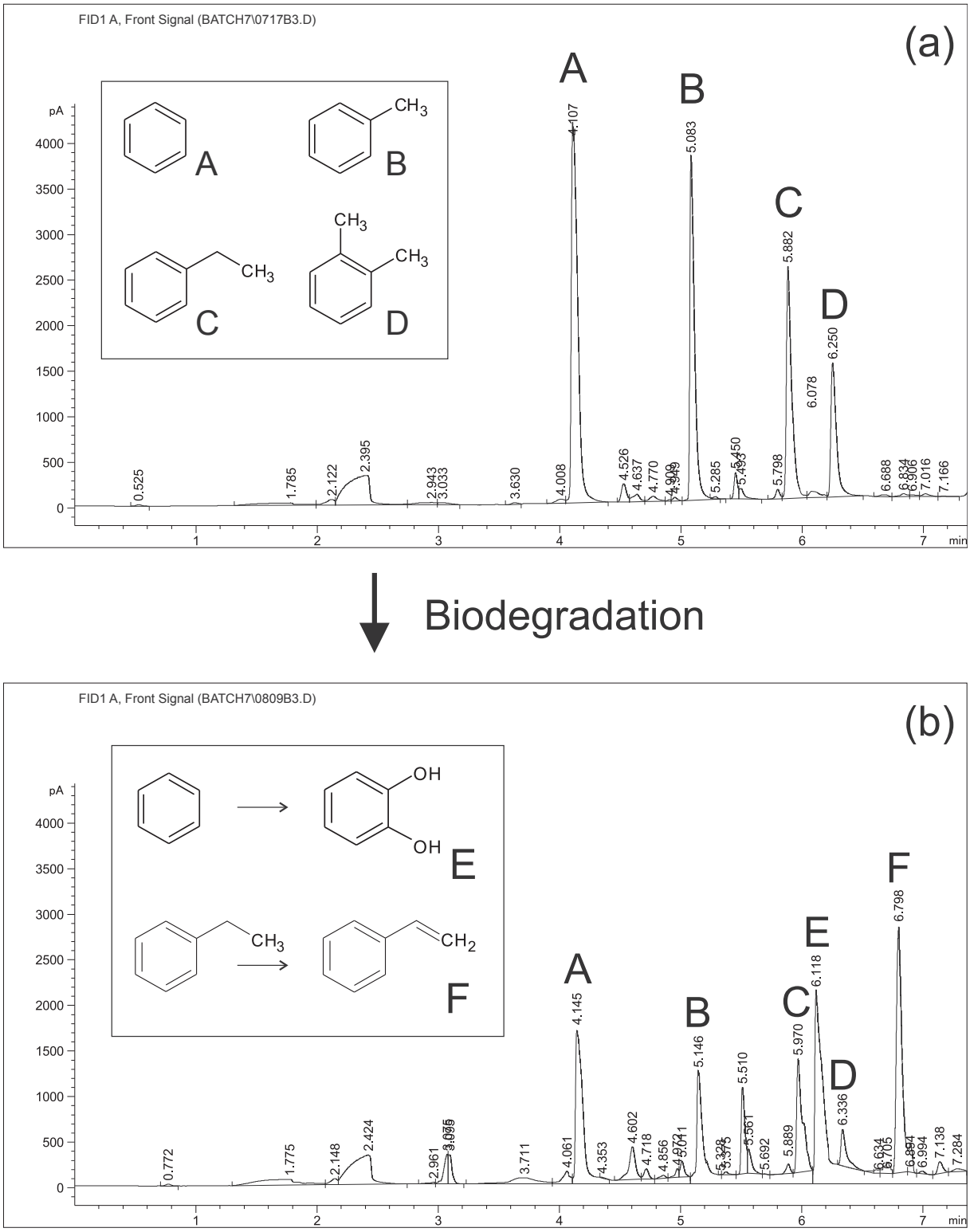


Fig. 3. Chromatogram of BTEX before (a) and after (b) biodegradation with benzene (A), toluene (B), ethylbenzene (C), xylene (D), catechol (E) and styrene (F) peaks.

changes in the micro-environment towards toxic compounds.

In general, assays containing PFCs presented lower average CO_2 output (131.23 ± 7.0 μmol CO_2) compared to assays without PFCs (372.61 ± 7.0 μmol CO_2). Although fluorocarbons are substantially inert from the biodegradability standpoint, the non-fluorinated portions introduced into the B1 microbiota, such as DBGE, were promptly consumed by the soil microorganisms. Since DBGE makes up most of the retail AFFF formulation, we find reasonable to argue that it is the source of the exceeding CO_2 production in B1-2 and B2-2 assays, instead of the PFCs.

The biodegradability of DGBE is already known in the literature, but not in co-contamination scenarios with BTEX or other

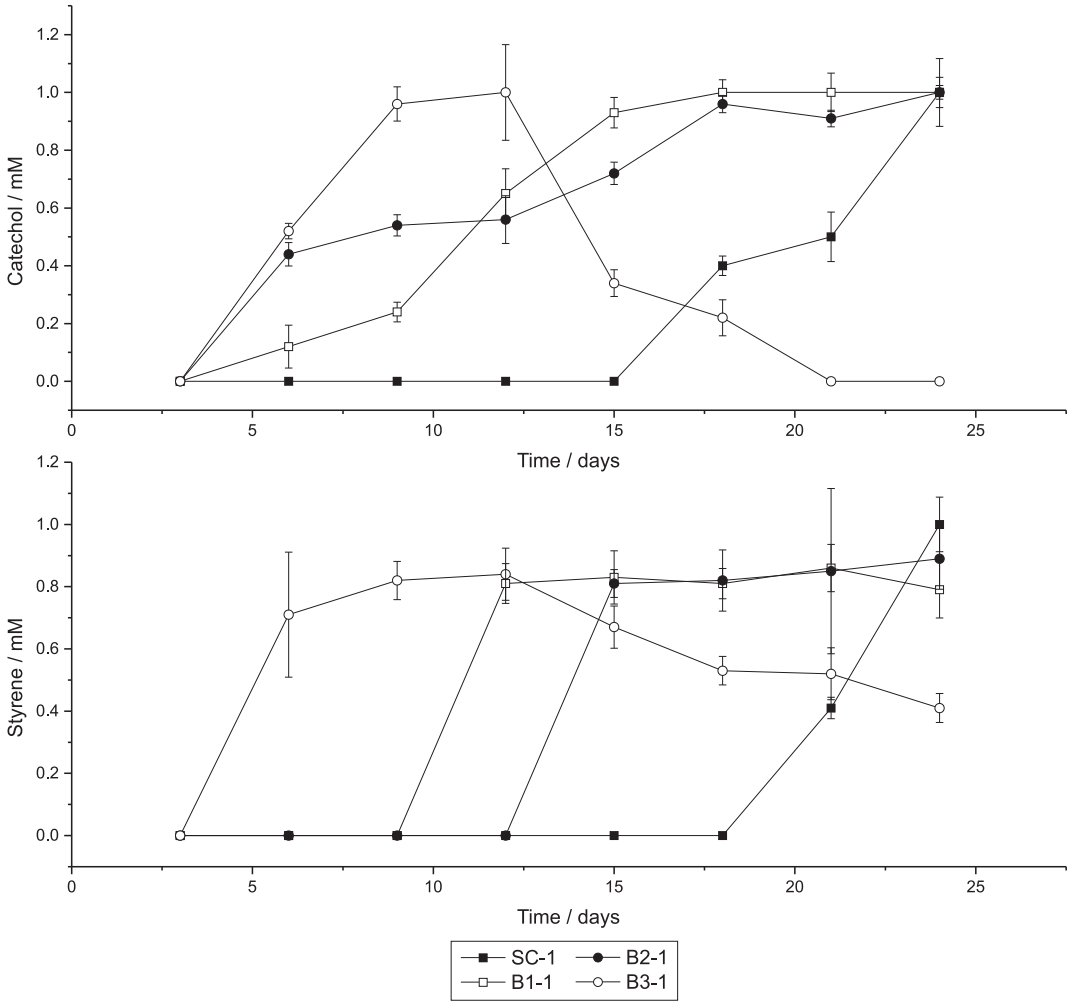


Fig. 4. Catechol and styrene concentration during monitoring degradation in soil control (SC-1) and in the biodegradation assays B1-1 (AFFF), B2-1 (DGBE only) and B3-1 (Lodyne only).

petroleum hydrocarbons. Our results increase the current knowledge of the co-contamination role individual AFFF components during bioremediation processes. The bioaugmented degradation of DGBE wastewater has not yet been investigated in depth, hence methods for improving the DGBE removal efficiency are needed. It is also important to mention that the glycol ethers are highly mobile and are not adsorbed to soil particles (CMA, 1994). These results suggest that DGBE biodegrade rapidly and must not persist in the environment.

When we observe the CO_2 production according to the total mineralization output, we observe that DGBE promotes the highest CO_2 mass yield (Fig. 6). Therefore, the largest obstacle towards total removal of AFFF from the environment still remains in the PFCs. Fig. 6 summarizes the total amount of CO_2 obtained at the end of the experiment at 105 days.

It is noticeable from Fig. 6 that the presence of PFCs compromises the substrate consumption during the biodegradation process, as seen in FC-2 and B3-2. Even though PFCs are present in B1-2, the CO_2 productions is a result of BTEX and non perfluorinated compounds metabolism. Therefore, the highest output observed in B2-2 is explained by the lack of PFCs and the presence of mostly biodegradable BTEX and DGBE compounds. In fact, the B2-2 total CO_2 output (6334.51 μmol) is roughly equivalent to the sum of both DC-2 (3517.49 μmol) and BC-2 (2354.54 μmol) values.

Another trend observed by our research regards the spiked CO_2 production in soil contaminations containing BTEX and no PFCs (CB-2) during in early biodegradation stages. However, the weekly rates did not reach values as high as B3-2 assays. Still, both curves present a similar CO_2 profile. We propose that the Lodyne mixture of fluorinated surfactants may have been responsible for improving the bioavailability of BTEX to soil micro-organisms. The BTEX components, along with many other petroleum derivatives, are mostly hydrophobic. Such property poses a major challenge in biodegradation, since micro-organisms access is compromised due to low dispersion in water. Therefore, emulsifying activity is an important characteristic in hydrocarbon biodegradation (Panda et al., 2016). An emulsion must have facilitated bioavailability occurs when fluorinated molecules with surface-active properties is dispersed BTEX as microscopic droplets in micro aqueous-phase regions. Besides CB-2 and B3-2 results, the possibility of perfluorinated compounds surface-active properties may also be considered and associated with increased CO_2 production by microorganisms in our assays. In summary, BTEX profits from fluorinated compounds, but overall CO_2 production in soil is negatively impacted by PFCs presence.

3.2.1. *Large-scale biodegradation prediction*

The cumulative CO_2 production data was adjusted according to

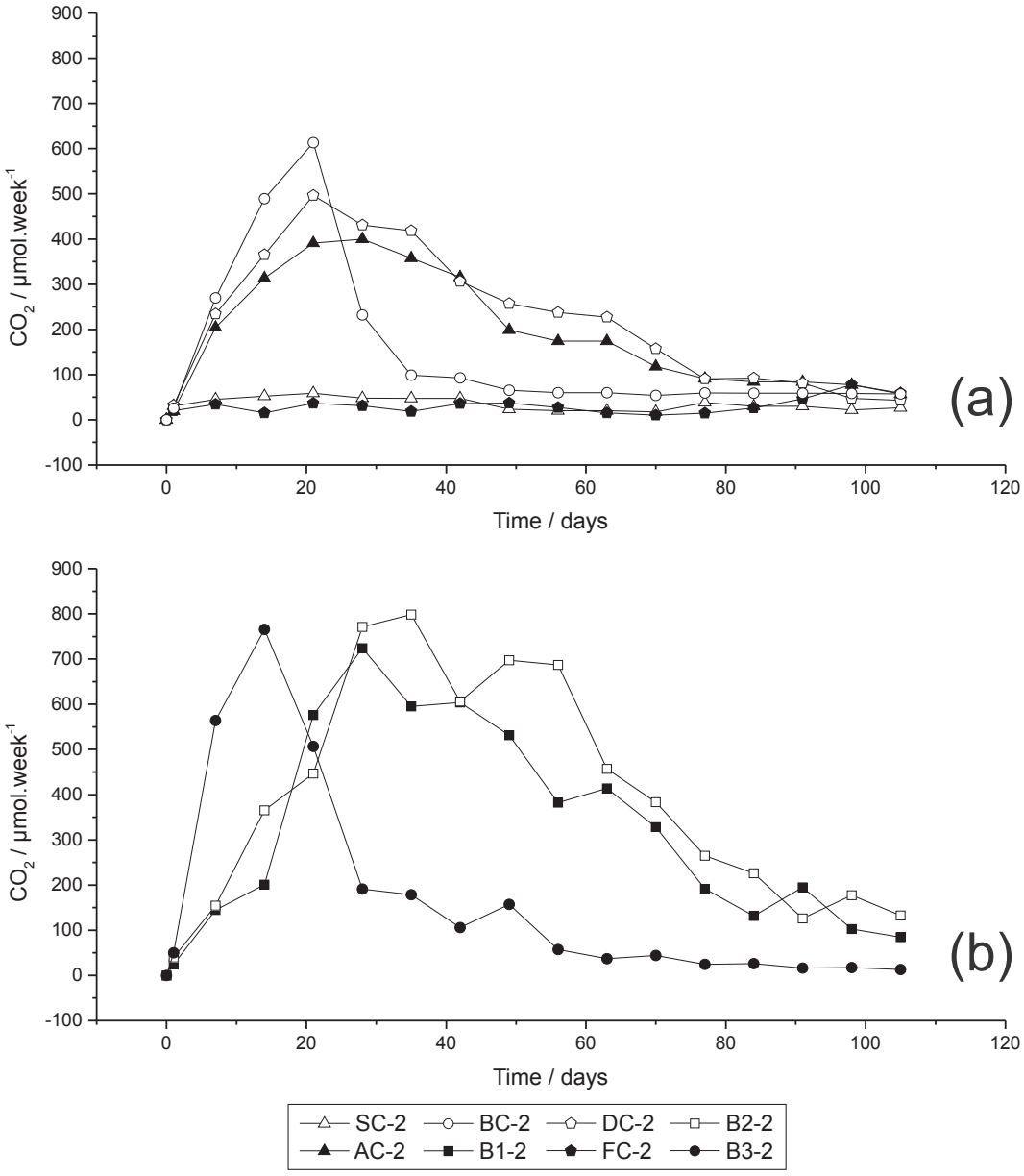


Fig. 5. CO_2 production rate in respirometric tests though 105 days in SC-2 (soil control), AC-2 (AFFF control), DC-2 (DGBE control), FC-2 (Lodyne control), BC-2 (BTEX control), B1-2 (AFFF), B2-2 (DGBE only) and B3-2 (Lodyne only).

an adapted Montagnolli et al. (2014) model using Equation (1) parameters. The modeling of data enabled a better understanding of biodegradation kinetics, allowing us to predict and extrapolate the process as a large scale contamination scenario with AFFF and BTEX. These mathematical and statistical tools were effective way to describe the influence of various parameters involved in the removal of pollutants during aerobic biodegradation (O'Neill et al., 2016) taking substrate input and mineralization output in consideration. The proposition of different models of biodegradation for each type of substance determined how the biodegradation profiles varies for each substance throughout the biodegradation process and how DGBE and PFCs may increase or decrease biodegradation rates in a set environment.

$$B=B_{max}/(1+[(B_{max}-B_o)/B_o]\exp^{-rt}) \quad (1)$$

Using the non-linear curve fitting algorithms in the cumulative production of CO_2, we were able to predict what mass percentage of mineralized substrate is expected to be obtained from microbial consumption. Fig. 7 shows the curves fitting profile.

The biodegradation profiles indicated an almost linear or exponential distribution pattern. Thus, the predictable profile allowed us to develop an adequate mathematical model. There was a shared deceleration trend in all cumulative CO_2 production after 40 days. Therefore, a non-linear logistic model was accurately adjusted to all datasets. Our strategy was to simulate as many models and iterations as possible to obtain at least 0.990 R^2 values to safely predict the maximum CO_2 amount from a given substrate. Two important parameters were obtained: maximum CO_2 of expected from biodegradation (B_{max}) and estimated time to the biodegradation to cease ($B_{max}T$). The predicted values are shown in Table 3.

The B_{max} value was established when the curve generated by the model stabilizes and no longer occurs significant an increase in

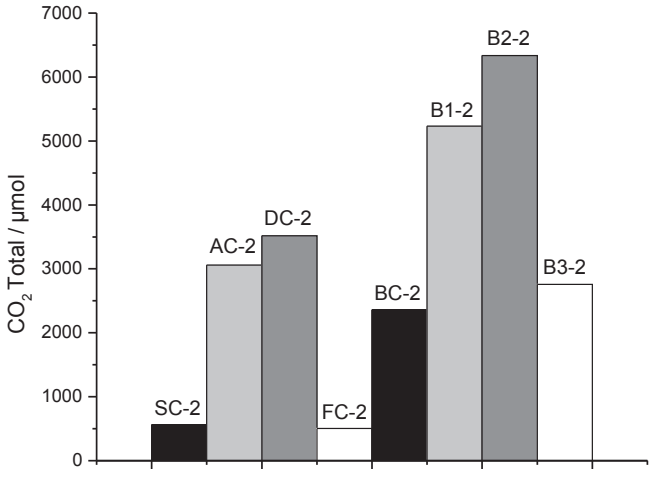


Fig. 6. Total CO_2 production in respirometric assays after 105 days in SC-2 (soil control), AC-2 (AFFF control), DC-2 (DGBE control), FC-2 (Lodyne control), BC-2 (BTEX control), B1-2 (AFFF), B2-2 (DGBE only) and B3-2 (Lodyne only).

parameter B, as defined in equation cumulative production of CO_2 over time. We defined B values < 0.99 x B_{max} as significant increase. The biodegradation cease time was defined as 0.95 x B = B_{max} (Montagnolli et al., 2009).

According to our model, a higher production of CO_2 is expected from systems containing PFCs and BTEX. The basal soil respiration from CS assays, showed that the soils organic matter accounts for 753.98 CO_2 throughout the experimental procedures. The expected B_{max} from CF assays were the lowest (470.23), confirming the impact of fluoride in soil microcosm.

The amount of time it takes for the B_{max} value to be reached ($B_{max}T$) is also important to determine microbial response to each given contamination scenario. According to the adjusted parameters, assays containing only soil or PFCs were the first ones to cease CO_2 production (Table 3). On the other hand, DBGE extended the biodegradation process, which always occurs over longer periods (up to 192.0 days in B2-2). Similar reports on substrates interfering with biodegradation length have also been reported in studies by Davis et al. (2013), LeFevre et al. (2012), Meynet et al. (2014) and Montagnolli et al. (2014).

The environmental behavior of AFFF and petroleum hydrocarbons in soil can be modeled in the laboratory using our approach.

Table 3
B_{max}, $B_{max}T$ and R^2 parameters from total CO_2 curve fit.

Assay ID and description	B_{max} (µmol CO_2)	$B_{max}T$ (days)	R^2
SC-2 (Soil control)	753.9875	80.2	0.9825
AC-2 (AFFF control)	2902.2521	132.5	0.9726
DC-2 (DGBE control)	3740.6744	173.7	0.9909
FC-2 (Lodyne control)	470.3463	98.2	0.9443
BC-2 (BTEX control)	2300.8653	129.2	0.9743
B1-2 (with AFFF + BTEX)	5050.2537	145.0	0.9846
B2-2 (DGBE only + BTEX)	6500.2654	192.0	0.9253
B3-2 (Lodyne only + BTEX).	2650.7596	110.1	0.9042

Although the studies discussed here are restricted to understanding to a lesser extent the respiration process of a microbial community in an environment with extremely controlled factors (temperature, field capacity, lighting), it was also shown that our respirometric assays were fit for the simulation of AFFF environmental behavior in co-contamination scenarios. Future scaled up experimental procedures are encouraged to enhance predictability of pollutants release and oil spills during improper disposal of perfluorinated contaminants.

The results provided by the measurement of CO_2 in respirometric assays are valuable from the environmental microbiology point of view. Although respirometric methodologies do not precisely describe byproducts formation nor identifies which microorganism are actively consuming PFC and BTEX, our models provided a reliable large-scale knowledge to fundamentally describe the behavior of pollutants release. The CO_2 production kinetics studies can be aimed towards efficiency of the biodegradation process, changing soil microflora and environmental parameters. Therefore, this is a base model to precisely predict the environmental behavior of contaminants towards higher removal ratios.

3.2.2. Microbiota inhibition by DBGE

The average chemical oxygen demand (COD) from the respirometric flasks was 5934.91 ± 1518.58 mg L^{-1}, and the pH ranged from 8.5 to 9. We found stable average values of COD in the different assays. No differences in COD removal efficiency was due to the use of different proportions of inoculum. Our results suggest 0.5% inoculum was sufficient for COD removal detection. The results show that the DBGE degradation pathways do not vary in function contaminants, unlike BTEX. The inoculum containing the DBGE assays demonstrated relatively high COD removal efficiency in the

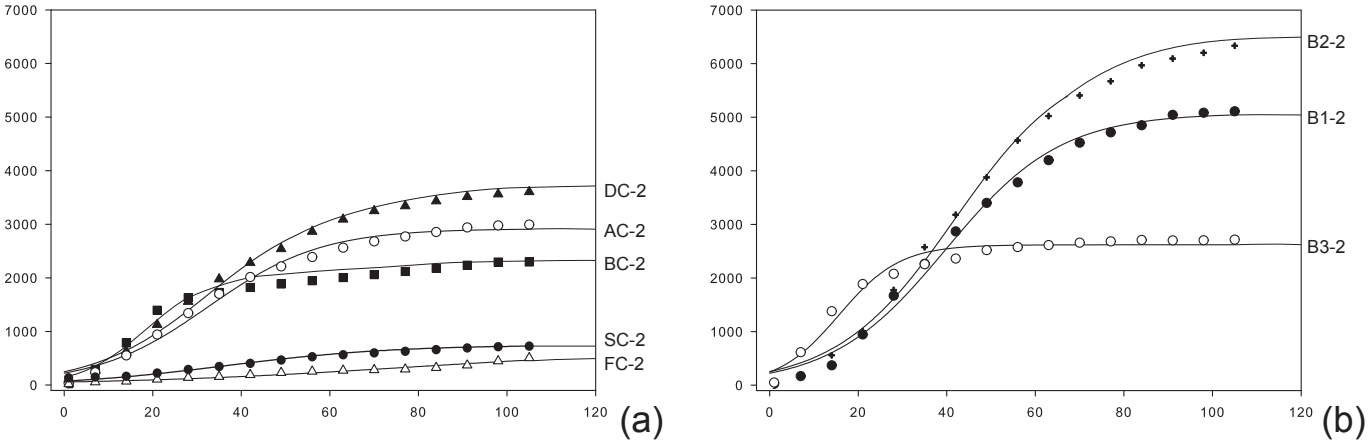


Fig. 7. Total CO_2 production curves fitted to Montagnolli et al. (2014) model in SC-2 (soil control), AC-2 (AFFF control), DC-2 (DGBE control), FC-2 (Lodyne control), BC-2 (BTEX control), B1-2 (AFFF), B2-2 (DGBE only) and B3-2 (Lodyne only).

DC-2, CA-2 and B2-2 assays in comparison to SC. The post-biodegradaton COD removal efficiencies were 67.80% and 59.44% in AC-2 and DC-2, respectively. These values were 74.5% higher than the values corresponding to the SC-2. Notably, we have achieved an efficiency of 96.92% for COD removal in B2-2 assays.

No inhibition mechanism caused by DGBE degradation was detected in this study. The COD removal rates were 152.14 $mg.L^{-1}.day^{-1}$, 161.10 $mg.L^{-1}.day^{-1}$ and 202.08 $mg.L^{-1}.day^{-1}$ in DC-2, AC-2 and B2-2 assays, respectively. This means that our DGBE adapted strains obtained by within respirometric assays showed better performance than other ether degrading bacteria reported by previous studies from other analogue esters from Blazo et al. (2012) and Young-Mo et al. (2007). Therefore, no inhibition was caused by increases in toxic metabolites produced during the degradation of DGBE that interfered in the biodegradation process.

4. Conclusions

This research suggests the discovery of a new metabolic pathway in aromatic hydrocarbons biodegradation that is directly affected by fluorinated substances. The results lead to a high sensitivity of microbial consortia due to PFCs in the AFFF formulation, therefore shifting their BTEX degradation routes in terms of intermediate products concentration. The release of AFFF compounds not only changes byproduct output but also drastically disturbs the soil microbiota according to highly variable CO_2 yields. Further studies are encouraged to survey the differential production of other possible BTEX intermediates and accurately determine the interference of individual PFCs during the biodegradation aromatic hydrocarbons.

Acknowledgements

We gratefully acknowledge the thoughtful review and important insights provided by Sarah Mae Wachlin and Brent Perumal, providing comments on the manuscript in its entirety and working with the authors as we made revisions to the text. We alone, of course, take responsibility for the final text and any errors that appear therein.

Our research group acknowledges CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CNPq (Conselho Nacional de Desenvolvimento Científico e Tecnológico), FUNDUNESP (Fundação para o Desenvolvimento da UNESP), PRH-ANP/MCT (Programa de Formação de Recursos Humanos em Geologia do Petróleo e Ciências Ambientais Aplicadas ao Setor de Petróleo e Gás) and UNESP (Universidade Estadual Paulista “Julio de Mesquita Filho”) for the financial support.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.chemosphere.2016.12.144.

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