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EFFECT OF THE WATER TYPE, THE INOCULUM AND THE CONCENTRATION ON THE PHENANTHRENE DEGRADATION IN A FLUIDIZED BED REACTOR USING ACTIVATED CHARCOAL AS A BACTERIAL SUPPORT

EFECTO DEL TIPO DE AGUA, EL INÓCULO Y LA CONCENTRACIÓN SOBRE LA DEGRADACIÓN DE FENANTRENO EN UN REACTOR DE LECHO FLUIDIZADO EMPLEANDO CARBON ACTIVADO COMO SOPORTE BACTERIANO

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Abstract

The phenanthrene degradation and microbial growth at short times (t ≤ 16 days) using a fluidized bed reactor (FBR) with activated charcoal (AC) as support for the bacterial consortium in different seawater types (sterile and non-sterile) and inoculum additions were studied. The effect of the reactor operating conditions (sterile and nonsterile seawater, addition of inoculum, phenanthrene concentration of 112 mg L^{-1}, and air flow of 1.5 L min^{-1}) and variations in phenanthrene concentration (60, 200, and 600 mg L^{-1} at an air flow of 2 L min^{-1}) were evaluated. Phenanthrene degradation at the maximum time was improved when an inoculum was added to the seawater (62.5%?69.5% of the initial concentration was degraded); the best microbial growth was observed when an inoculum was added to sterile seawater (constant concentration) and when phenanthrene concentration was 200 mg L^{-1}; however, the highest degradation rate values (mg CFU^{-1}) were obtained for a phenanthrene concentration of 600 mg L^{-1}. Operating conditions and phenanthrene concentration had significant impacts on phenanthrene degradation and microbial growth ($P < 0.05$). The findings show that the FBR efficiency in phenanthrene degradation responds to multifactorial operating conditions; however, high efficiencies can be reached if the operating conditions, including inoculum addition, are carefully controlled.

Keywords: phenanthrene degradation, microbial growth, fluidized bed reactor, inoculum, operating conditions.

Resumen

El estudio evaluó la degradación de fenantreno y el crecimiento microbiano a tiempos cortos (t ≤ 16 días) en un reactor de lecho fluidizado (FBR) con carbón activado (AC) como soporte bacteriano para diferentes condiciones de operación (agua de mar estéril y no estéril, adición de inóculo, concentración de fenantreno de 112 mg L^{-1} y flujo de aire de 1.5 L min^{-1}) y variaciones en la concentración (60, 200 y 600 mg L^{-1} de fenantreno a un flujo de aire de 2 L min^{-1}). La degradación del fenantreno al tiempo máximo incrementó al añadirse inóculo al agua de mar (de 62.5% a 69.5%); el inóculo favoreció el crecimiento microbiano en agua de mar estéril (concentración constante) especialmente cuando la concentración de fenantreno fue de 200 mg L^{-1}. La tasa de degradación más alta (mg CFU^{-1}) se observó para una concentración de fenantreno de 600 mg L^{-1}. Las condiciones de operación y la concentración tuvieron un impacto significativo en el proceso de degradación y el crecimiento microbiano ($P < 0.05$). Los resultados muestran que la eficiencia del FBR en la degradación del fenantreno responde a condiciones de operación multifactoriales, pero se pueden alcanzar altas eficiencias si las condiciones operativas, incluida la adición de inóculo, se controlan cuidadosamente.

Palabras clave: degradación de fenantreno, crecimiento microbiano, reactor de lecho fluidizado, inoculo, condiciones de operación.

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1 Introduction

The importance of the petrochemical industry worldwide is undeniable. In recent years, there has been developed in various productive sectors. In the maritime sector, there has been an increase in the movement of cargo and cruise ships, as well as the activity on oil rigs. However, such an increase in activity has led to spills and leaks of petroleum-derived hydrocarbons, such as total petroleum hydrocarbons (TPH), diesel and gasoline fractions, benzene, toluene, ethylbenzene, xylene (BTEX), and polycyclic hydrocarbon aromatics (PAHs) (Canul 2014). Due to this, marine oil pollution is related to ships or vessels accidents during their operation, the shipwreck, and the incidents at the oil exploration well or the illegal bilge discharges of water; additionally the physical and chemical properties of oil spill and oil slick affect significantly marine life, natural structure of ocean or water source, tourism and entertainment activities (Hoang *et al*. 2018).

The oil pollution directly impacts human health and biodiversity, and its harmful effects include interference with cellular processes, food seeking, and carcinogenesis (Carrillo 2009).

In Mexico, there are remnants of a significant oil spill that occurred in 1979, when more than one million barrels were left in the ocean (Botello *et al*. 2007); by 2010, 1518 oil spills had been recorded in Mexico as a result of the increased marine oil activity (SEMARNAT & INECC 2012).

Of the oil derivatives, polycyclic aromatic hydrocarbons (PAHs) are a group of high-priority environmental pollutants of the soil and sediment, due to their toxic, mutagenic, and carcinogenic effects. They are ubiquitous compounds and originate from natural and anthropogenic pyrolysis of organic material from forest fires, automobile exhaust, coal refining practices, the oil industry, etc. (Hughes *et al*. 1997). The Environmental Protection Agency in North America (EPA) considers 16 PAHs to be of high importance, which include phenanthrene, benzofluoranthene, and benzo (?) anthracene (Wong *et al*. 2004; Chavez *et al*. 2005). Among PAHs, phenanthrene is often studied by researchers because it both contains K regions and bay regions, which are considered as the basic carcinogenic and mutagenic molecular structure in most of the high molecular weight PAHs (Rodriguez *et al*. 2017).

Phenanthrene is a three-ring PAH found as a pollutant in the soil, estuarine waters, sediments, and other terrestrial and aquatic sites. This compound is normally utilized as a model compound in PAH biodegradation studies (Tang *et al*. 2005; Lors *et al*. 2010).

Because phenanthrene is lipid-soluble, when it is ingested it is easily absorbed by the human body. During metabolism, phenanthrene breaks down into epoxy compounds that are highly mutagenic and carcinogenic, affecting the lungs, liver, and skin (Tian *et al*. 2002).

In order to minimize the effects of PAHs in the environment, including phenanthrene, several technologies have been developed during the last few decades, including physicochemical and biological methods, with biological processes using microbial consortia being used the most. These methods are promising for the bioremediation of PAHs and are environmentally friendly (Janbanghu & Fulekar 2011).

Most research has focused on degradation efficiency. Among the studied variables, the degradation of mixtures of different PAHs or the degradation of specific PAHs, as well as the effect of inoculation on phenanthrene degradation in batch reactors, has been reported (García & Peralta 2008; Madueño *et al*. 2009). Phenanthrene degradation at short times has been demonstrated (Acosta-Rubí *et al*. 2017; Agrawal *et al*. 2018). Recent studies have shown that phenanthrene can be successfully degraded by a bacterial consortium using several strains including *Sphingobacterium* sp., *Bacillus cereus*, *Achromobacter insolitus* MHF ENV IV (Janbanghu & Fulekar 2011), *Marinobacter hydrocarbonoclasticus*, *Roseovarius pacificus*, *Pseudidio marina sediminum* (Moghadan *et al*. 2014), *Corynebacterium urealyticum* (Abdul-Talib *et al*. 2015), *Pseudomonas putita* (Pantsyrnaya *et al*. 2011), *Sphingobium* sp. (Cai *et al*. 2017) and *Bacillus* sp.(Liu *et al*. 2019). Some studies report phenanthrene degradation at short times; Gao *et al*. (2013) reported a complete phenanthrene degradation using *Stenotrophomonas maltophilia* strain C6 at 14 days. Similarly, Acosta-Rubí *et al*. (2017) (15 days) and Agrawal *et al*. (2018) (14 days) reported phenanthrene degradation using inoculum and *Ganoderma lucidum* to degrade phenanthrene under different conditions. Additionally, utilizing support materials where microorganisms can grow also influences degradation, and different studies have shown that organic compounds may adhere to organic colloids in marine waters, groundwater, and soil (Chin

& Gschwend 1992; Shimizu *et al*. 1998; Villholth 1999).

Bioreactors are widely used to treat various contaminants including PAHs. The efficiency of PAHs treatment using fixed-bed reactors (Tziotzios *et al*. 2007) and fluidized bed reactors (FBR) (Quintero & Cardona 2011) has been tested. The support materials used in bioreactors play an important role. Among the most used are perlite and sepiolite (Arnaiz *et al*. 2006), silica gel (Martínez & García 2012), activated charcoal (Moreno *et al*. 2007, Flores *et al*. 2016), and kaolinite and quartz (Gong *et al*. 2018). However, although support materials are important, many other variables should be considered; metabolic activity, contaminant concentration in the environment, and metabolites that produce degradation (García & Peralta 2008; Rosales 2008), temperature and pH (Liu *et al*. 2018) are important to account for in the design of bioreactors to determine the appropriate conditions for microbial activity. Additionally, the inoculum effect, water type and the effect of the interaction of these factors during phenanthrene degradation have not been studied deeply.

Based on the FBR widely used, the question is, what effect will have the operating conditions (type of water and inoculum effect) and high phenanthrene concentrations have on the treatment of water containing phenanthrene using an FBR as a realistic approach in the phenanthrene decontamination? To address this question, the aim of this research was to evaluate a phenanthrene treatment system consisting of an FBR using activated charcoal (one of the most used materials) as support for degrading microorganisms in different water types. The degrading capacity of a mixed microbial consortium adapted to phenanthrene as the only source of carbon under the influence of native microorganisms of seawater as well as the effect of the variation in phenanthrene concentration on microbial growth and degradation were evaluated.

2 Materials and methods

2.1 Chemicals, seawater, and materials used

2.1.1 Phenanthrene

Phenanthrene at 98% purity (Sigma Aldrich®) with a molecular weight of 178.23 g MoL^{-1} and a density of 1,063 mg mL^{-1} at 25°C according to the manufacturer was used in this investigation.

2.1.2 Seawater

A total 56 L of seawater was used for this research; the water was collected at the dock area of the port of Telchac, Yucatan, Mexico using amber glass bottles with a capacity of 4 L. The bottles were sealed with parafilm paper and placed in an ice box for transfer to the laboratory. The seawater was passed through a filter (Whatman GFC 47 mm) to remove sand and impurities. After filtration, the seawater (14 L) was sterilized at 121°C for 15 min in an autoclave to remove the microorganisms present in the sample. Seawater was characterized physicochemically (Table 1) by measuring the parameters of dissolved oxygen (OD), pH, conductivity, temperature, and salinity using the multi-parameter device HIDROLAB model Quanta G (Gutiérrez 2014). Additionally, the total organic carbon (TOC) was determined by the method APHA 5310 (APHA 2005a). Biochemical oxygen demand (BOD5), chloride, sulfate, calcium, potassium, magnesium, and sodium were determined according to the Mexican standard NMX-AA-028-SCFI -2010 (SE 2011). Total Kjeldahl nitrogen and total sulfates were determined by methods 4500-N_{org} and 4500-SO_4^{2-}, respectively (APHA 2005b, c). Finally, the seawater was stored at 4°C for later use in the filling of the reactors.

2.1.3 Activated charcoal

The support medium for bacteria growth used in the reactor was a 4-mm microporous charcoal pellet because of its ideal characteristics for microbial support (Fernández 2014). The charcoal was activated in order to increase its porosity, achieve greater contact area, and favor the growth of bacteria. For this purpose, the chemical method was used as follows: 100 g of pellet-type charcoal was weighed and a series of washes were carried out, firstly with tap water and then with distilled water until the water was colorless. The charcoal was then treated with 300 mL of concentrated HCl and maintained under stirring conditions for 24 hours. Then, it was washed with distilled water and placed in an oven at a temperature of 110°C for 8 h and then in porcelain capsules in the muffle at a temperature of 350°C for 2 h.

Table 1. Physicochemical parameters of seawater.

Physicochemical parameters							
Temperature (°C)	Conductivity (μS cm^{-1})	TDO[a] (mg L^{-1})	pH	Salinity g L^{-1} (PSU*)	BOD5 (mg L^{-1})	TOC[b] (mg L^{-1})	TKN[c] (mg L^{-1})
23.4	54000	7.41	8.02	37.02	3.7	22.12	0.32

Chloride (mg L^{-1})	Sulfate (mg L^{-1})	Total phosphorus (mg L^{-1})	Calcium (mg L^{-1})	Potassium (mg L^{-1})	Magnesium sodium (mg L^{-1})	Sodium (mg L^{-1})
22057	2775	0.3	458.03	4 23.30	1355.91	11574.63

*Practical salinity units. [a]Total dissolved oxygen; [b]Total organic carbon; [c]Total kjeldahl nitrogen

2.1.4 *Inoculum*

The inoculum used consisted of a bacterial consortium containing the species *Nocardioides lentus*, *Bacillus pumilus*, *Bacillus* sp YXA2-9, and *Halomonas* sp N34. These bacterial strains were previously isolated from seawater; they were available in the Environmental Engineering Laboratory of the Engineering Faculty of the Autonomous University of Yucatan; this consortium was selected because it was previously used in phenanthrene degradation tests and proved its capacity to grow in presences of phenanthrene in miniaturized reactors. For their application, the strains were again isolated using nutrient medium tripticasein soybean agar (TSA). The strains were subjected to a growth process in 250 mL of nutrient broth for three days. Subsequently, the process of enrichment and generation of the mixed consortium began. This process consisted of pouring 50 mL of nutrient broth containing each of the strains and 25 mg of phenanthrene into a flask of 500 mL of capacity. The flask was filled to a volume of 300 mL by adding 250 mL of sterile synthetic sea water (24.5 g of NaCl + 11.1 g of $MgCl_2$ + 4.1 g of Na_2SO_4 + 1.54 g of $CaCl_2$ + 0.7 g of KCl + 200 mg of $NaHCO_3$ + 100 mg of KBr + 30 mg of H_3BO_4 + 20 mg of $SrCl_2$ + 3 mg of NaF) to obtain a final concentration of 100 mg L^{-1} phenanthrene The enrichment process was developed over three weeks by repeating the enrichment process weekly until its application to the reactors.

2.2 *Fluidized bed reactor design*

The reactors used (at laboratory level) consisted of an acrylic cylinder 10.7 cm (4 inches) in diameter and 95.8 cm (37.7 inches) in height, containing a concentric glass tube 5.1 cm (2 inches) in diameter by 79.3 cm (31.5 inches) in height and an aeration flow of 1.5 L min^{-1} for the first stage of the experiment and 2 L min^{-1} for the second stage.

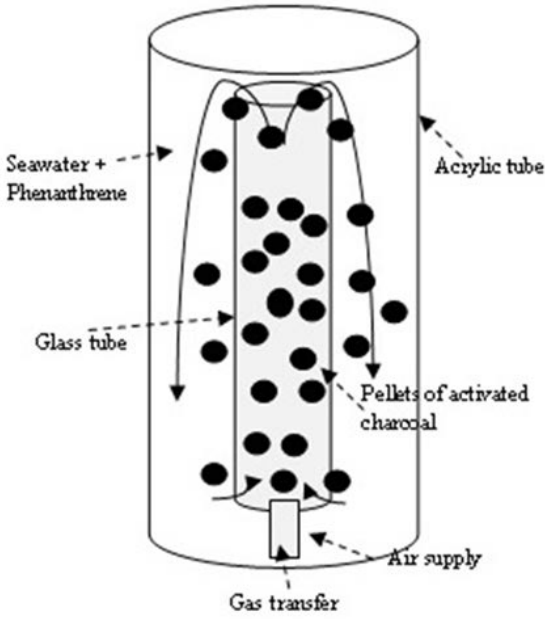

Fig. 1 Operating model of the fluidized bed reactor.

The flow rates were determined as a function of the optimum rate at which the activated charcoal pellets remained in suspension. The total volume of the reactors was 7 L. The transfer of gases to the reactors was performed by the lower base by means of air diffusers at the aforementioned flow rate. Oxygen transfer tests by the transient regime were performed in each reactor using the APHA method 4500-O (APHA 2005d). The design of the reactor is shown in Fig 1.

2.3 *Phenanthrene degradation process (PDP) and microbial growth in the FBR*

Phenanthrene degradation in FBR and the microbial concentration therein were determined in two stages at short times (t ≤ 16 days). The effect of operating

conditions on the phenanthrene degradation process is poorly understood. For this reason, this study focused on FBR operating conditions and their effect on phenanthrene degradation. The first stage was developed to determine the effect of the inoculum and the conditions of operation using sterile or non-sterile sea water on the degradation of a single concentration of phenanthrene. The second stage was developed to determine the effect of the variation of phenanthrene concentration on degradation thereof and microbial concentration.

2.3.1 Effect of operating conditions of FBR and inoculum on PDP

Three experiments were carried out: one using nonsterile seawater without inoculum (NSSWOI), a second with sterile seawater with the inoculum (SSWI), and one last experiment using non-sterile seawater with the inoculum (NSSWI). Additionally, one experiment using a reactor with sterile seawater and 800 mg of phenanthrene previously diluted by mechanical agitation (final concentration of 112 mg L^{-1}) and 100 g of activated charcoal without inoculum as a control was implemented to indicate phenanthrene volatilization and possible microorganism growth by contamination.

For the NSSWOI experiment, 100 g of activated charcoal was used, which was contaminated with 800 mg of phenanthrene (previously dissolved in 5 mL of acetone) in a volume of 500 mL of seawater, as appropriate for the experiment. The mixture was stirred for 12 h at 150 rpm; the acetone used was evaporated by a filter air pump to maintain sterile conditions. Subsequently, the mixture of activated charcoal, phenanthrene, and seawater was completely poured into the reactors. For the SSWI and NSSWI experiments, the activated charcoal (100 g) was combined with 500 mL of the bacterial (inoculum) consortium by shaking at 150 rpm for 24 hours. Then, contamination with 800 mg of phenanthrene via 12 hours of agitation before inoculating the reactor was performed. For all cases, each reactor was filled to 7 L with seawater, as appropriate for each experiment. The final estimated concentration of phenanthrene for each reactor was 112 mg L^{-1}. For all cases, sterile air filters with 0.2 μm pore size were placed to avoid contamination by bacteria from the outside environment.

2.3.2 Effect of concentration on PDP

Three experiments were performed, one for each concentration. The final concentrations of phenanthrene in the reactors used for this stage were 60, 200, and 600 mg L^{-1} in sterile seawater. For the first stage, 100 g of activated charcoal was used, which was contaminated with phenanthrene (429 mg, 1429 mg and 4286 mg previously dissolved in 5 mL of acetone) according to the final concentration required in the reactor (60, 200, and 600 mg L^{-1}) in a volume of 500 mL of sterile seawater (two parts to complete 1 L). The acetone used was evaporated by a filter air pump to maintain sterile conditions. This mixture was stirred for two hours at 150 rpm. The mixture of activated charcoal, phenanthrene, and seawater was completely poured into the reactors, and 1 L of the inoculum was added. The final volume of each reactor was 7 L. As in the first stage, sterile air filters with a 0.2 μm pore size were placed to prevent bacterial contamination from the outside environment.

An additional reactor consisting of sterile seawater, charcoal, and phenanthrene (similar to the first stage) at the concentration used in each experiment was implemented at this stage to determine the percentage of phenanthrene lost in the absence of the inoculum, which could be interpreted as volatilized phenanthrene. For both experiments, the purity of the phenanthrene (98%) was considered to determine the quantity to apply and reach the final concentration in the reactors.

2.4 Sampling and analyses

2.4.1 Sampling

To determine the amount of phenanthrene in the charcoal and liquid in both stages of the experiment, 20 mL of the liquid was collected from each reactor and passed through filter paper (Whatman GFC 47 mm). The collected liquid was subjected to liquid-liquid extraction to determine the phenanthrene remnants in the reactor water. Similarly, by decantation of a known volume of collected water (100 mL, returning the water to the reactor after decantation), approximately 250 mg of charcoal was obtained. The filter paper and charcoal were subjected to solid-liquid extraction to determine the phenanthrene contained in the material. These procedures were performed in triplicate. In all cases and for all sampling times, the pH of the water was determined. The total concentration in reactors was determined by the sum of the phenanthrene measured

in charcoal and liquid.

2.4.2 *Growth in plaque and bacterial count*

At each sampling, a 1 ml aliquot was taken and placed in an isotonic solution to determine growth in the liquid phase of the reactor. Dilutions were performed; 100 μl of this solution was poured into Petri dishes with TSA culture medium, and the sample was homogeneously expanded using a sterile glass loop. This procedure was performed in triplicate for each sampling while maintaining the sterile conditions.

For the mobile phase in the reactor, 250 mg of the charcoal in each sample was taken and placed in an isotonic solution. It was shaken in a vortex for 3 minutes and 1 mL was used to make dilutions. Then, 100 μl of each dilution was poured into Petri dishes with TSA agar and the same procedure as described above was followed. Finally, the samples were incubated at 37°C and counted at 48 hours to determine the colony forming units (CFU).

2.5 *Phenanthrene residues determination*

2.5.1 *Liquid-liquid extraction*

For this procedure, analytical grade dichloromethane was used as a selective solvent. As previously stated, 20 mL of water from each reactor was collected and filtered using filter paper (Whatman GFC 47 mm). The filtrate was placed in 250 mL separatory funnels. Subsequently, 15 mL of dichloromethane was added to the filtrate in 3 steps (5 mL per step). The funnel was stirred for 2 min. After 10 min, the organic phase was collected in 50 ml flasks. The extract was evaporated in rote-vapor, leaving the phenanthrene in the flask. The sample was reconstituted with 1 ml of analytical grade acetonitrile, placed in glass vials, and stored at 4°C for further chromatographic analysis. The extraction efficiency was 81.25 ± 6.62%.

2.5.2 *Solid-liquid extraction*

After filtration and decantation of the water, the charcoal and filter paper were placed inside Teflon tubes and 25 mL of hexane-acetone solution (1:1, V/V) was added to each tube. Microwave-assisted extraction was performed using the EPA method 3546 (EPA 2000). For this purpose, a temperature ramp starting at 80°C maintained for 10 min was used, followed by an increase up to 115°C maintained for 15 min. After extraction, samples were filtered using glass wool previously washed in acetone and placed in 50 mL flasks. The samples were rotary evaporated and reconstituted in 1 mL of analytical grade acetonitrile. Finally, they were placed in glass vials and stored at 4°C for chromatographic analysis. The extraction efficiency was 94.43 ± 13.62%.

2.5.3 *Chromatographic method*

The phenanthrene residues in both stages of research were performed using a gas chromatograph Trace 1300 GC Ultra (Thermo Scientific®). A capillary column Perkin Elmer® Elite-17, 30 m × 0.25 mm × 0.25 μm using BIP helium as carrier gas at a constant pressure of 100 kPa was used. For the analysis, an injection temperature of 250°C with the detector and the transfer line at 270°C were used. The ramp used in the furnace was at an initial temperature of 50°C for 1 minute, with temperature increases of 15°C min^{-1} to 225°C. The furnace temperature was brought to 300°C with an increase of 30°C min^{-1}; a temperature of 300°C was held for 1 minute. Finally, 1 μl of the sample was injected in a splitless mode using helium as the carrier gas with a flow of 1 mL min^{-1}. The limits of quantification (LC) and detection (LD) were 0.03 and 0.02 mg L^{-1}, respectively.

2.6 *Data analyses*

For the study and analysis of phenanthrene degradation, the first-order kinetic model (Eq. 1) was used and the half-life (Eq. 2) was determined for both stages of the experiment.

$$C = C_0 e^{-kt} \tag{1}$$

$$DT_{50} = \ln(2)/k \tag{2}$$

C_0= Initial concentration of phenanthrene (mg L^{-1})
k= dissipation rate (day^{-1})

Multifactorial analyses of variance (ANOVA) at 95% confidence were performed in order to determine the significance of the time, operating conditions, and concentration on the degradation of phenanthrene in the reactors and the microbial growth. A multiple rank test at 95% confidence according to the least significant differences (LSD) was performed to determine homogeneous groups. Principal components analysis (PC) was developed in order to know the relationship of factors involved in the operating conditions of the reactors on the microbial growth and the remaining phenanthrene. Additionally, an analysis of variance component (AVC) at 95% confidence was performed to determine

the contribution of each factor to the variability of phenanthrene concentration and microbial growth in the reactors. The statistical program Statgraphics Centurion® XVII was used.

3 Results and discussions

3.1 *Effect of operating conditions of the FBR and inoculum on phenanthrene degradation*

As shown in Fig. 2a, 2b, and 2c, the phenanthrene was not completely degraded in the different experimental set-ups, but a greater degradation of phenanthrene in the reactors where the inoculum was added was observed. Fig. 2c illustrates that the best degradation was recorded in the reactor using nonsterile seawater with the inoculum (NSSWI) (69.5% of the initial concentration was degraded); this can be attributed to the positive interaction of seawater microorganisms with the inoculum. Liu *et al*. (2017) report that microorganisms such as bacteria whit high efficiency for PAHs and heavy metals bioremediation will compete with aborigines organisms and weaken the ability to reduce pollution. Thus, finding out an appropriate way to increase the competitiveness and aggrandize activity of the screened strains, in reality, is very essential. In this research, the fact that inoculum used consisted of bacteria isolated from marine environment seems to reduce the competition between autochthonous organisms in seawater and the strains and results support that. The interaction between microorganisms in the biodegradation process which result in the combined potential of the consortia to produce catabolic enzyme activities have been reported before (Mikesková *et al*. 2012). Although this was not studied in this research can?t be ruled out.

For the control reactor, it was observed that 22.5% of the initial amount of phenanthrene was lost at 16 days without a significant difference with a previous time of 12 days. However, this cannot be attributed to a biodegradation process, since no microbial growth was recorded at any sampling time; it is important to highlight that 31.8% and 45.7% of the initial concentration of phenanthrene in this reactor stayed adsorbed on the activated charcoal and dissolved in water respectively. This loss can be attributed to volatilization and chemical degradation. Irwin (1997) reported that phenanthrene can volatilize up to 50% of its concentration in water within 9.4 to 13.5 hours under normal environmental conditions. For this experiment, the lower volatility observed can be attributed to a significant fraction of phenanthrene adsorbed on the activated charcoal, limiting its availability, as well as the operating conditions of the reactors. García-Uitz *et al*. (2016) reported losses due to volatilization and chemical degradation of phenanthrene of up to 11%, which are lower than the losses reported in this experiment; this difference can be attributed to the fact that they did not use aeration, and the experiments were carried out on a smaller scale.

During the NSSWOI experiment, a reduction of 37% of the initial concentration of phenanthrene was observed at the end of the 14 days of sampling. Part of this reduction can be attributed to degradation by indigenous organisms, and this is supported by the growth of bacterial colonies observed in the reactor, specifically in the charcoal used (Fig. 2a). The maximum bacterial growth was reached at day 11 (2.2E5 CFU mL^{-1}), but after this time growth decreased significantly; this may be due to the production of intermediary molecules as a result of phenanthrene biodegradation, which may inhibit population growth (Hongwei *et al*. 2002). Taking into account the dissipation attributable to the volatilization or chemical degradation obtained in the control experiment (22.5%), it can be deduced that 14.5% of the phenanthrene dissipated in this experiment was degraded biologically.

The phenanthrene concentration reduction reached during the SSWI was 85%; considering that 22.5% was due to volatilization, it can be inferred that 62.5% was degraded by microorganisms, and this is attributable to the inoculum supplied to the reactor. Tam *et al*. (2002) reported 40% phenanthrene degradation after six days in inoculated synthetic seawater; this is comparable to the 49% degradation that has been observed after six days in this experiment under similar conditions, which supports the degrading action of the inoculum. Because of dilution when applying sterile seawater in the reactor, the initial concentration of microorganisms was observed to be lower in this experiment than in the experiment with nonsterile seawater. However, the highest microbial growth was observed for the SSWI. The maximum microorganism growth was observed on the sixth day, a result similar to the seven days reported as the time to reach maximum microorganism growth by García-Uitz *et al*. (2016) using phenanthrene as the sole carbon source in synthetic seawater at microcosms level.

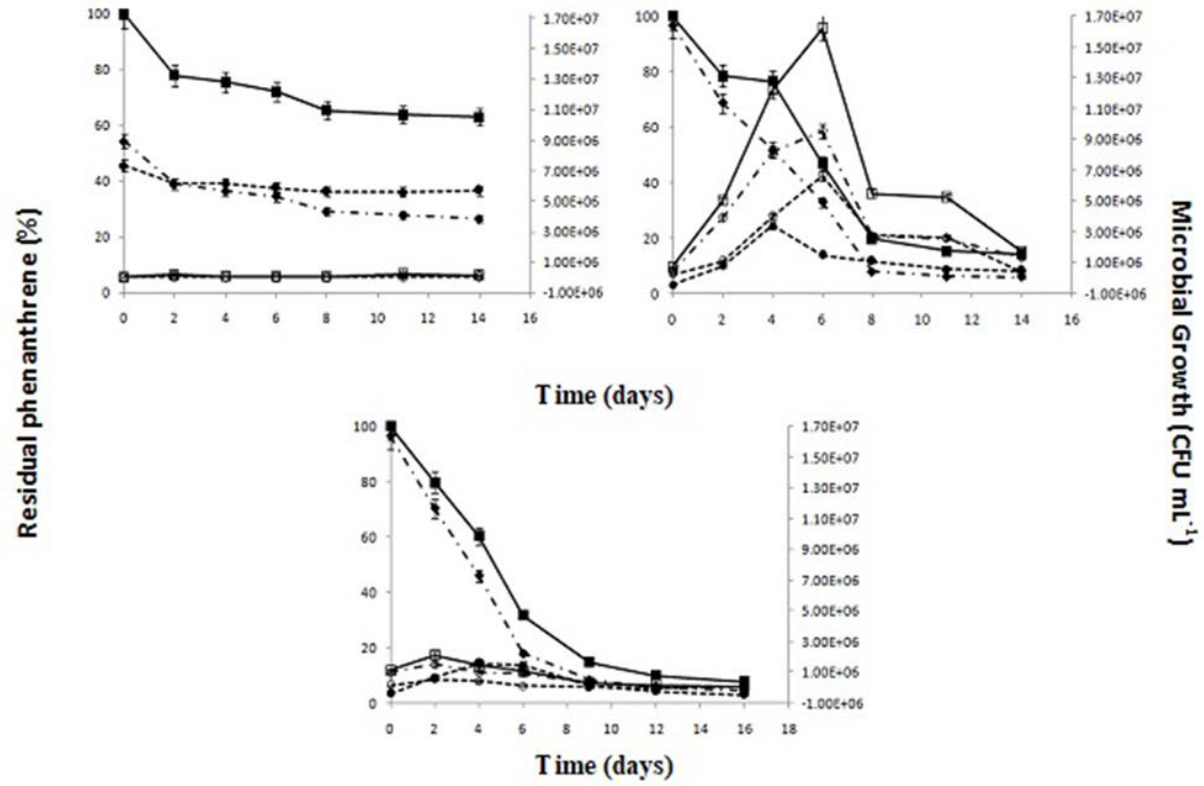

Fig. 2 Degrading kinetics of phenanthrene and growth kinetics of microorganisms in different FBR. (a) Nonsterile seawater without inoculum (NSSWOI); (b) Sterile seawater with inoculum (SSWI); (c) Nonsterile seawater with inoculum (NSSWI). −■− Total phenanthrene in the reactor; −−●−− phenanthrene in liquid phase; − − ♦ − − phenanthrene recovered from charcoal; −□− CFU mL^{-1} in the reactor; − − o − −CFU mL^{-1} in liquid phase; − − ◊ − −CFU mL^{-1} in the charcoal.

It is noteworthy that in this experiment, the greatest amount of phenanthrene was found in charcoal and not in seawater (Fig. 2b); this can be attributed to the adsorption of phenanthrene on the surface of the charcoal boosted by the presence of a biofilm product of the pretreatment with strains and phenanthrene. It is well known that activated charcoal is also a good support media for microbial growth. Thus, biologically activated charcoal with attached biomass can effectively remove organic pollutants both by adsorption and biodegradation (Xing *et al*., 2008).

The maximum phenanthrene dissipation was observed in the final experiment (NSSWI), with values of 90% and 92% dissipation recorded after 12 and 16 days, respectively. Considering the volatilized percentage, 69.5% represents the maximum degradation of phenanthrene due to a positive interaction of the native bacteria of the seawater and the consortium of pure strains; this is slightly low in comparison with a phenanthrene degradation of 75-100% using bacterial consortia as inoculum under different conditions reported by previous researchers (Nasrollahzadeh *et al*. 2010; Janbanghu & Fulekar 2011; Pinyakong *et al*. 2012; Fernández 2014; Umar *et al*. 2017) and quite similar to reported by Hernández-Martínez *et al*. (2019) in degradation of crude oil using biomass (~70% of degradation) at 14 days; however, volatilization was not considered and no report about microorganisms identification was presented. Fig. 2c shows that the maximum bacterial growth occurred on the second day, after which the concentration of microorganisms decreased. This can be explained by the fact that the microorganisms rapidly degrade phenanthrene, which is their only source of energy, and decreasing the amount of available phenanthrene leads to increased mortality of the microorganisms. Other authors have reported the complete degradation of phenanthrene in the liquid medium by using methods of immobilization of the microorganisms to natural or

Table 2. Adjusted parameters for first-order kinetic model.

Experiment	Parameters				
	Co (mg L^{-1})	**k ($days^{-1}$)**	**DT_{50} (days)**	**R^2**	**SEE**
Nonsterile seawater without inoculum (NSSWOI)	99.83	0.03	23.11	74.89	7.13
Sterile seawater with inoculum (SSWI)	110.63	0.15	4.62	90.97	11.09
Nonsterile seawater with inoculum (NSSWI)	108.36	0.18	3.85	96.9	6.7
Sterile seawater with inoculum (60 mg L^{-1})	63.47	0.06	11.55	90.6	4.37
Sterile seawater with inoculum (200 mg L^{-1})	213.64	0.07	9.9	93.23	13.26
Sterile seawater with inoculum (600 mg L^{-1})	583.3	0.04	17.32	95.2	20.56

Co: Initial concentration of phenanthrene; k: dissipation rate; DT_{50}: phenanthrene half life; R^2: square correlation coefficient; SEE: standard error of estimation

artificial supports (Xue-Qin *et al*. 2009, 2010). However, these authors do not consider the fraction of phenanthrene that can be volatilized. It is important to note that the microbial growth represented in CFU mL^{-1} was higher in the SSWI experiment, but the best phenanthrene dissipation was observed in the NSSWI experiment. This can be explained by the fact that colonies observed in the later experiment were bigger than in the other two experiments, which means that colonies were denser, and more microorganisms were involved in the degradation process.

The ANOVA showed that both the time and the operating conditions (type of water used with/without inoculum) had a significant effect at 95% confidence ($P < 0.05$) on the dissipation of phenanthrene, with time being the most significant [TIME ($F = 6.78$, $P = 0.0044$), OPERATING CONDITIONS ($F = 4.80$, $P = 0.0381$)]. The multiple rank test showed two homogeneous groups, one formed by the SSWI and NSSWI experiments and another one by NSSWOI. It is also evident that the minor phenanthrene remnants coincide with those reported in the first group, which corresponds to both reactors inoculated. Finally, the analysis of variance components showed that the time and the operating conditions contribute to 62.09% and 37.91%, respectively, of the phenanthrene remnant variation, which supports the greater significance of the time and the fact that operating conditions are also important.

The multifactor analysis (ANOVA) at 95% confidence showed that the reactor operating conditions (which ones) had a significant effect on microbial growth ($P < 0.05$), but time did not. The multiple rank analysis showed two groups with respect to operating conditions, one group made up of the NSSWOI and NSSWI experiments and another one with the SSWI experiment, the latter having the highest significant microbial growth. The analysis of variance components showed that the operating conditions contribute to 51.55% and the time to 48.45% of the variation in the microbial growth.

The DT_{50} values (Table 2) support the fact that the addition of the inoculum accelerates phenanthrene dissipation. An important aspect was that the reactors were operated under pH conditions from 6.4 to 8; the lowest pH was recorded in the NSSWOI experiment (mean= 6.96) and the highest in SSWI (mean= 7.8), which could influence the dissipation of phenanthrene. According to PC analysis (Fig. 3), pH has a regular positive relationship with the microbial growing (CFU) and good negative relationship with the remaining of phenanthrene (%RP) (because of the cousin of the angle between the two vectors), this explains why the highest CFU observed was presented in SSWI, and the lowest phenanthrene remainings were observed in NSSWI, this last means that inoculation plays an important role in phenanthrene degradation in the absence of autochthonous microorganisms. Umar *et al*. (2017) established that 6.5 is the optimum pH for phenanthrene degradation using *Cronobacter sakazakii*, and a greater value inhibits bacterial growth, while lower values favor the hydroxyl radicals? production that de-activated the PAHs degrading enzymes. This translates into a decrease in the degradation efficiency. Although for this research *Cronobacter sakazakii* was not present in the inoculum, PC analysis support that increments (between 6.4 - 8) of pH favor phenanthrene degradation, which implies that according to the microorganism type present, the pH can affect the phenanthrene degradation in different ways. PC analysis shows that time and CFU have a good negative relationship with %RP; this means that

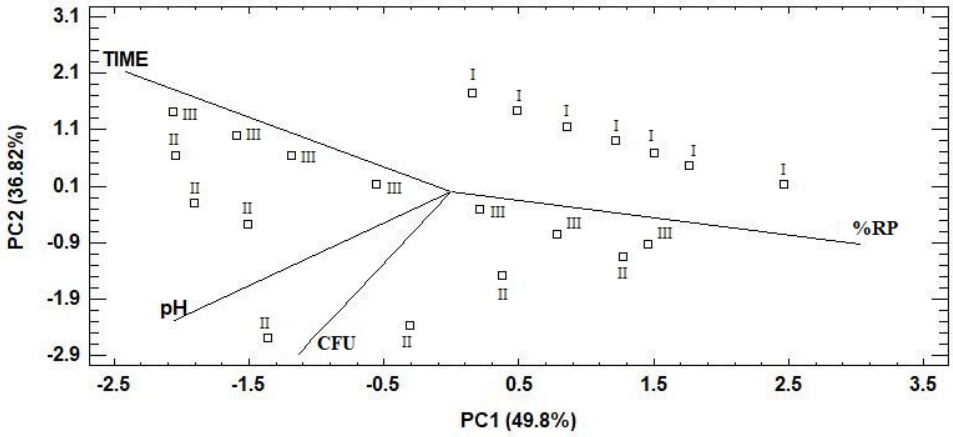

Fig. 3. Bi-graphic of component weight and relationship of remaining phenanthrene and pH, TIME and colony forming units (CFU). I: no sterile seawater without inoculum (NSSWOI); II: sterile seawater with inoculum (SSWI); III: no sterile seawater with inoculum (NSSWI).

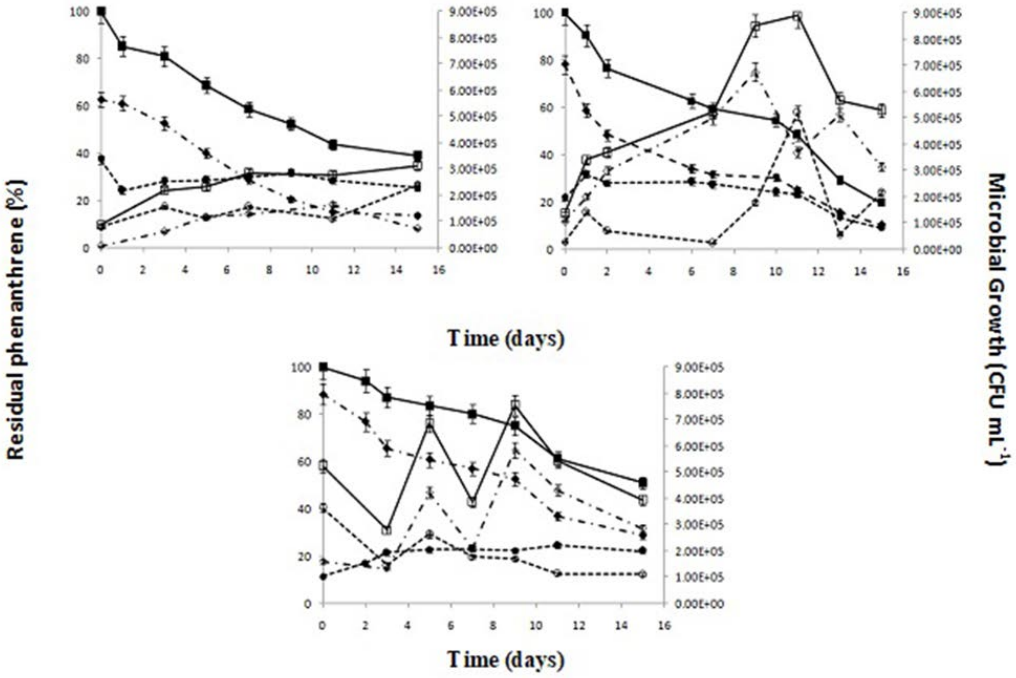

Fig. 4. Degrading kinetics of phenanthrene and growth kinetics of microorganisms in different FBR. (a) 60 mg L^{-1}; (b) 200 mg L^{-1}; (c) 600 mg L^{-1}. –■– Total phenanthrene in the reactor; ––●–– phenanthrene in liquid phase; – – ♦ – – phenanthrene recovered from charcoal; –□– CFU mL^{-1} in the reactor; – – o – –CFU mL^{-1} in liquid phase; – – ◊ – –CFU mL^{-1} in the charcoal.

increments in CFU and residence time increase the phenanthrene degradation in the reactor. Distribution of sampling points (Fig. 3) shows that for NSSWOI and NSSWI reactors time is the main factor affecting pesticides remaining (because of the small perpendicular distances to the TIME vector); however, for SSWI reactor, although time shows to be important, some point are more related with CFU and pH, two factors that have proved the negative or positive effect on PAHs degradation (Fahy *et al*. 2008).

Table 3. Rates of phenanthrene degradation for both stages.

Time (days)	Rates of phenanthrene degradation (mg CFU-1)					
	First stage			Second stage		
	NSSWOI[a]	SSWI[b]	NSSWI[c]	E1[d]	E2[e]	E3[f]
0	0	0	0	0	0	0
1	UD	UD	UD	UD	6.13E-08±6.80E-09	UD
2	1.52E-07±5.79E-09	4.48E-09±5.53 E-12	9.89E-09±1.10E-09	UD	1.40E-07±1.83E-08	UD
3	UD	UD	UD	4.76E-08±6.24E-09	UD	2.62E-07±2.91E-08
4	3.79E-07±2.12 E-08	2.01E-09±2.31E-10	2.80E-08±3.37E-09	UD	UD	UD
5	UD	UD	UD	5.08E-08±2.28E-09	UD	1.36E-07±1.78E-08
6	8.33E-07±5.27E-08	3.43E-09±2.00E-09	6.50E-08±2.92E-09	UD	UD	UD
7	UD	UD	UD	4.24E-08±4.93E-09	1.72E-07±7.70E-09	2.92E-07±1.31E-08
8	9.05E-07±8.93E-08	1.53E-08±5.51E-10	UD	UD	UD	UD
9	UD	UD	2.72E-07±3.17E-08	UD	UD	1.89E-07±2.20E-08
11	1.84E-07±1.67E-08	1.69E-08±1.76-09	UD	1.25E-07±4.96E-09	1.29E-07±1.50E-08	4.08E-07±1.62E-08
12	UD	UD	5.54E-07±2.20E-08	UD	UD	UD
13	UD	UD	UD	UD	2.76E-07±1.09E-08	UD
14	3.22E-07±8.88E-08	5.19E-08±1.47E-08	UD	UD	UD	UD
15	UD	UD	UD	1.15E-07±1.68E-08	3.36E-07±4.91E-08	7.10E-07±1.04E-07
16	UD	UD	1.16E-06±1.70 E-07	UD	UD	UD

a: no sterile seawater without inoculum; b: sterile seawater with inoculum; c: no sterile seawater with inoculum; d: 60 mg L^{-1}; e: 200 mg L^{-1}; f: 600 mg L^{-1}. UD: undetermined. CFU: colony forming units.

3.2 Effect of initial concentration of phenanthrene on the degradation

For this experiment, losses of 6% by volatilization were observed in the control. Similar to the first stage, a fraction of the initial concentration of phenanthrene stayed adsorbed on charcoal (63%) and dissolved in water (31%). The concentration played an important role in phenanthrene degradation. The phenanthrene dissipations were 60.78%, 80.38%, and 48.97% for reactors working at concentrations of 60, 200, and 600 mg L^{-1}, respectively. Taking into account that 6% of phenanthrene was volatilized in the control reactor, we obtained final results of phenanthrene degraded of 54.78%, 74.38%, and 42.97% for the 60, 200, and 600 mg L^{-1} concentrations, respectively. The results show that phenanthrene reached its maximum degradation at 200 mg L^{-1}, which in turn favored microbial growth, and this degradation was higher than that observed in the reactors working with the other concentrations of phenanthrene (60 mg L^{-1} and 600 mg L^{-1}) (Fig. 4). Abdul-Talib *et al*. (2015) showed that increasing the concentration of phenanthrene in a liquid medium reduces its degradation efficiency.

From the multifactorial variance analyses (ANOVA) at 95% confidence, it was observed that the time and the phenanthrene concentration had a significant effect on phenanthrene dissipation ($P < 0.05$), but only the phenanthrene concentration had a significant effect on microbial growth ($P < 0.05$). This explains why the microbial growth (CFU mL^{-1}) for the reactor at a concentration of 600 mg L^{-1} presented a decrement in function of time compared to the two smaller concentrations. This coincides with the results obtained by Nasrollahzadeh *et al*. (2010), who reported a decrease in the growth of microorganisms when the concentration of phenanthrene increased. Similarly, other studies have shown that phenanthrene concentrations greater than 230 mg L^{-1} have inhibitory effects on microorganisms (Madueño *et al*. 2009; Lu *et al*. 2012). Multiple rank analyses determined two groups for phenanthrene degradation, one formed by reactors working at 60 and 200 mg L^{-1} and the other for the reactor working at 600 mg L^{-1}; the phenanthrene degradation was significantly lower at the 600 mg L^{-1} concentration. Similarly, for microbial growth, two homogeneous groups were differentiated by concentration, one being formed by the concentration of 60 mg L^{-1} and the other one by the other two concentrations; the microbial growth was significantly lower at the 60 mg L^{-1} concentration of phenanthrene. The analysis of variance components showed that 93% of the phenanthrene remnants variation and 65% of the microbial growth variation are attributed to the time in the reactor; however, for the latter case, the variation in function of time was not significant according to ANOVA.

The DT_{50} values (Table 2) showed faster phenanthrene dissipation for the reactor operated at 200 mg L^{-1} and longer residence times are observed for the two other concentrations applied. These observations suggest that microorganisms take longer to acclimate and therefore to degrade phenanthrene at lower concentrations; while at higher concentrations, longer residence time can be interpreted as saturation

of phenanthrene. The phenomena of adsorption and desorption of phenanthrene on charcoal seem to play an important role (Li *et al*. 2012). The microbial growth in the reactor can also be inhibited by the presence of toxic metabolites, which are the products of phenanthrene degradation and their accumulation in the medium. This may also affect the degrading behavior of the function of the phenanthrene concentration (Moreno *et al*. 2009; Lu *et al*. 2012).

The rate of phenanthrene degradation is shown in Table 3. The results show a high phenanthrene degradation rates; for NSSWI, degradation rate always increased compared with the other two experiments in the first stage. This supports the fact that maximum phenanthrene degradation (69.5%) was observed in the NSSWI experiment. For the second stage, in which the rate of phenanthrene degradation was a function of the concentration, the highest rates of degradations were observed in the reactor working with 600 mg L^{-1} of phenanthrene; however, the best growth of microorganisms was observed in the experiment working at 200 mg L^{-1} (Fig. 4b). How was discussed previously, presence of metabolites can inhibit the bacterial growth; some metabolites such as 2-hydroxy-1-naphthoic acid and 1-hydroxy-2-naphthoic acid have been reported in phenanthrene degradation process by fusant strain F14, being the last metabolite the most toxic (Lu *et al*. 2012); so, high phenanthrene concentration can result in high phenanthrene degradation rates but implies high metabolite presence which affects the microbial growth of the strains used in this research.

It is important to note that the rate of degradation in E3 shows fluctuations in time, while in E2 this rate was always increasing. This led us to infer that 200 mg L^{-1} of phenanthrene was the optimum concentration for microbial growth. Kong *et al*. (2017) report that 33.23% of the phenanthrene (initial concentration of 20 mg L^{-1}) can be degraded under low oxygen conditions at 24 hours; in the present research, the highest phenanthrene removal at one day was over 40% for a phenanthrene concentration of 200 mg L^{-1} and aeration supply of 2 L min^{-1}. These results for the two stages of the experiment show the ability of the inoculum to degrade phenanthrene even at high concentrations when aeration is supplied.

The results obtained in this research show that phenanthrene dissipation by microbial degradation using FBR depends on a multifactorial behavior, with the conditions under which these processes are developed, the phenanthrene concentrations to be treated, and the inoculum playing very important roles. This is supported by previous studies about the microbial biodegradation of phenanthrene (Umar *et al*., 2017).

These results show that volatilization of phenanthrene was higher in the first stage with experiments at a low airflow compared with experiments in the second stage. Although phenanthrene tends to float in seawater because of its density, airflow seems to have contributed to the loss by volatilization of phenanthrene (Loya, 2013); at a lower airflow (1.5 L min^{-1}) phenanthrene is less distributed in water, so it is more available for volatilization.

Conclusions

The degradation of phenanthrene in FBR using AC as support and natural seawater at short times was directly affected by the reactor operating conditions and the inoculum, with the latter being the most important. The results show that at a constant phenanthrene concentration of 112 mg L^{-1}, the maximum phenanthrene degradation (69.5%) was observed for the NSSWI reactor, inferring that autochthonous microorganisms and inoculum had a cooperative interaction. Similarly, across different phenanthrene concentrations, the maximum degradation (74.38%) was observed at the optimal concentration of 200 mg L^{-1}, this concentration was optimal to microorganisms growth, showing an important relationship between microorganisms presence and phenanthrene degradation. FBR was shown to be efficient (degradation >69%), but the appropriate operating conditions are the key to reach the highest efficiency. These results are conclusive, and an FBR using AC as support for degrading phenanthrene microorganisms can be used at industrial level to treat effluents contaminated with phenanthrene, even in bioremediation processes focused on microorganisms.

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Nomenclature

FBR	fluidized bed reactor
PDP	phenanthrene degradation process
AC	activated charcoal
CFU	colony forming units
%RP	percentage of remaining phenanthrene
NSSWOI	nonsterile seawater without inoculum
SSWI	sterile seawater with inoculum
NSSWI	non-sterile seawater with the inoculum
V	volume, mL
C_o	initial concentration of phenanthrene, mg L^{-1}
C	concentration at time t, mg L^{-1}
K	dissipation rate, day^{-1}
T	time, day
DT_{50}	phenanthrene half-life, day

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