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RAW GLYCEROL AS COSUBSTRATE ON THE PAHs BIODEGRADATION IN SOIL

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ABSTRACT

The purpose of this investigation was to evaluate the efficacy of raw glycerol obtained as a by-product from the biodiesel manufacturing industry on the bioremediation of a soil contaminated with 8.43mg/kg of the USEPA 16 priority PAHs. Experiments were carried out using raw glycerol at 0.63, 0.32 and 0.16mg/kg. The second condition resulted in the best PAH biodegradation in 60 days, 68.0±0.1%, confirming that this compound may be used as a cosubstrate and adding value to this residue. Germination Index assay on soil samples proved that bioremediation eliminated the phytotoxic effects.

Keywords: Bioremediation, polycyclic aromatic hydrocarbons, raw glycerol, cosubstrate, germination index.

INTRODUTION

Polycyclic Aromatic Hydrocarbons (PAHs) are the primary organic contaminants in nature and are mainly found in soil. The hydrophobic characteristic of these molecules allows them to strongly bind to particles present in the soil, thus hindering their bioavailability and, consequently, their biodegradation. In addition, the mutagenic and carcinogenic potential of PAHs is well known and widely reported in the literature (Sanchés-Guerra *et al.*, 2012; Gong *et al.*, 2007; Mater *et al.*, 2006). PAHs originates mainly from vegetable, geochemical, and anthropogenic sources. Chemically, PAH molecules present two or more aromatic rings and cyclopentanes that are condensed and arranged in linear form, angular form, or in groups, and they are formed from saturated hydrocarbons under oxygen-deficient conditions through pyrosynthesis or pyrolysis catalyzed by constituents present in the medium (Muckian *et al.*, 2007).

Due to the complexity of the soil as well as the diversity and nature of the contaminants, several technologies have been developed and employed for the removal of polluting chemicals. Among these methods, bioremediation has been highlighted. The use of microorganisms for the removal of petroleum hydrocarbons from soil has been widely documented in the literature. This technique is environmentally friendly and is well-accepted by the public (Jacques *et al.*, 2007; Supaphol *et al.*, 2006).

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The biodegradation of PAHs in soil is slower than other hydrocarbons. These molecules are not the preferred sources of carbon, and so the microbiological removal process occurs through cometabolic pathways. Based on this principle, the addition of cosubstrates, e.g., glycerol, to the contaminated soil can contribute to the process of biodegradation. Glycerol is one of the adjuvants most widely used as a cosubstrate in various industrial bioprocesses, and it has been successfully used in the biodegradation of raw oil and in the process of denitrification of wastewater (Bodík *et al.*, 2009; Zhang *et al.*, 2005).

Glycerol has several advantages over other cosubstrates due to its osmoregulatory properties and the fact that it can serve as the preferred source of carbon in the synthesis of biosurfactants. In addition, glycerol is widely available in the market (Batista *et al.*, 2006; Nevoigt and Stahl, 1997). In this context, raw glycerol, a byproduct of the biodiesel industry, acquires an added value because it can be reused in the oil and gas industry. Glycerol production and supply have been increasing over the past years, thus stimulating research aimed at developing functional applications for the product (Ayoub and Abdullah, 2012).

Raw glycerol is regarded as an industrial waste because of its concentration of alkali metals, soaps, and other impurities derived from the process of biodiesel production. Even the raw form of glycerol can be used as a carbon source for energy and biomass production, with similar results compared to glycerin (Liu *et al.*, 2009). Because research using glycerol is a relatively new topic,

there are no reports describing its application as a cosubstrate during bioremediation of soils contaminated with PAH.

MATERIALS AND METHODS

Soil

The soil samples used in the present study were collected from a coastal region of Brazil. The samples were subjected to physicochemical and microbiological characterization, and the results are shown in table 1.

Raw glycerol

Raw glycerol was kindly provided by the Instituto Virtual Internacional de Mudanças Globais associated with the Instituto Alberto Luiz Coimbra de Pós-graduação e Pesquisa em Engenharia da Universidade Federal do Rio de Janeiro – IVIG-COPPE/UFRJ). The raw glycerol was coproduced during the process of biodiesel production from soybean (*Glycine max*, L. Merrill) by the transesterification reaction via the ethyl route using potassium hydroxide as a catalyst. The compound had the following characteristics: density 0.0011 g/cm^3, pH 9.9, ethanol content (2.8% w/w), water content (9.3% w/w), KOH content (2% w/w), glycerol content (84.4% w/w), diglyceride content (1.0% w/w), triglyceride content (<0.5% m/m), and potassium content (50.7 mg/g).

Biodegradation experiments

The biodegradation experiments were conducted for a period of 60 days in reactors with the following dimensions: 0.22 m width, 0.22 m length, and 0.09 m depth, containing 2 kg of soil. The effect of raw glycerol addition as a cosubstrate, using 0.63, 0.32, and 0.16 mg/kg of the compound, was analyzed.

To ensure adequate oxygen supply, the soil was regularly aerated by rotating it every 72 hours with a glass rod. The concentration of heterotrophic bacteria and filamentous fungi were determined from samples collected at 0, 7, 15, 30, and 60 days by the pour plate technique using Nutrient Agar (Merck, Darmstadt, Germany) containing 50 mg/L nystatin suspension at 10.000 UI/mL (Sigma, St. Louis, USA) for heterotrophic bacteria and Sabouraud Agar containing 2% glucose (Vetec, Rio de Janeiro, Brazil) and 50 mg/L ampicillin trihydrate at 96.0 to 100.5% purity (Sigma, St. Louis, USA) for filamentous fungi. Bacteria and fungi were incubated at 28±1°C for 48 hours or 72 hours, respectively. Abiotic loss was calculated using a reactor containing the same amount of soil added with a solution of 10% silver nitrate (Vasconcelos *et al*., 2011).

Ecotoxicological assay

Seed germination and root elongation tests were performed to determine the germination index (G_I), as described by Tiquia *et al*. (1996), using the following equation:

$$G_I = [(S_1/S_2) \times (R_1/R_2)] \times 100$$

Where S1 is the number of germinated seeds in the soil extract, S2 is the number of germinated seeds in the control, R1 is the average root length in the soil extract, and R2 is the average root length of the control. Seeds of *Allium porrum*, L., *Cucumis anguria*, L. and *Cucumis melo*, L. were used in this study. Incubation was performed at 22±1°C in the dark for 5 days. The test was conducted in triplicate using ten seeds of each species. The Germination Index takes into consideration control assays carried out with distilled water.

RESULTS AND DISCUSSION

The effect of the addition of raw glycerol as a cosubstrate in the biodegradation of the 16 priority PAHs from USEPA was analyzed. Degradation of the contaminants was observed in all tested concentrations. The average pH of 7.4±0.1 and soil moisture of 17.0±0.1% ($p < 0.05$) were favorable conditions for the degradation process. The percentage of biodegradation of each PAH is shown in table 2.

The addition of 0.63 mg/kg of raw glycerol promoted an approximately 64% reduction in PAH; however, the concentration of the compound that most highly stimulated the process was 0.32 mg/kg, resulting in a 6% increase in biodegradation and a 15% increase compared to the control reactor. A significant increase in the percentage of PAH removal, attributed to reduced raw glycerol content, was also observed in previous studies using the compound as a cosubstrate during the biodegradation of toxic compounds (Easterling *et al*., 2009; Chen *et al*., 2007). Attempts to reduce the concentration of raw glycerol to 0.16 mg/kg had no significant effect, yielding a value very close to that observed in the control reactor.

There was almost 100% removal of PAHs with two or three aromatic rings in all tested conditions. This was due to the simplicity of the molecular arrangement of these compounds and their low concentrations in soil. In contrast, the presence of raw glycerol negatively influenced the biodegradation of anthracene and fluorene, which corroborates the findings of Bengtsson and Zerhouni (2003). Moreover, degradation of these PAHs were inversely proportional to the concentration of raw glycerol, as observed by Baboshin *et al*. (2003), possibly related to the preferred choice of carbon source by telluric microorganisms.

Phenanthrene had the lowest percentage of removal in all reactors, including the control. The result is supported by the findings of Viñas *et al*. (2005) who observed that incomplete degradation of phenanthrene was related to

Table. 1 Characterization of the soil samples.

Analysis	Results	Reference
Texture (%)		ABNT (1984)
Clay (<0.02mm)	9±5	
Silt (0.002-0.02mm)	33±1	
Fine sand (0.02-0.2mm)	22±2	
Medium sand (0.2-0.5mm)	19±1	
Coarse sand (0.5-1.0mm)	12±4	
Gravel (>1.0mm)	5±5	
Total Organic Carbon (mg/kg)	23,000±1,000	USEPA[a] 9060
Total N (mg/kg)	1,397±30	USEPA 315.2
Total P (g/kg)	777±11	USEPA 365.3
16 priority PAHs[b] (mg/kg)	8.43±590	USEPA 8270C, 3540C e 3630C
pH	7.8±0.1	EMBRAPA (1979)
Water-Holding-Capacity (%)	21.4±0.1	Vasconcelos *et al.* (2011)
Water content (%)	10.0±0.1	EMBRAPA (1979)
Heterotrophic bacteria (UFC/g)	$34\pm1x10^6$	Vasconcelos *et al.* (2011)
Filamentous Fungi (UFC/g)	$22\pm1x10^4$	Vasconcelos *et al.* (2011)

[a] United States Environmental Protection Agency. [b] Polycyclic Aromatic Hydrocarbons

Table 2. Percentage of the USEPA 16 priority PAHs biodegradation (abiotic loss = 10±0.1%)[a]

PAH (# rings)	Raw glycerol concentration (mg/kg)			
	0.00	0.16	0.32	0.63
Naphtalene (2)	>99.9±0.1	>99.9±0.1	>99.9±0.1	>99.9±0.1
Acenaphthene (3)	>99.9±0.1	>99.9±0.1	>99.9±0.1	>99.9±0.1
Acenaphtilene (3)	>99.9±0.1	>99.9±0.1	>99.9±0.1	>99.9±0.1
Fluorene (3)	>99.9±0.1	88.0±0.1	86.0±0.1	76.0±0.1
Anthracene (3)	>99.9±0.1	55.0±0.1	46.0±0.1	44.0±0.1
Phenanthrene (3)	18.0±0.1	30.0±0.1	43.0±0.1	38.1±0.1
Fluoranthene (4)	51.8±0.1	51.8±0.1	59.7±0.1	59.5±0.1
Pyrene (4)	60.9±0.1	33.7±0.1	69.4±0.1	54.4±0.1
Benzo[a]anthracene (4)	52.2±0.1	56.3±0.1	77.2±0.1	66.8±0.1
Chrysene (4)	45.2±0.1	46.6±0.1	70.2±0.1	56.3±0.1
Benzo[b]fluoranthene (5)	65.1±0.1	67.5±0.1	79.7±0.1	73.6±0.1
Benzo[k]fluoranthene (5)	70.5±0.1	73.0±0.1	93.0±0.1	90.5±0.1
Benzo[a]pyrene (5)	52.8±0.1	55.0±0.1	85.1±0.1	81.9±0.1
Dibenz[a,h]anthracene (5)	43.5±0.1	52.5±0.1	70.7±0.1	66.2±0.1
Benzo[g,h,i]perylene (6)	85.1±0.1	85.6±0.1	85.1±0.1	74.5±0.1
Indeno[1,2,3-c,d]pyrene (6)	85.3±0.1	90.7±0.1	84.4±0.1	84.4±0.1
Biodegradation (%)	58.1±0.1	59.4±0.1	68.0±0.1	63.9±0.1

USEPA – United States Environmental Protection Agency. PAHs –Polycyclic Aromatic Hydrocarbons. [a] initial concentration = 8.43mg/kg (sum of the USEPA 16 priority PAH)

the low bioavailability of the compound when nutrient supplementation in relation to the degradation of several PAHs was analyzed.

With regard to PAHs with four to six aromatic rings, the percentage of biodegradation was between 33.7 and 93.0±0.1%. The reduction in six of the ten PAHs of this group, benzo[a]anthracene, chrysene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene and dibenzo[a,h]anthracene, was due to the raw glycerol concentration used. Successful biodegradation can possibly be attributed to higher bioavailability of these compounds in the soil, favored by the emulsifying property of raw glycerol. High values were observed in the PAHs with five and six aromatic rings; these values were significantly higher than those

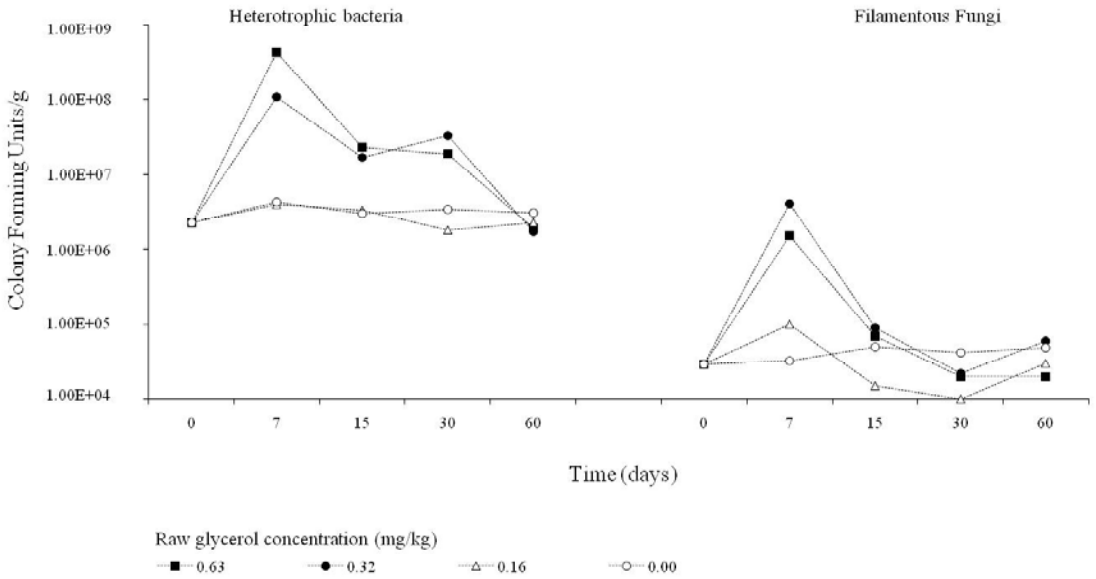

Fig. 1. Microbial concentration during PAHs biodegradation.

Table 3. Range of PHAs biodegradation (mg/kg/day) ($p \leq 0,05$)

Time (days)	Raw glycerol concentration (mg/kg)			
	0.00	0.16	0.32	0.63
0-30	0.05±0.01	0.10±0.01	0.18±0.10	0.15±0.11
30-60	0.04±0.01	0.02±0.01	0.04±0.11	0.03±0.10

PAHs – Polycyclic Aromatic Hydrocarbons

Table 4. Germination Index ($p \leq 0,05$).

Plants	Raw glycerol concentration (mg/kg)			
	0.00	0.16	0.32	0.63
Allium porrum	40.6±4.5[a]	41.9±7.4[a]	121.9±13.4[c]	108.4±15.1[c]
Cucumis anguria	50.3±5.5[a]	51.9±5.8[b]	85.1±4.9[c]	83.6±2.7[c]
Cucumis melo	73.5±6.1[b]	74.3±3.8[b]	101.9±11.2[c]	88.3±3.8[c]

[a] High phytotoxicity. [b] moderate phytotoxicity. [c] no phytotoxicity

reported in the literature (Lei *et al.*, 2005; Joner *et al*., 2004; Xu and Obbard, 2004).

In addition to raw glycerol, other PAHs may work as cosubstrates during the removal of certain PAHs with high molecular weights. This metabolic strategy has previously been investigated through kinetic studies in which removal of pyrene in the presence of fluoranthene and phenanthrene was observed (Hwang and Cutright, 2003; Dean-Ross *et al*., 2002). This observation may explain the highest percentage removal of pyrene at 69.4±0.1%, coinciding with the highest percentage removal of fluoranthene at 59.7±0.1% and phenanthrene at 43.0±0.1% in the reactor with the addition of 0.32 mg/kg raw glycerol. An important observation concerns the second highest percentage of degradation of pyrene that occurred in the reactor without the addition of raw glycerol. In this case, the microbiota used two distinct classes of cosubstrates for the removal of these PAHs.

When raw glycerol was not included in the reactor, the biodegradation percentages of PAHs with four to six aromatic rings were between 43.5 and 85.3±0.1%. The greatest reductions were observed in highly persistent molecules of benzo[g,h,i]perylene and indeno[1,2,3-c,d]pyrene. The high removal percentages can be explained by the distribution of PAHs in soil aggregates, thus favoring bioavailability.

As shown in figure 1, the presence of raw glycerol allowed for the development of a population of heterotrophic bacteria and filamentous fungi in the soil, depending on the concentration of cosubstrate employed. Furthermore, the levels of nitrogen and phosphorus ensured the necessary supply of these nutrients, which was verified by the stability of the microbial population in the reactor without raw glycerol.

The addition of 0.32 and 0.63 mg/kg of raw glycerol increased the population of heterotrophic bacteria and filamentous fungi by two orders of magnitude during the first seven days of the experiment. In the reactor containing 0.16 mg/kg of the glycerol compound, a slight increase was observed in the microbial population during the same period, possibly due to the low concentration of raw glycerol used. Over time, the microbial density decreased, and the concentration was reestablished to a similar level to that determined at the beginning of the experiment under all glycerol conditions tested.

The experimental time required for the reestablishment of heterotrophic bacteria was between 30 and 60 days. The concentration of filamentous fungi was reduced twice as fast as the bacterial concentration. Such behavior was also observed by Ballaminut and Matheus (2007) and may be attributed to the higher sensitivity of fungi to nutrient depletion. The reduction in the microbiota in this type of research is a predicted event and may also be related to the presence of toxic metabolites and the phenomenon of tolerance, in which environmental stresses, such as the petroleum hydrocarbon contamination, promote compensatory effects, causing the microbiota to balance and stabilize (Igwo-Ezikpe *et al*., 2010; Ayotamuno *et al*., 2007).

The microbial communities in soil are structured in a way that they can eliminate the xenobiotic compounds, using a mechanism involving metabolic cooperation, chemotaxis, and cometabolism. However, supplementation of the medium with a soluble carbon source at non-inhibitory concentrations directly influences the bioprocess through the induction of growth and increased metabolic rate (Pizzul *et al*., 2007; Grabowski *et al*., 2005).

Table 3 shows that the highest rates of biodegradation occurred in the first half of the experiment in systems where raw glycerol was added, coinciding with the period having higher microbial density. With the depletion of the cosubstrate, the degradation rate was significantly decreased, revealing the contribution of raw glycerol in the process, in agreement with previous studies in which glycerin was administered as a carbon source (Vasconcelos *et al*., 2011; Baboshin *et al*., 2003).

It is possible that with the depletion of raw glycerol, other molecules such as low molecular weight PAHs and light oil fractions may have been used as cosubstrates, which explains the significant decrease in the rate of daily PAH reduction in the second half of the bioprocess; however, this did not lead to the end of the bioprocess, as seen in the reactor without raw glycerol.

The raw glycerol concentration also affected the reduction of the phytotoxic effect determined by the germination index (G_I) shown in Table 4. The G_I is the result of seed germination and the rate of elongation of the root extract in contaminated soil and in control soil. For any plant, the phytotoxicity of the soil constituents is considered moderate when the G_I ranges between 50 to 80%. A G_I below 50% indicates high phytotoxicity, and a G_I above 80% indicates the lack of a phytotoxic effect (Anastasi *et al*., 2009).

An 8.43 mg/kg concentration of PAH promoted moderate (*C. anguria* and *C. melo*) to high (*A. porrum*) phytotoxicity. This result is in disagreement with the results observed by Debiane *et al*. (2008), in which a phytotoxic effect of the tested hydrocarbons was detected at concentrations above 10 mg/kg. The decrease in the inhibitory effect on plants coincided with a significant reduction in the content of the contaminant. The best condition for the PAH biodegradation test for the elimination of phytotoxicity was observed at raw glycerol concentrations of 0.63 and 0.32 mg/kg. Despite the high content of potassium and other impurities in raw glycerol, in two of the three plants tested, the G_I reached values above 100%, which indicates the presence of nutrients in the soil extract (Paradelo *et al*., 2008).

At the lowest concentration of cosubstrate tested (0.16 mg/kg), the result showed similarity to that seen in the assay with untreated soil sample, i.e., a moderate to high phytotoxicity. This result is most likely related to the cosubstrate concentration employed, which had no significant effects on the microbiota, and consequently, on the degradation of PAHs. This suggests that raw glycerol in this condition could have been used only as a preferred source of carbon and not as a cosubstrate, in agreement with the results presented by Duquenne *et al*. (1999).

The results presented in this study contribute to the understanding of raw glycerol application as a cosubstrate during the biodegradation of PAHs in soil, ensuring an efficient strategy for the removal of these contaminants as well as in reducing the toxic effects on the plants studied. In this context, the reuse of waste in the biodiesel industry can become economically viable and impart an added value to raw glycerol. Failure to remove the impurities originating during the transesterification process, due to the low volume of cosubstrate required, corroborates that assertion. In addition, future impacts to the soil due to the accumulation of potassium from the catalyst may be

avoided by employing glycerol produced by new generations of biodiesel production technologies.

CONCLUSION

Under the conditions established by the present study, the best strategy for the biological removal of PAHs was obtained by adding 0.32 mg/kg of raw glycerol, which also contributed to the elimination of phytotoxic effects in the tested plants. Most importantly, the assignment of functionality to raw glycerol reflects on the valuation of the product and contributes positively to discussions on the reuse of waste from industrial processes.

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