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Effect of exogenous phenazine addition on crude heavy oil degradation by *Pseudomonas aeruginosa* TGC04

Efecto de la adición de fenazina exógena sobre la transformación de petróleo crudo pesado por *Pseudomonas aeruginosa* TGC04

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Abstract
Previous studies have shown that phenazines contribute to hydrocarbons (HC) transformation by *Pseudomonas aeruginosa.* The rod is capable of degrading HC shortly after exposure to oil; and this study aimed to assess the effect of phenazine methosulfate (PMS), on the removal of HC by bioaugmentation with pyocyanin-producing *P. aeruginosa* TGC04. Microcosms were prepared containing fine sand and oil-contaminated beach sand (1:4). The pre-inoculum was prepared with pasteurized sand enriched with 10 μmol/L of PMS; supplemented with barley malt bagasse and incubated at 29±1°C for 10 days. Afterwards, portions of the inoculum were added to the microcosms (1:10; 1:100 and 1:1000). The total petroleum hydrocarbons (TPH) were reduced by up to ≈49%, while the 16 USEPA priority polycyclic aromatic hydrocarbons (PAHs) were reduced by ≈37 and 56%. *P. aeruginosa* TGC04 preferentially degraded 4-6 ring PAHs (80-89%). The 2-3 ring PAHs were removed by up to ≈37%. In the presence of PMS, there was a significant reduction in HC; the highest rates of degradation, however, were observed without PMS (1:100). As a contribution, this study expands the knowledge that the hydrocarbonoclastic activity of *P. aeruginosa* is not increased by addition of exogenous phenazines but favors the removal of 4-6 ring PAHs.
Keywords: Bioremediation, Allochthonous bioaugmentation, Encompassed inoculum, Phenazine Methosulphate, Polycyclic Aromatic Hydrocarbons.

Resumen
Estudios anteriores han demostrado que las fenazinas contribuyen a la transformación de hidrocarburos (HC) por *Pseudomonas aeruginosa.* La bacteria degrada el HC poco después de la exposición al petróleo. Este estudio objetivó evaluar el efecto del metosulfato de fenazina (PMS) sobre la eliminación de HC mediante bioaumentación con *P. aeruginosa* TGC04 productora de piocianina. Se prepararon microcosmos que contenían arena fina y arena de playa contaminada con petróleo pesado (1:4). El preinóculo se preparó con arena pasteurizada enriquecida con 10 μmol/L de PMS; suplementado con bagazo de malta de cebada e incubado a 29±1°C durante 10 días. Posteriormente se agregaron porciones del inóculo a los microcosmos (1:10; 1:100 y 1:1000). Los hidrocarburos totales de petróleo (TPH) se redujeron hasta en ≈49%, mientras que *P. aeruginosa* TGC04 degradó preferentemente los hidrocarburos aromáticos policíclicos (HAP) de 4-6 anillos (80-89%). Los HAP de 2-3 anillos se eliminaron hasta en un ≈37%. En presencia de PMS, hubo una reducción significativa de HC; Sin embargo, las tasas más altas de degradación se observaron sin PMS (1:100). Como contribución, este estudio amplía el conocimiento de que la actividad hidrocarbonoclástica de *P. aeruginosa* no aumenta con la adición de fenazinas exógenas, sino que favorece la eliminación de HAP de 4-6 anillos.
Palabras clave: Biorremediación, Bioaumentación alóctona, Inóculo abarcado, Metosulfato de fenazina, Hidrocarburos aromáticos policíclicos.

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1 Introduction

Although different energy alternatives have been developed and disseminated over the last few decades, dependence on fossil fuels is still imperative in modern society with significant leaks still a sad and dramatic reality (Silva *et al*., 2021). For almost ten months between 2019 and 2020, ≈ 5,300 tons of crude oil were spilled approximately 3,000 km off the Brazilian coast (Araújo *et al*., 2021; Lessa *et al*., 2021). The oil shared similar properties to extra-heavy oil (Nobre *et al*., 2022; Zacarias *et al*., 2021; Oliveira *et al*., 2020). This accident caused multifactorial losses and is considered the largest in the history of Brazil and the South Atlantic (Estevo *et al*., 2021; Pena *et al*., 2020), as well as one of the greatest disasters involving a crude oil spill in the world, with consequences to be felt for many decades (Anjos *et al*., 2023).

After a large oil spill, a change in the C:N:P ratio can be observed at the site where the accident occurred (Jacques *et al*., 2008). Exposure to hydrocarbons promotes drastic changes in the autochthonous microbiota (Sarkar *et al*., 2016) and a hydrocarbonoclastic community later becomes dominant (Teramoto *et al*., 2013). Microorganisms are principally involved in the transformation of hydrocarbons in nature (Dourado *et al*., 2017). The option for this natural detoxification process, however, does not seem to be the most appropriate decision since coastal areas are very fragile areas (Disner and Torres, 2020).

In this context, emergency intervention may require an inoculation of high-density cells of competent allochthonous hydrocarbonoclastic microbes when the indigenous population is not able to maintain efficient degradation of the contaminant (Brzeszcz *et al*., 2020). Bioaugmentation is based on the survival and maintenance of the catabolic activity of the inoculant against petroleum hydrocarbons (Nowak and Mrozik, 2016). The technique is very effective in the initial stages of the process (Woź niak-Karczewska *et al*., 2019). Around 70 to 75% of petroleum hydrocarbons can be transformed within approximately 30 up to 100 days of treatment (Cavalcanti *et al*., 2019).

Due to their constitution, Gram-negative bacteria are the most promising microbes in terms of bioaugmentation treatment (Abena *et al*., 2019). *Pseudomonas aeruginosa* is one of the most important hydrocarbonoclastic bacteria (Ojewumi *et al*., 2018). It is abundant in soils contaminated by petroleum hydrocarbons, playing a key ecological role in soil detoxification (Huang *et al*., 2021; Crone *et al*., 2019). In addition, *P. aeruginosa* exhibits versatility in physiological and metabolic terms, which results in increased bioavailability of contaminants (Zhao *et al*., 2018) and capacity to transform paraffins (Karamalidis *et al*., 2010), naphthenes (Shekhar *et al*., 2015), aromatics (Zhang *et al*., 2015) and polycyclic aromatics (Filinov *et al*., 2010).

P. aeruginosa is proficient in the production of a myriad of secondary metabolites (Depke *et al*., 2020). Some of them, such as phenazines, are synthesized under situations of environmental stress, including nutrient-limiting conditions, exposure to complex molecules, oxidative stress, and competition (Arruda *et al*., 2019). The specific phenazine of *P. aeruginosa* pyocyanin (PYO) is an important signaling molecule in quorum-sensing systems (Gonçalves and Vasconcelos, 2021). This property is closely associated with the response mechanism of *P. aeruginosa* to high selective pressures exerted by the environment (Bahari *et al*. 2017). Given this, PYO and other phenazines may participate in the processes involving the degradation of oil based on the premise that PYO acts as an electron carrier in the presence of oxygen and in the formation of free radicals to react with hydrocarbons (Abdelaziz *et al*., 2022).

Interest in the role of phenazines in hydrocarbon transformation processes is very recent and still little explored (Mangwani *et al*., 2015; Wu et al., 2014). As an example, there are reports of the use of hexane and toluene as substrates for the synthesis of PYO (Ozdal *et al*., 2019). Additionally, the correlation between PYO synthesis and biosurfactant production was only first reported by Das and Ma in 2013. *P. aeruginosa* grown in mineral medium produced ≈10 μg/mL of PYO. This coincided with high emulsification indices of three petroderivatives (60-75 %). A second strain produced ≈5 μg/mL and reached an emulsifying index of 25 and 40%. In addition, our group observed that the difference of approximately 60 times in the concentrations of PYO resulted in an increase of 65% in the removal of pyrene and 45% of anthracene from samples of sandy soil. Additionally, a high correlation was observed between the synthesis of PYO and the emulsification of a lubricating oil mixture (Viana *et al*., 2018).

Therefore, the aim of this study was to evaluate the effect of adding exogenous methosulfate phenazine (PMS), associated with allochthonous bioaugmentation with *P. aeruginosa* TGC04 on the reduction of the total petroleum hydrocarbons (TPH) and the 16 USEPA priority polycyclic aromatic hydrocarbons (PAH) from oil-contaminated beach sand.

Table 1. Characterization of sand and barley malt bagasse samples.

Parameters	Results			Reference
	BS	FS	BMB	
Total organic carbon (% w/w)	25,976	0.74	1,200.00	USEPA 9060
Total nitrogen (mg/Kg)	35.2	31.8	497.1	USEPA 315.2
Total phosphorus (mg/Kg)	< 1	3.04	421	USEPA 365.3
Clay (%)	0.99	0.8	—	ISO 13320: 2020
Silt (%)	1.29	6.4	—	
Coarse sand (%)	1.86	60.1	—	
Fine sand (%)	95.86	32.7	—	
Water holding capacity (%)	16.2	30.1	—	Cavalcanti et al. (2019)
[1]TPH (mg/Kg)	25,900.00	—	—	USEPA 8015
Gasoline (mg/Kg)	< 134.00	—	—	
Kerosene (mg/Kg)	320	—	—	
Diesel (mg/Kg)	5,160.00	—	—	
Lubricant oil (mg/Kg)	20,400.00	—	—	
16 USEPA priority [2]PAHs (mg/Kg)	75.83	—	—	USEPA 8270
Acenaphthene (mg/Kg)	< 0.27	—	—	
Acenaphytilene (mg/Kg)	< 0.27	—	—	
Anthracene (mg/Kg)	3.47	—	—	
Phenanthrene (mg/Kg)	25.6	—	—	
Fluorene (mg/Kg)	4.13	—	—	
Naphthalene (mg/Kg)	2.67	—	—	
Benzo[a]anthracene (mg/Kg)	7.94	—	—	
Benzo[a]pyrene (mg/Kg)	3.36	—	—	
Benzo[b]fluoranthene (mg/Kg)	< 0.27	—	—	
Benzo[g,h,i]perylene (mg/Kg)	< 0.27	—	—	
Benzo[k]fluoranthene (mg/Kg)	< 0.27	—	—	
Chrysene (mg/Kg)	13.5	—	—	
Dibenzo[a,h]anthracene (mg/Kg)	< 0.27	—	—	
Fluoranthene (mg/Kg)	< 0.27	—	—	
Indeno[1,2,3, c-d]pyrene (mg/Kg)	< 0.27	—	—	
Pyrene (mg/Kg)	13	—	—	

BS: oil-contaminated beach sand; FS: Uncontaminated fine sand; BMB: barley malt bagasse; [1]TPH: Total Petroleum Hydrocarbons; [2]PAH: Polycyclic Aromatic Hydrocarbons

2 Material and methods

2.1 *Sand and contaminant*

Oil-contaminated beach sand was collected from the foreshore of the municipality of Tamandaré (Pernambuco, Brazil) in September 2019 and kept stored in an open area away from access to bathers. The sand appeared as a dense, dark and oily material. The study also used samples of fine beach sand, free from hydrocarbon contamination as described in section 2.5. The physicochemical characteristics of the sands used in this study are summarized in Table 1. Also summarized is the characterization of barley malt bagasse (BMB) used in the preparation of the pre-inoculum.

2.2 *Pseudomonas aeruginosa TGC04*

The strain was originally recovered from sand soil located in a gas station and is registered in the Brazilian registry of genetic heritage and associated knowledge (#A404D65) and in the UFPEDA culture collection (1063B). For acclimation to the contaminant, a suspension of the strain was prepared in 0.85% NaCl, with turbidity standardized at an optical density (OD) of 0.4 at 600 nm (Cawley *et al.*, 2019). Then, 1 mL of the suspension was transferred to 100 mL of mineral medium, composed of (mg/L): K_2HPO_4 (500); $(NH_4)_2SO_4$ (500); $MgSO_4$ (500), $FeCl_2$ (10); $CaCl_2$ (10); NaCl (25); $MnCl_2$ (0.1), $ZnSO_4$ (0.01), yeast extract (500) and two drops of complex B solution, pH 7.2±0.2 (Del'Arco and de França, 2001), supplemented with 10 μg/mL PMS (Sigma-Aldrich, China) and completed with contaminated sand 1% (w/v). The system was incubated under shaking at 150 rpm, at 29±1°C for 5-7 days. After the oil emulsification, a new 1 mL

aliquot was transferred to a new bottle until reaching 10% of contaminated sand (w/v).

2.3 PYO production and quantification

Two synthetic exogenous phenazines were tested: PMS (Sigma-Aldrich China; batch #SYN1371310, purity 90%) and phenazine (PNZ) (Sigma-Aldrich Ukraine; batch #MKCG5144, purity 98%). The respective stock solutions were prepared in DMSO 1%. Three concentrations were tested: 1, 5 and 10 μmol/L. The solutions were added to 20 mL of cetrimide agar in Petri dishes and then the strain was inoculated by spreading and incubating for 72h at 29±1ºC.

PYO quantification was performed by solid-base extraction (Abou *et al*., 2018), with modifications. Briefly, the agar was cut into small cubes and transferred to tubes containing 20 mL of chloroform. After stirring for 10 min, the organic phase was separated and 10 mL of 0.2 mol/L HCl solution was added. Followed by further stirring, the phase containing protonated PYO (pink) was carefully neutralized with 1.5 mol/L Tris-HCl until it turned blue. The concentration of PYO (μg/mL) was determined by applying the value of the OD value at 580 nm of the neutralized phase to the equation:

$$PYO = [OD_{580} - 6 \times 10^{-4} \div 14.026] \quad (1)$$

2.4 Pre-inoculum

The pre-inoculum was prepared with adaptations to the strategy described by Innemanová *et al*. (2018) and previous findings from our group. Initially, 100 g of fine sand were pasteurized at 60ºC for 30 min (Franco-Hernández *et al*., 2003). Afterwards, BMB 0.5% (w/w) was mixed with the oil-contaminated beach sand (Oliveira *et al*., 2021) and 5 mL of the inoculum (≈10^4 CFU/mL), prepared in MWY broth (500 mg/L of yeast extract and mineral water *quantum sufficit*) (Viana *et al*., 2017) added or not to 0.1 mL of 10 μmol/L PMS. The mixture was incubated at 29±1°C for 10 days with the water content maintained at 60-70% of water holding capacity, corrected with sterilized distilled water (Innemanová *et al*., 2018). At the end of ten days, the cell density (CFU/g) had increased by two log units.

2.5 Bioaugmentation assay

Microcosms with a capacity of 400 mL were prepared, filled with 200 g of pasteurized sand, distributed in two layers: 50 g of fine sand as a base, completed with 150 g of oil-contaminated beach sand (Sundaram *et al*., 2013). Then, portions of the pre-inoculum were transferred to the microcosms and mixed again (inoculum:sand ratio 1:10; 1:100 and 1:1000). The microcosms were incubated at 29±1ºC for 21 days and the residual TPH concentrations of the 16 USEPA priority PAHs were determined by gas chromatography coupled with mass spectrometry using the USEPA 8015 and USEPA 8270 methods, respectively. Abiotic losses, i.e., the percentual of organic material degraded by non-living factors, were known calculated in microcosm a containing the same amount of sand, kept sterilized by adding silver nitrate 10% (m/w) (Vasudevan and Rajaram, 2001). Cell quantification in sandy soil was performed by pour plate and expressed in CFU/g.

2.6 Statistical treatment

All experiments were performed in triplicate and results expressed as the mean ± standard deviation. To verify the normal distribution of the data, the values were analyzed using the Shapiro-Wilk test. Homoscedasticity was tested and checked using the Levene test and when the data did not follow a normal distribution, the Kruskal-Wallis test was used, followed by the Conover test, adjusted by the Hochberg test.

3 Results

Initially, the PYO production capacity of *P. aeruginosa* TGC04 was verified in the presence of exogenous phenazines (Fig. 1). There was only PYO production in media containing 10 μmol/L of PMS (7.37±0.10 μg/L) and PNZ (0.54±0.10 μg/L). Without addition of exogenous phenazines, *P. aeruginosa* TGC04 produced 1.90±0.10 μg/L of PYO. Therefore, PMS was chosen for the bioaugmentation assay.

The initial C:N:P ratio in microcosms at the time of *P. aeruginosa* TGC04 inoculation was 100:0.1:0.003 (Table 1). After the treatment performed to test the effect of the addition of PMS on hydrocarbons degradation, there was a reduction between ≈37 and 56% of the 16 USEPA priority PAHs (abiotic loss =10%) in the ratios of the three tested inoculum:sand samples (Fig. 2 I). There was a preferential degradation of 4-6 ring PAHs (≈80-89%, p=0.02) and there was no enhancement of treated compared to microcosms without PMS (Fig. 2 III). Benzo[a]fluoranthene was the least assimilated PAH, while all the others were degraded, in particular, chrysene and pyrene, the two 4-6 ring PAHs most concentrated in the sand.

For the 2-3 ring PAHs (Fig. 2 IV), the size of the inoculum and the presence of PMS were important factors that influenced the removal of these hydrocarbons, ≈14 and 38% (p=0.01). The most prevalent 2-3 ring PAHs were phenanthrene, fluorene and anthracene; virtually, all of them were removed.

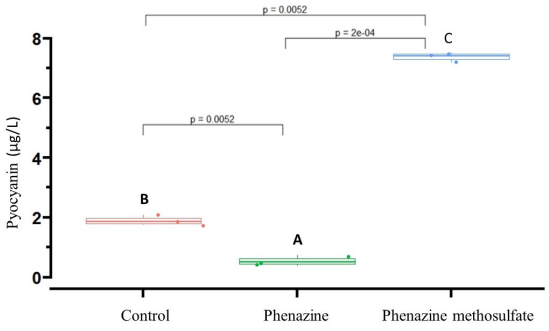

Figure 1. Pyocyanin production by *Pseudomonas aeruginosa* TGC04 in the presence of exogenous phenazines. Distribution other than normal (p = 0.007). Means of the triplicate different from each other (p = 0.027). Post-hoc result according to the Conover test: Both control (B) vs phenazine (A) and control vs Phenazine Methosulfate (C) (p = 0.0052); Phenazine (A) vs Phenazine Methosulfate (C) (p = 0.0002).

On the other hand, naphthalene was not transformed, suggesting that it was not bioavailable (Table 2). Additionally, the results with 2-3 ring PAHs (Fig. 2 IV) were similar to those observed for all of the 16 PAHs (Fig. 2 I).

TPHs were less degraded by the *P. aeruginosa* TGC04 strain (abiotic loss =40%) (Fig. 2 II). Only in the 1:100 condition without PMS was there a higher percentage of degradation (≈50%). Additionally, the most significant results were observed in treatments without PMS, and the 1:100 inoculant: sand ratio favored the greatest reduction in TPHs content.

Abiotic losses were observed at 10 and 40%, respectively, for the 16 USEPA priority PAHs and TPH, suggesting that *P. aeruginosa* TGC04 handled the heaviest fractions of the petroleum. Additionally, the cell density in the sand at the end of the treatment was ≈10^6 CFU/g. Possibly these were not altered because they were still in the log phase after the inoculation.

Table 3 shows that among the microcosms containing PMS, the 4-6 ring PAHs had the highest daily removal rates, under conditions 1:1000 and 1:10. Under this condition, the daily TPH removal rate was 3.3 times higher than in the microcosms lacking PMS. On the other hand, under conditions without PMS, when the ratio was 1:1000, removal rate was 15.6 times higher in TPH removal when compared to microcosms with PMS. For the 1:100 ratio, there was a 5.8-fold difference. At this ratio, the highest daily degradation rates of all tested hydrocarbons were observed.

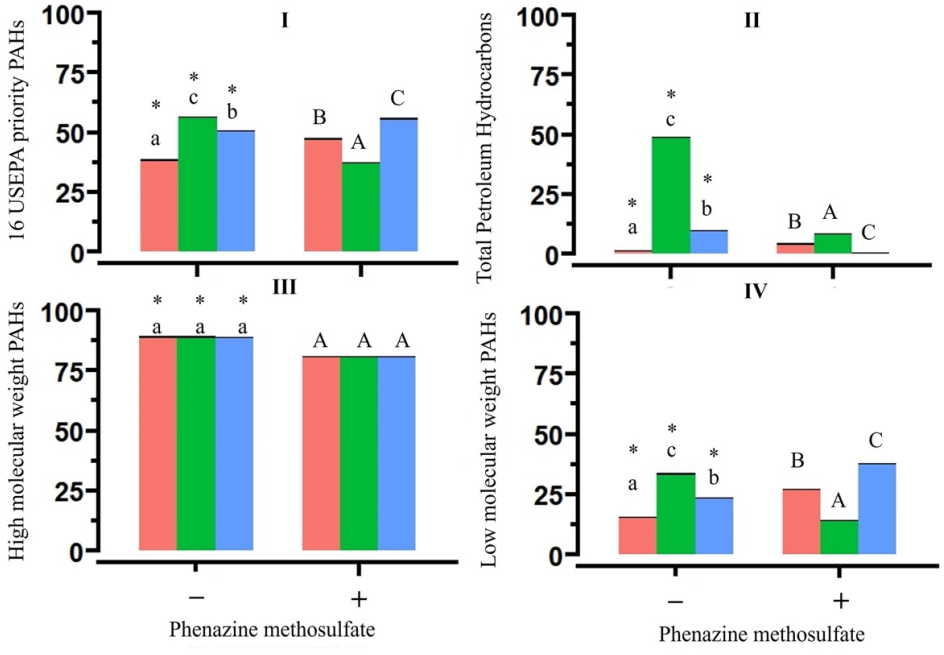

Figure 2. Effect of PMS on hydrocarbon reduction (%). Inoculum: sand ratio of 1:10 (red), 1:100 (green) and 1:1000 (blue). (+): presence and (-): absence of PMS in the microcosms: I (p = 0.01); and II (p = 0.01); III (p = 0.01); IV (p = 0.02).

Table 2. Hydrocarbon reduction by *Pseudomonas aeruginosa* TGC04 (p = 0.02).

Treatments	∑16 priority [1]PAHs	∑ 4-6 ring PAHs	∑ 2-3 ring PAHs	[2]TPH	[3]PYO (μg/L)
1:10 ([4]PMS)	47.3±0.1	81.0±0.1	27.1±0.1	4.2±0.1	
1:100 (PMS)	37.4±0.1	81.0 ±0.1	14.2±0.1	8.4±0.1	7.37±0.10
1:1000 (PMS)	55.8±0.1	80.9±0.1	37.8±0.1	0.6±0.1	
1:10	38.4±0.1	89.1±0.1	15.6±0.1	1.3±0.1	
0.11111111	56.4±0.1	89.1±0.1	33.5±0.1	49.0±0.1	1.90±0.10
0.73611111	50.7±0.1	89.0±0.1	23.7±0.1	9.8±0.1	

[1]PAH: Polycyclic Aromatic Hydrocarbons; [2]TPH: Total Petroleum Hydrocarbons; [3]Pyocyanin. [4]Phenazine Methosulfate. (1:10; 1:100, and 1:1000: inoculum: sand ratio).

4 Discussion

4.1 Allochthonous bioaugmentation

Hydrocarbon biodegradation is a complex process that requires metabolically capable microbes (Canul-Chan *et al.,* 2023). The hypothesis of this work was that exogenous phenazines can stimulate the production of PYO and, therefore, increase the degradation of petroleum hydrocarbons by acting on the metabolism of *P. aeruginosa* TGC04 in the presence of oil in an oxygen-rich environment. The hydrocarbonoclastic potential as well as the use of *P. aeruginosa* in the bioremediation of oil-contaminated soils is widespread (Suwardi *et al*., 2021; Wu *et al*., 2019). There is very little information, however, about the association between the degradation of hydrocarbons and phenazines (Viana *et al*., 2018, Das and Ma, 2013).

After an oil spill, the increase in organic matter in the soil caused by hydrocarbons results in nutrient imbalance and new generations of hydrocarbonoclastic microbes may be prevented from growing, enabling bioaugmentation treatment (Leys *et al*., 2005). The growth rate, the ability to use specific substrates and to overcome natural competition, however, are determining factors of the microbiota for the positive results of bioaugmentation of contaminated soil (Zhu *et al*., 2015; Duquenne *et al*., 1999). New bioaugmentation models can minimize certain limitations in this process (Fernandez *et al*., 2019). Some adopted strategies, such as allochthonous bioaugmentation, guarantee good results (Chen *et al*., 2019), as demonstrated by the present study.

Bioaugmentation is not indicated for prolonged treatments; it is an effective strategy, however, if applied in the initial and most critical phase of the intervention after the oil spill (Woź niak-Karczewska *et al*., 2019). It is reported that PAHs can be removed by up to 75% within 30 to 100 days by applying bioaugmentation (Cavalcanti *et al*., 2019). Thus, since the *P. aeruginosa* TGC04 strain removed more than 80% of 4-6 ring PAHs after 21 days, the selective use of this microorganism is considered fundamental for successful treatment. Brzeszcz *et al*. (2020) observed a reduction of almost 87% in oil, attributing the result to the hydrocarbonoclastic potential of this microorganism, as well as its persistence under hostile conditions. Because the composition of the microbiota can vary between soil types, as well as throughout the phases of bioremediation of oil-contaminated sites, the most appropriate choice of an added agent is a critical decision in the process (Radwan *et al*., 2019). Therefore, the introduction of a pre-adapted microbe increases the chances of positive results.

Pseudomonas spp. are microbes of particular interest in bioaugmentation because they exhibit characteristics crucial to the outcome of the process: 1) they are abundant in the soil; 2) easy to cultivate and with a high growth rate, even in the presence of unconventional substrates; 3) easy to manipulate and reintroduce into the soil, and 4) have notable metabolic versatility and production of active metabolites (Chin-A-Wong *et al*., 2003). Reports in the literature are that *P. aeruginosa* has crucial requirements in terms of oil catabolism and has shown highly promising traits during the screening of hydrocarbonoclastic populations (Chikere and Fenibo, 2018).

Although some *P. aeruginosa*'s metabolites such as PYO can alter the composition of microbial diversity and may result in a reduction in the percentage of hydrocarbon removal, the use of axenic culture of *P. aeruginosa* produces very positive results, no less effective than mixed cultures (Norman *et al*., 2004). Ilori and Amund (2000) described *P. aeruginosa* as the only microbe able to degrade 13 hydrocarbons relative to the 4 species they investigated. Mittal and Singh (2009) observed that 4 among 20 strains of *P. aeruginosa* degraded 20% of aromatic compounds in 60 days. Shekhar *et al.* (2014) described the growth of *P. aeruginosa* in the presence of 5 types of aromatic hydrocarbons in concentrations of up to 5% for 10 days, observing a more that were found to have a toxic, but not biocidal effect on the cell. Belo-Akinosho *et al*. (2016) discussed that *P. aeruginosa* best contributed to the return of fertility of agricultural soils contaminated by hydrocarbons (ratio

Table 3. Daily hydrocarbon removal rate (p= 0.01).

Treatments	Degradation rate (±0.10 mg/Kg)			
	∑ 16 priority [1]PAHs	∑ 2-3 ring PAHs	∑ 4-6 ring PAHs	[2]TPH
1:10 ([3]PMS)	2.25	1.29	3.86	0.2
1:100 (PMS)	1.78	0.68	3.86	0.4
1:1000 (PMS)	2.66	1.8	3.85	0.03
1:10	1.82	0.74	4.24	0.06
0.11111111	2.69	1.6	4.24	2.33
0.73611111	2.41	1.13	4.23	0.47

[1]PAH: Polycyclic Aromatic Hydrocarbons; [2]TPH: Total Petroleum Hydrocarbons; [3]PMS: Phenazine Methosulfate

inoculum:sand 1:20 and 1:200), among 44 isolates tested.

In the present study, a high rate of daily degradation of PAHs by *P. aeruginosa* TGC04 was observed. This occurred possibly because the inoculum remained longer in the stationary phase of bacterial growth as it uses different alternative metabolic pathways that interact with the basic core metabolism (Frimmersdorf *et al*., 2010). As a result, the inoculant can tolerate more toxic compounds in the oil, such as 4-6 ring PAHs, and these molecules commonly become preferred carbon sources (Vasconcelos *et al*., 2013). Karamalidis *et al*. (2010), unlike our study, observed a preference for 3 ring PAHs; only from the 21st day onwards, the other PAHs began to be degraded. On the other hand, Salam *et al*. (2011) found that two strains of *P. aeruginosa* degraded 90-92% of different hydrocarbons in 21 days, demonstrating more affinity for pyrene and crude oil; a daily removal rate of 4.32 to 4.38% was achieved, values approximate to what we found in our study with the 4-6 ring PAHs (Table 3).

The degradation of more recalcitrant molecules, such as 4-6 ring PAHs, may occur via cometabolism, where bioavailable 4 ring PAHs or 2-3 ring PAHs can be used as the cosubstrate (Vasconcelos *et al*., 2013). The degradation rate of PAHs is greater when contamination occurs in mixtures of PAHs. Sawulski *et al*. (2015) observed that within 20 days, the assimilation of phenanthrene had contributed to the removal of fluoranthene. Similarly, benzo[a]pyrene was removed in the presence of 4 ring PAHs. The 4-6 ring PAHs are more toxic than the 2-3 ring PAHs, but the induction of enzymes that degrade the heavier hydrocarbons may serve to remove the lighter hydrocarbons. This phenomenon has been described for treatments using pure cultures (Sawulski *et al*., 2015). Silva *et al*. (2009) observed that after 12 weeks (abiotic loss =20%), pyrene was rapidly consumed and participated in the removal of > 5 ring PAHs. On the other hand, although more naphthalene, anthracene and phenanthrene were removed than the 4-6 ring PAHs, they found residual concentrations of naphthalene. This was also observed in the present study, an event attributed to the fact that this compound had become less bioavailable.

Innemanová *et al*. (2018) described a 72% reduction of 5-6 ring PAHs in 4 months. As also observed in the present work, the more concentrated inoculant generated the least positive results in terms of degradation. This was because the inoculum was poor, but the authors were unable to explain its mechanism. In another investigation, it was found that there was no difference between free or encapsulated inoculum of the *P. aeruginosa* Spet strain since, in both cases, the PAHs were reduced by up to 89% within 191 days (Karamalidis *et al*., 2010). This shows that the methodology used in the present study was economical.

TPHs on the other hand, were not consumed in the same proportion as the PAHs; our findings, however, were similar to a 90-day study that assessed the addition of surfactant and the moisture content for stimulating growth of the autochthonous microbiota and favor the removal of TPH in biopile systems (Cisneros-de la Cueva *et al*., 2024). On the other hand, Haghollahi *et al*. (2016) found a 70% reduction in TPH in sandy soil only after 270 days. The authors assumed that the result obtained was c aused by the fact that TPHs degradation rates are higher in sandy soils since sand is a porous soil system, which facilitates oxygen transfer as well as microbe access to the pollutant. In pores < 3 mm, however, this property practically disappears. Given this, we assume that the lower degradation of TPHs in the present study was due to the characteristics of the oil mass encrusted in the agglomerates. The chemical complexity of extra-heavy crude oil is an even greater challenge due to the low availability of hydrocarbons. It is worth noting that *P. aeruginosa* TGC04 achieved 50% TPHs reduction in a significantly shorter time than previous studies (Haghollahi *et al*., 2016; Lladó *et al*., 2012).

4.2 Role of phenazines

Beach sands are sites where microbiota plays a key role in the balance of the coastal ecosystem (Disner and Torres, 2020). Oil contamination drastically

changes the C:N:P ratio of the soil and in response to nutritional stress, *P. aeruginosa* synthesizes different phenazines using intracellular levels of ATP (Özcan and Kahraman, 2015). In addition to generating energy for the cell, phenazines confer a selective growth advantage to their producers (Blankenfeldt *et al*., 2004). This contributes to phenazine-producing microbes, such as *P. aeruginosa*, becoming dominant following an oil spill episode (Norman *et al*., 2004).

Phenazines participate in biological fitness regulation across species (Fitzpatrick, 2009) and protect cells from hydrocarbon toxicity, through different mechanisms (Costa *et al.*, 2015). PYO is the main phenazine produced by *P. aeruginosa*, and although there is no correlation between pigment synthesis and resilience, the strains that produce the most PYO appear to be more significantly resistant to toxic compounds (Finlayson and Brown, 2011). In addition, the mechanisms by which PYO participates in the transformation of hydrocarbons have not yet been fully revealed. Two hypotheses have been proposed: the first involves the generation of reactive oxygen compounds that react with hydrocarbon molecules (JabÍ oń ska *et al*., 2023). The second hypothesis attributes to PYO the role of a terminal signaling factor in the quorum sensing of *P. aeruginosa.* This acts in the synthesis of biosurfactants that increase the oil contact area, its assimilation and metabolization into intermediates of the citric acid cycle, important for biomass production. and energy (Dietrich *et al*., 2006). In addition, the sugars required in the process come from the gluconeogenesis process (Das and Chandran, 2011).

PYO also participates in the formation of biofilms that protect *P. aeruginosa* from the toxic effects of hydrocarbons (Das *et al*., 2013). Biofilm growth depends on iron and redox active compounds such as phenazines can increase Fe^{2+} bioavailability (McRose *et al*., 2023). Iron is usually found in coastal areas and it acts as a barrier retaining and accumulating chemical species such as phosphorus (Charette and Sholkovitz, 2002). Furthermore, the limestone content of sand helps in the deposit and transport of iron, providing a scenario that enables microbe survival. It is important to note that a previous study had identified the iron content in the accident affected area as *circa* 5,000 mg/kg (Mirlean *et al*., 2013).

PMS was added to make phenazine available at the beginning of pre-inoculum growth, as well as to stimulate PYO production by *P. aeruginosa* TGC04. The introduction of an exogenous phenazine redox mediator was used because, in the log phase of bacterial growth, the production of phenazines is low due to catabolic repression resulting from the depletion of C and N sources; as well, there is a switch from planktonic to sessile lifestyle (Denning *et al*. 2003). PMS is an analogue of the PYO intermediate 5-methyl phenazine-1-carboxylic acid (5-Me-PCA), and in low concentrations it is non-toxic, enabling the development of colonies, tolerance to hydrocarbons and the formation of biofilms of *P. aeruginosa* in oxygen-limited environments (Sporer *et al*., 2018).

The function of PMS is to serve as a primary electron acceptor in a redox reaction, resulting in the generation of superoxide anion (O_2·) and hydroxyl radical (·HO) (Jahn *et al*., 2020). These free radicals, in addition to reducing intracellular NAD(P)H into NAD(P) (Yamaki and Muratsubaki, 2012), can also act on sensitive cells, reducing the growth of competitors (Wang and Coates, 2017).

Free radicals promote nucleophilic attack on hydrocarbons, especially aromatic and condensed aromatic ones, destabilizing the molecule (Unglaube *et al*., 2020). This may explain the greater reduction in PAH relative to TPHs. Our results supported this hypothesis, which should be further investigated in future work. Free radicals are products generated in aerobic processes in *P. aeruginosa*. A redox cycle mediated by NADPH is regulated by phenazines, reducing the redox destructive potential of free radicals. Therefore, PYO also regulates primary metabolism during the log phase of *P. aeruginosa* growth and keeps the cell stable in the environment, prolonging the stationary phase. The added PMS may have promoted nucleophilic attack on hydrocarbons and possibly increased oxidative stress. In order to neutralize the free radicals formed, *P. aeruginosa* TGC04 may have used PMS as an electron acceptor for NADPH, thus obtaining the means to guarantee biomass and energy production.

Additionally in soil systems, residual phenazines are degraded by some amidase-producing autochthonous microorganisms under aerobic and anaerobic conditions (Zhu *et al.*, 2023). These enzymes participate of the breakdown of amide bonds and may form products with little or no toxicity (Kapitanov *et al.*, 2023). Amidase is one of the representative groups of hydrolases necessary for ring cleavage in the biodegradation of oil by hydrocarbonoclastic microorganisms (Ramdass and Rampersad, 2023). Thus, further studies with consortia may unveil whether exogenous phenazines are effective in hydrocarbon-contaminated soil bioremediation in terms of optimization of bioaugmentation and reduction of abiotic losses.

Conclusion

In summary, *P. aeruginosa* is a species with high potential for use as a bioremediation agent for sandy soil systems polluted with heavy crude petroleum. The addition of exogenous PMS did not enhance the hydrocarbonoclastic activity of *P. aeruginosa*

TGC04, in terms of reducing PAHs and TPHs, nor did it accelerate the biodegradation process. The most dramatic observation of the present work was the fact that PMS participated in the removal of 4-6 ring PAHs and to a lesser extent in removal of TPHs, which contains many other heavy chain hydrocarbon fractions, but not those composed of aromatic or polycyclic compound. Overall, these results cannot be used to predict field performance, but they indicate that the best approach to remove PAHs appears to be allochthonous bioaugmentation, stimulating PYO production.

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Nomenclature

BMB	Barley Malt Bagasse
CFU/g	Colony Forming Unit per gram of soil/sand
PAHs	Polycyclic Aromatic Hydrocarbons
PMS	Phenazine Methosulphate
PNZ	Phenazine
PYO	Pyocyanin
TPH	Total Petroleum Hydrocarbons

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