

# Correlation between pyocyanin production and hydrocarbonoclastic activity in nine strains of *Pseudomonas aeruginosa*

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**Abstract**— The more pyocyanin produced by the hydrocarbon degrading *Pseudomonas aeruginosa* strains, the more hydrocarbons would be assimilated and transformed. To evaluate this assumption, we assessed the potential use of nine wild types of pyocyanin-producing *P. aeruginosa* in bioremediation. They exhibit concentrations of pyocyanin ranging from 0.08 to 28.68 µg/mL. Hydrocarbonoclastic activity in the presence of pyocyanin was determined by two protocols. First, a high correlation was found between the synthesis of pyocyanin and the emulsification index of lubricating oil. Second, two strains were tested for their ability to degrade anthracene and pyrene in soil by the concentration of pigment produced. Microcosms were filled with 250g of sterile sandy soil, supplemented with glycerin and then contaminated with a 20 mg/kg of mixture of the two compounds. The volume of the inoculum suspension ( $\approx 10^8$  CFU/mL) was equivalent to 5% of the soil mass contained in the microcosms. Static incubation lasted 60 days at 25°C. A roughly 60-fold difference between the pigment concentrations produced by the two strains resulted in an increase of 65 and 45% in the pyrene and anthracene biodegradation, also indicating that the molecule served as a co-substrate of pyrene degradation.

**Keywords**— Bioremediation; Biodegradation; Petroleum hydrocarbons; Pyocyanin; Pyrene.

## I. INTRODUCTION

*Pseudomonas aeruginosa* is a ubiquitous aerobic Gram-negative rod, measuring 0.5 µm in width by 1.5 µm in length and endowed with a single polar flagellum, whose preferential habitat is the soil (Abdul-Hussein and Atia 2016). The bacterium exhibits remarkable metabolic versatility, ensuring its persistence in environments with different physicochemical conditions, which may exert different degrees of selective pressures (Maia et al. 2009; Pirnay et al. 2005). In addition, *P. aeruginosa* can utilize more than 90 molecules as a source of carbon and energy (Scott-Thomas et al. 2010), among which are paraffinic (Karamalidis et al. 2010), naphthenic (Shekhar, Godheja, and Modi 2015), aromatic (Zhang et al. 2005) and polycyclic aromatic hydrocarbons (Filinov et al. 2010). This characteristic makes the bacterium an excellent choice as an agent of removal of these compounds in the environment (Salam 2016; Atzél et al. 2008).

*P. aeruginosa* synthesizes at least six different pigments: fluorescein (Allydice-Francis et al. 2012), pyoverdine (Yin et al. 2017), pyorubin A and B (Abu et al. 2013), pyomyelin (Ferguson, Cahilli, and Quilty 2007) and pyocyanin (Viana et al. 2017). The latter, a fluorescent phenazine compound of exuberant blue-greenish coloration is synthesized exclusively by 90-95% of the strains (Mavrodi et al. 2001) and its main function is to

participate in reactions involving the production of reactive oxygen species (Muller and Merret 2014) and in the reduction of the  $\text{Fe}^{3+}$  ion, an important growth factor for bacteria (Jayaseelan, Ramaswamy, and Dharmaraj 2009).

Most of the studies involving pyocyanin have focused on clinical issues, generally related to antibiotic multi-resistance (Kerr and Snelling 2009), antimicrobial activity (Martins et al. 2014) and immunological changes or responses caused by opportunistic infections (Sales-Neto et al. 2016). However, the synthesis of pyocyanin is associated to a response mechanism to environments with high selective pressures as hydrocarbon-contaminated soils (Bahari et al. 2017), allowing *P. aeruginosa* to persist as well as to use hydrocarbons as the sole source of carbon. This could be applied to the bioremediation processes.

Interest in the role of pyocyanin in the degradation of hydrocarbons is very recent and the topic is still little explored (Mangwani, Kumari, and Das 2015; Wu et al. 2014). The correlation with pyocyanin and biosurfactant synthesis was first reported in this decade (Das and Ma 2013). The production of 10.89  $\mu\text{g/mL}$  of the pigment reflected emulsification indices ( $E_n$ ) of three fuels between 60 and 75%, while another strain, whose production of pyocyanin was significantly lower, 4.81  $\mu\text{g/mL}$ , the  $E_{24}$  obtained were between 25 and 40%, reflected in a biosurfactant synthesis that was ten-fold less.

In order to verify the importance of the pigment in the hydrocarbonoclastic activity of *P. aeruginosa* strains and to assume that the more pyocyanin produced, the more hydrocarbons would be assimilated and transformed, we chose as the main objective of this study to evaluate the biodegradation of pyrene and anthracene in soil by wild type strains of *P. aeruginosa* and to correlate this with the concentration of pyocyanin.

## II. MATERIAL AND METHODS

### *Pseudomonas aeruginosa*

Nine wild type strains were tested. They were taken from the collection of the Laboratory of Environmental Microbiology of the Federal University of Paraíba, the samples having been collected by our research group from highly selective pressure environments, such as gas stations, activated sludge and wastewater, all in the city of João Pessoa-PB, Brazil (Cavalcanti et al. 2017). Some characteristics of these isolates are summarized in Tab. 1.

### Production of fluorescent pigments and determination of the concentration of pyocyanin

The production of pyocyanin and fluorescein was stimulated using traditional media (King, Ward, and Raney 1954). The inoculum was prepared with 0.85%

NaCl solution, standardized with tube No. 1 of the MacFarland scale, from fresh culture of each strain of *P. aeruginosa* on nutrient agar (APHA, AWWA, and WEF 2012). For the synthesis of pyocyanin, 1 mL of the suspension was transferred to conical flasks containing 200 mL of King A broth. Incubation occurred under 150 rpm shaking at  $29 \pm 1^\circ\text{C}$  for 72h (El-Fouly et al., 2015). To detect fluorescein, King B agar was used. Static incubation was conducted under the same temperature and time conditions as described above.

Pyocyanin extraction was conducted by combining the two methodologies (Nowroozi, Sepahi, and Rashmonejad 2012; Hassani et al. 2011). A 10 mL volume of the King A broth containing the cells and the pigment dissolved after 72h was transferred to test tubes and then 3 mL of chloroform was added. After vigorous vortexing, the tubes were allowed to stand for 2 hours and the pyocyanin in the protonated form was retained in the chloroform phase, which turned blue. Then, 1.5mL of the blue phase was transferred to another tube and 1 mL of a 0.2 mol/L HCl solution was added. After vigorous stirring, pyocyanin was obtained in its red acidic form. The tubes were also allowed to stand for 2 hours. The concentration of pyocyanin ( $\mu\text{g/mL}$ ) was estimated by measuring the optical density of the acidified supernatant at  $\lambda=520\text{nm}$ . The measured value was multiplied by the molar extinction coefficient 17.072 (Das and Ma 2013) and subsequently corrected for the ratio between the spent volume of chloroform in the extraction and the rate for acidification. The assay was performed in duplicate.

### Index of emulsification of hydrocarbons ( $E_n$ )

Each strain was inoculated as previously described in nutrient broth. After incubation under agitation of 150 rpm for 24 h at  $29 \pm 1^\circ\text{C}$ , a volume of 2 mL was transferred to tubes, to which same volume of gasoline, kerosene and lubricating oil was added. The control of the test was performed with 1% SDS solution. The mixture of the aqueous and oily phases was accomplished by vigorous vortexing for 2 minutes and then allowed to stand.  $E_{24}$ - $E_{72}$  were calculated from the height measurement of the emulsion layer, divided by the height of the total volume in the tube, multiplied by 100 (Naem et al. 2017). The test was performed in duplicate.

### Biodegradation tests for polycyclic aromatic hydrocarbons (PAH)

The tests were conducted in sealed microcosms of polyethylene, filled with 250g of sterile soil contaminated with 10 mg/kg of both PAH anthracene and pyrene (Merk, Darmstadt, Germany), to which had been added 0.25 mL/kg glycerol (Vasconcelos, Oliveira, and de França 2013). The function of glycerin was to serve as an

alternative source of carbon for the production of biomass as well as co-substrate of the PAH removal process, representing 0.32 mg/kg of carbon. The PAHs dissolved in acetone were sprayed on the soil (Eom et al. 2007), which was then stirred and allowed to stand for 30 minutes in a sterile environment until solvent volatilization. The soil characterization is summarized in Tab. 2.

Two strains were tested: TGC07 (PYO+) and TGC06 (PYO-). The pre-inoculum was prepared from the cultures of the King A broth, incubated under agitation at 150 rpm for 72 h at  $29 \pm 1^\circ\text{C}$ . Then, a volume of the bacterial suspension containing approximately  $10^8$  CFU/mL was added to the microcosms, equivalent to 5% of the soil mass contained in the microcosms (Palittapongampim et al. 1998) and obtaining moisture content of about 20%. Incubation occurred for 60 days at  $25^\circ\text{C}$ . The abiotic losses were estimated in the microcosms containing the contaminated sterilized soil, added with 10% (w/v) silver nitrate solution (Vasconcelos, de Frsança, and Oliveira 2011). The test was conducted in duplicate. The levels of anthracene and pyrene were detected by GC-MS (Method 8270C) (USEPA 1996). The extracts were obtained by soxhlet extraction using dichloromethane (Method 3540C) (USEPA 1996) and the preconcentration of the samples was conducted under  $\text{N}_2$  atmosphere.

#### Complementary tests

The biomass value was determined by the dry weight technique (Olsson and Nielsen 1997). The *P. aeruginosa* strains were incubated with 150 rpm shaking in 200 mL of King A broth for 72h. After, a 10 mL volume was centrifuged at 10,000 rpm for 15 minutes. The cells were rinsed three times with a 0.85% NaCl solution and at the end, 10 ml were resuspended and incubated at  $80^\circ\text{C}$  for 24h. The biomass, in dry weight (mg/L), was calculated from the difference between the masses before and after the incubation. The test was performed in duplicate.

The estimate of the mineralized  $\text{CO}_2$  in the microcosms was performed as described by Severino et al. (2004) each tendays during the biodegradation tests. A vessel containing 25 mL of the 0.5 mol/L NaOH solution was left inside the microcosms. NaOH was titrated with 0.5 mol/L HCl solution in the presence of phenol red. The amount of  $\text{CO}_2$  (mg/kg of soil) produced by the strains was estimated by the difference between the spent volume of acid to neutralize the base in the control and in the treatment, using equation Eq. (1):

$$\text{CO}_2 = [(V_1 - V_0) \times 44] \div 0.25 \quad (1)$$

Where  $V_1$  - volume (mL) of HCl required to neutralize NaOH in the treatment;  $V_0$  - volume (mL) of HCl required to neutralize the base in the microcosm control; 44 - the molecular weight of  $\text{CO}_2$ ; and 0.25 - mass (kg) of the soil in the microcosm. The focus of the

following methodology was to estimate gas emission in order to discount the estimated values between only two conditions: in the first condition, we aimed to detect microbiological stimulation due to the addition of glycerol rather than to verify from which specific carbon source the  $\text{CO}_2$  had been produced. In the second condition, we wanted to find out the amount of  $\text{CO}_2$  produced by abiotic reactions in order to avoid overestimating gas emission. Biotic activities in uncontaminated soil were not considered in this case because only microbiota were used in the microcosm tests.

#### Statistical treatment

The Pearson correlation between the concentration of pyocyanin and biomass produced with  $E_{72}$  was verified, compared to low-pyocyanin-producing strains. We used the IBM® SPSS® Statistics version 21 program, considering significant if  $p < 0.05$ .

### III. RESULTS

#### Production of pyocyanin and correlation with emulsification index

The concentration of pyocyanin produced ranged from 0.08 to  $28.68 \mu\text{g/mL}$  on average. Alteration of the medium staining occurred in the 48-72h-incubation interval. The strains that produced less than  $0.80 \mu\text{g/mL}$  were coded as PYO- and the others as PYO+. This value represents 10 times more than the lowest concentration detected. Regardless of the higher or lower concentration of the pigment, the determined biomass values were similar, ranging from 8 to  $10 \text{ mg/L}$ , as presented in Tab.3.

Throughout the emulsification test, when the indices were registered, they increased, reaching the maximum in  $t=72\text{h}$ . These values are presented in Table 4. The strains were better able to emulsify the kerosene and especially the lubricating oil, to the detriment of the gasoline.

In descending order, the percentages of strains that emulsified the fuels were: 100% (lubricating oil), 89% (kerosene) and 22% (gasoline). The  $E_{72}$  in the control was higher than the maximum obtained by the *P. aeruginosa* strains with the gasoline, but lower than that verified in the lubricating oil, except for the PYO- strains. In relation to kerosene, the results were quite different:  $E_{72}$  was the same as that determined by five strains, inferior to three (TGC01, TGC03 and TGC09) and superior to one (TGC06).

Among the PYO+ strains, a 96.7% correlation was found between the synthesis of pyocyanin and  $E_{72}$  of the lubricating oil ( $p=0.07$ ). A high correlation (92.6%) was also obtained between  $E_{72}$  of the lubricating oil and kerosene ( $p=0.023$ ). Although statistically non-significant ( $p=0.18$ ), there was a 70.7% correlation between biomass production and pyocyanin synthesis. The same pattern,

however was observed among PYO<sup>-</sup> (72.2%,  $p = 0.28$ ). No other comparisons were statistically significant.

#### Biodegradation of anthracene and pyrene

The percentages of removal of the two PAH by TGC07 (PYO<sup>+</sup>) and TGC06 (PYO<sup>-</sup>) were different, in terms of the pyocyanin concentration. TGC07 produced approximately 60 times more pyocyanin than TGC06 and archived higher biodegradation rates of the anthracene and pyrene. After 60 days of processing, the performance of TGC07 in both compounds degradation was about 45 and 65% higher than TGC06. There was a preferential degradation of the anthracene to the detriment of pyrene in both strains, and TGC07 produced 25% more CO<sub>2</sub>, compared to TGC06. The results obtained are presented in Tab. 5.

### IV. DISCUSSION

#### Production of pyocyanin

The particular nutritional and metabolic versatility presented by *P. aeruginosa* makes the bacteria common to different environments (Bellin et al. 2014). In some of these environments, the nutritional shortage especially related to PO<sub>4</sub><sup>-3</sup> and Ca<sup>+2</sup> ions, forces the pyocyanin-producing strains to exhibit the pigment (Whooley and McLoughlin 1982). The synthesis of pyocyanin can be a sign of a resistance factor to certain compounds present in the environment, which may be toxic to other microorganisms, such as heavy metals (Muller and Merrett 2004), degermants (Lefebvre et al. 2017), dyes (Sarioglu et al. 2017) and petroleum hydrocarbons (Mittal and Singh 2009).

Although organic matter is widely available in these environments, the selective pressure exerted on the microbiota forces *P. aeruginosa* to combine mechanisms to persist (Deng 2012), thus enabling the ability to triumph over competing organisms (Özcan and Kahraman 2015). On the other hand, factors other than pyocyanin, such as alginate production, rhamnolipids and adhesins, among others (Winstanley, O'Brien, and Brockhundst 2016; Das et al. 2014), favor the resilience of *P. aeruginosa* since the pigment is not synthesized by 5 to 10% of the strains (Finlayson et al. 2011).

In aqueous media, concentrations of pyocyanin may range from 0.31 to 80 µg/mL (Hassani et al. 2011; El-Shouny, Al-Bidani, and Hamza 2011). *In vitro* production display of the pigment occurs within 48 hours if specific conditions of temperature and agitation are offered, i.e., 30°C and 150rpm (Agrawal and Chauhan 2016). With the King A medium, the pyocyanin is synthesized during the final part of the log phase and at the beginning of the stationary phase (Cabeen 2014). This time is dependent on the generation time of the strains. Under these cultivation

conditions, *P. aeruginosa* tends to have a generation time ranging from 3 to 6 h (Vasconcelos, Lima, and Calazans 2010; Tamagnini and Gonzales 1997), which justifies the appearance of the blue-greenish coloration of the strains of the present study, between 48 and 72 hours after the beginning of the incubation. However, the culture medium also influences this result. In mineral broth and GSNB, for example, pyocyanin was detected diffused in the medium, after 96h of incubation at 37°C (El-Fouly et al. 2015).

Three strains exhibited concentrations higher than 20 µg/mL of pyocyanin after 72h of incubation and the maximum value obtained was TGC02 (28.68±0.05 µg/mL). This concentration was similar to that obtained in a recent study, using the same incubation conditions, 26.12 µg/mL, and when the main source of nitrogen was 13 g/L peptone (Agrawal and Chauhan 2016). On the other hand, studies that used some modifications of the traditional methodology obtained amounts of the pigment between 9.3 and 42.0 µg/mL (Barakat et al. 2015; El-Fouly et al. 2011). Thus, for optimization in the process, aiming towards the use of PYO<sup>+</sup> in the bioremediation of hydrocarbons, higher concentrations of pyocyanin might be obtained in these strains, suggesting future research on the subject.

#### Correlation of E<sub>n</sub> and biomass production with the synthesis of pyocyanin

In the environment, one of the main roles played by *P. aeruginosa* includes the mineralization of several natural or synthetic compounds (Frimmersdorf et al. 2010). Due to the ability to transform organic matter into biomass and energy, the bacterium represents a potential bioremediation agent for soils contaminated by hydrocarbons (Das and Chandran 2007; Zhang et al. 2005). Ecologically, pigment synthesis guarantees many advantages to *P. aeruginosa*, even though antimicrobial activity against bacteria (Jayaseelan, Ramaswamy, and Dharmaraj 2014) and fungi (Sudhakar and Karpagam 2011) is still the most investigated property of pyocyanin.

In recent years, there has been a growing interest in the role of pigment in the biodegradation of hydrocarbons (Das and Das 2015; Das et al. 2013). Although very recent, the question is important for the petroleum industry, as it provides a better understanding of the participation of the pyocyanin in the processes of oil removal, given the potential of the application of *P. aeruginosa* in the interventions for the removal of oil at certain sites.

Previous studies have shown that the production of higher concentration of pyocyanin in PPGAS medium was proportional to the increase of E<sub>24</sub> in gasoline, diesel oil and hexadecane. The pyocyanin was shown to assist in the process of synthesis of tensioactive molecules,



indispensable for assimilation of these hydrocarbons by the bacterium. The highest index obtained was 50%, coinciding with the higher concentration of biosurfactant synthesized in PPGAS medium. It is important to note that pyocyanin has no emulsifying properties but may serve as a cellular signal for the synthesis of surfactant compounds (Das and Das 2015; Das et al. 2013).

In the present study, there was a high correlation between lubrication oil and kerosene  $E_{72}$ . The most statistically significant correlation, however, occurred with the  $E_{72}$  of the lubricating oil and pyocyanin synthesis. This indicated that the type of fuel had the highest influence as well as suggested that pyocyanin might be a species-specific factor involved in the degradation of the oil by *P. aeruginosa*.

The low  $E_{72}$  of gasoline and diesel possibly can be explained by the toxicity of these fuels. Both are very volatile, a characteristic that makes them more harmful to the cells, when compared to the hydrocarbons endowed with longer carbon chains, as in the case of kerosene (C11-C14) and lubricating oil (C20-C40) (Adam and Duncan 2002).

There were no significant differences in the determined values of biomass produced. It is known that one of the functions of pyocyanin is to participate in the process of assimilation of growth factors in environments with nutrient scarcity (Tredget et al. 2004). The production of pyocyanin in traditional media used in the routine of a Microbiology laboratory is based on the energy state of *P. aeruginosa*, which is reduced under conditions of low nutrient concentration, resulting in a decrease in the growth rate and an increase in the concentration of pigment. On the other hand, under favorable nutritional conditions, the energy generation capacity increases, reflecting in the growth rate, with repression of the synthesis of pyocyanin (Whooley and McLoughlin 1982).

This fact could not be observed in the present study. In theory, the biomass values of the five PYO+ strains should have been lower than the other four PYO- strains. However, the medium used in the test had peptone and glycerol in its composition, factors that stimulate pigment synthesis, as well as being responsible for the development of biomass (Norman et al. 2004). This association of nutrients justifies the high percentage of correlation between these variables.

To reinforce this observation, a recent study on the production of biosurfactants by *P. aeruginosa* strains showed a significant increase in microbial biomass when the peptone content was increased 3.5-fold and the glycerol concentration reduced to half of the content of the LB broth composition. Under these conditions, pyocyanin was also synthesized, because the oligopeptides present in

peptone serve as essential nutrients for the synthesis of fluorescent pigments (Das et al. 2015).

This opens new horizons to try to identify whether the  $E_n$  of certain hydrocarbons correlates better with the biomass or the concentration of pyocyanin or both. The findings of this work suggest higher correlations with the production of pyocyanin and, thus, indicate *P. aeruginosa* as one of the species with potential for applications in bioremediation.

### Effect of the concentration of pyocyanin on the biodegradation of PAH

PAHs are compounds with high mutagenic and carcinogenic potential, formed in incomplete combustion processes and released into the environment, the vast majority as a result of human activity (Romero et al. 2010). The physicochemical properties of PAH give these compounds a recalcitrant nature when present in soil (Van Herwijnen et al. 2003). When they have 2 and 3 rings, such as anthracene, they are referred to as low molecular weight PAH. Those containing 4 rings, such as pyrene, or above, are classified as PAH of high molecular weight (Daugulis and McCracken 2003). The assimilation and mineralization of PAH in soils is only possible for certain organisms that exhibit hydrocarboclastic activity, as in the case of *P. aeruginosa* (Bello-Akinoso et al. 2016).

Preferred degradation of a certain microbe by a class of PAH or to a specific PAH can be verified from the determination of the ratio of the initial and final concentrations of the high and low molecular weight of the PAHs. The negative value indicated that the preferential consumption of *P. aeruginosa* strains was by anthracene (Tolun et al. 2006). In a previous study, anthracene was also the most consumed PAH when mixed with pyrene (Dean-Ross, Moody, and Cerniglia 2002). However, in the literature the preferential consumption by high molecular weight PAH has been well documented (Cavalcanti et al. 2017; Vasconcelos, Oliveira, and de França 2013; Vasconcelos, de França, and Oliveira 2011; Bengtsson and Zerhouni 2003). The greater consumption of anthracene by TGC06 and TGC07 strains may be justified by some characteristics of the molecule: solubility in water (0.7 mg/L) and vapor pressure ( $2.55 \times 10^{-5}$  mmHg), higher than pyrene, which presents 0.145 mg/L and  $4.25 \times 10^{-6}$  mmHg, respectively (Bojes and Pope 2007; Mrozik, Piotrowska-Seget, and Łabużek 2003).

The degradation of the anthracene by *P. aeruginosa* begins with the oxidation at the 1,2-position of the molecule and in the sequence fission of the ring occurs, producing salicylate and catechol, which undergoes ortho- or meta-cleavage form intermediates of the tricarboxylic acid cycle (Yong and Zhong 2013). From these intermediates, the energy required for the anthracene to be

used as the co-substrate in the removal of the pyrene is provided. It is possible that with the depletion of glycerol, the anthracene assumed the co-substrate function, justifying its greater reduction by the two tested strains.

Cometabolism is one of the microbial strategies for the removal of recalcitrant compounds in soil. Cavalcanti et al. (2017) described the event by investigating the role of cakes from the processing of oleaginous plants as co-substrates during the removal of phenanthrene and pyrene, employing consortia composed of different pseudomonad strains. After 60 days, there was a preference for pyrene and part of this removal, about 80%, was aided by the consumption of phenanthrene, since there was no supplementation of the soil with another carbon source.

The co-substrates are also important for the maintenance of the biomass throughout the hydrocarbon removal bioprocess. In addition, an inoculum with high cell concentration ensures that the expected adaptation events, subsequent to the introduction of the strains in the soil, are not significantly affected and therefore do not reflect a reduction of the biodegradation rate in the first days (Baggi 2000).

Although the nutritional versatility of *P. aeruginosa* may simplify the reason for the removal of approximately 30% of the two PAH by TGC06, the literature proposes that the mechanism that can maintain the hydrocarbonoclastic activity in PYO- strains is based on the hypothesis that *P. aeruginosa* synthesizes other bioactive phenazines, including the final intermediate of pyocyanin biosynthesis, MPCAB (5-methylphenazine-1-carboxylic acid betaine), even if PYO- strains do not encode the conversion-related genes in pyocyanin (Chieda et al. 2008). In addition, most of phenazinic intermediates may act as auto-inducers for the synthesis of tensoactive compounds by *P. aeruginosa* (Bahari et al. 2017; Mangwani, Kumari, and Das 2015).

The concentration of glycerol employed may also have governed cell growth after the addition of the inoculum to the soil. A previous study evaluated different compounds as additional sources of carbon for the production of biomass in soils and glycerol exerted this function from the concentration of 0.07%. After 24h, more than 80% of the compound had diffused into the soil, particularly stimulating the cells near the diffusion regions (Duquenne et al. 1999).

The concentration of glycerol employed in the experiments resulted in a mass ratio of 100:3 between each PAH, individually, with glycerol. The literature reports that in a mass ratio of 100:8, there was preferential consumption of glycerol by a consortium made up of bacteria and fungi (Vasconcelos, Oliveira, and de França 2013). This interfered negatively in the removal

percentage of 16 priority PAHs in 60 days. When the mass ratio was reduced to 100:3, glycerol could be used as a co-substrate during the bioremediation process, resulting in a removal of about 70% of the same 16 priority PAHs after 60 days. It should be noted that the concentrations of anthracene and pyrene were ten and one hundred times lower than those used in the present study, but the percentages of removal of the two PAHs were about 40 and 71%, which reinforces the function of glycerol as an adjuvant and not the preferential source of carbon in both that and in our study.

The moisture content in the microcosms was appropriate for the biodegradation process of PAH. In addition to being essential for metabolic reactions to occur, the water extends the solid/liquid interface, increasing oxygen diffusion, as well as mass transfer ratio. In addition, water competes for the same PAH adsorption site in the soil, allowing an increase in the degree of removal of these compounds (Bengtsson and Zerhouni 2003; Ettrema and Wardle 2002).

The estimation of CO<sub>2</sub> produced is a simple alternative monitoring tool and can be applied without the obligation of quantification of the cultivable biota (Amadori, Fumagalli, and Mello 2009). Under the conditions offered in the experiments, the emitted concentrations of CO<sub>2</sub> were compatible to those determined in a natural soil, of semi-arid region, with moisture content similar to the one used in this work (Zhang et al. 2003). This indicates that there was a growth stimulation in TGC7 and TGC06 by the addition of glycerol. Other supplements available in the literature produced distinct responses. While the addition of 40 mg/kg of peanut and sesame cakes contributed to the generation of about 500 mg/kg of CO<sub>2</sub> in 60 days (Cavalcanti et al. 2017), much lower values (between 2.4 and 35 mg/kg) were obtained by using 10% (w/w) of sugarcane bagasse, manure and castor cake (Severino et al. 2004).

The diffusion constant of pyocyanin in the soil is  $0.5 \times 10^{-9} \text{ m}^2/\text{s}$ , which in solid media represents a diffusion time of 3 minutes between two points measuring 1  $\mu\text{m}$ , distant from each other, 1 mm (Bellin et al. 2014). It is important to emphasize that pyocyanin, as a bioactive compound, the *in situ* application of pyocyanin-producing strains, may lead to disturbances of the microbiota present in the area to be treated. The same can be intuited when *P. aeruginosa* is investigated on a laboratory scale. A study on the subject was conducted and verified that some members of the microbial community were inhibited in the presence of 9.5M of pyocyanin, and even then, after 50 days of the process, there was specific removal of dibenzothiophenes, naphthalene, and C29-C30 Hopanes (Norman et al. 2004). It was concluded that the

use of pyocyanin-producing strains is possible, since the excreted pyocyanin may affect other microorganisms, even though this inhibition will be limited to the space surrounding the *P. aeruginosa* colony in the soil (Ajello and Hoadley 1976).

Because pyocyanin assumes the role of auto-inducer in cell signaling processes that result in the synthesis of surfactant compounds, the molecule emerges as an interesting research target, among the possible metabolic strategies employed by *P. aeruginosa* in hydrocarbon mineralization (Vinckx et al. 2010). Biosurfactants secreted in the soil favor the displacement of the bacterium towards PAH and consequently its assimilation (Kaskatepe and Yildiz 2016; Alsohim et al. 2014). In addition, pyocyanin when involved in the *P. aeruginosa* density sensing mechanism may also participate in the expression of genes involved in the development of biofilms and favor the degradation of some PAHs, including pyrene (Mangwani, Kumari, and Das 2015). If we consider that similar results by PYO- in the anthracene and pyrene degradation, it is possible that other phenazines rather than pyocyanin may have been involved in the metabolism of hydrocarbons by *P. aeruginosa*. A deepening of these new fronts of research that have emerged from this work guides and encourages future studies that will try to better understand the mechanisms of this process.

## V. CONCLUSION

Under the conditions established by the present study, pyocyanin was involved in the emulsification of lubricating oil and biodegradation of two PAH by *P. aeruginosa*. However, the strains continued to exert hydrocarbonoclastic activity even when pyocyanin was synthesized in low concentrations. The preliminary results suggest that the phenotypic criterion of pigment production may be relevant in the choice of *P. aeruginosa* aiming complex hydrocarbons biodegradation propouses.

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## TABLES

Table.1: Phenotypic characteristics and origin of *Pseudomonas aeruginosa* strains

Strain	Isolation site	Pyo	Flu	Growth at 42°C	Act	Cet
TGC01	Soil (gas station)	+	—	+	+	+
TGC02	Soil (gas station)	+	+	+	+	+
TGC03	Soil (gas station)	+	+	+	+	+
TGC04	Soil (gas station)	+	+	+	+	+
TGC05	Soil (gas station)	+	+	+	+	+
TGC06	Soil (gas station)	+	—	+	+	+
TGC07	Soil (gas station)	+	+	+	+	—
TGC08	Activated sludge	+	—	+	+	+
TGC09	Wastewater (pigsty)	+	—	+	+	+

Ace – acetamide utilization test; Cet – resistance to ceftriaxone; Pyo – pyocyanin; Flu – fluorescein.

Table.2: Physical and chemical characterization of soil

Parameter	Result	Reference
pH	7.7±0.2	(EMBRAPA 1979)
Holding water capacity (%)	30.1±0.7	(Watwood, White, and Dahn 1991)
Humidity (%)	0.31±0.01	(EMBRAPA 1979)
Grain size distribution (%)		(ABNT 1984)
Clay (< 0.02 mm)	0.99	
Silt (0.002-0.02 mm)	1.29	
Fine sand (0.02-0.2 mm)	21.42	
Medium sand (0.2-0.5 mm)	41.51	
Coarse sand (0.5-1.0 mm)	32.93	
Gravel (> 1.0 mm)	1.86	
Total organic carbon (mg/Kg)	7.4	(USEPA 1996)
Total N (mg/Kg)	31.8	(USEPA 1993)
Total P (mg/Kg)	3.4	(USEPA 1978)

Table.3: Pyocyanin concentration and culture biomass (72h)

Strains	Pyocyanin( $\mu\text{g/mL}$ )	X (mg/L)
TGC01	8.02 $\pm$ 0.01	8.0 $\pm$ 0.1
TGC02	28.68 $\pm$ 0.05	10.0 $\pm$ 1,4
TGC03	8.99 $\pm$ 0.38	8.0 $\pm$ 0,2
TGC04	20.33 $\pm$ 1.98	8.0 $\pm$ 0.1
TGC05	0.13 $\pm$ 0.01	8.0 $\pm$ 0.1
TGC06	0.36 $\pm$ 0.02	9.0 $\pm$ 0,2
TGC07	21.54 $\pm$ 0.33	8.0 $\pm$ 0,1
TGC08	0.08 $\pm$ 0.04	8.5 $\pm$ 0.7
TGC09	0.10 $\pm$ 0.01	8.5 $\pm$ 0.1

Table.4:  $E_{72}$  (%) for fuels from *Pseudomonas aeruginosa* strains

Strains	Fossil fuels		
	Gasoline	Kerosene	Lubricating oil
TGC01 (PYO+)	–	5.0 $\pm$ 0.0	50.0 $\pm$ 0.2
TGC02 (PYO+)	–	10.0 $\pm$ 0.0	100.0 $\pm$ 0.0
TGC03 (PYO+)	–	7.5 $\pm$ 0.1	55.0 $\pm$ 0.5
TGC04 (PYO+)	–	10.0 $\pm$ 0.0	87.5 $\pm$ 0.3
TGC07 (PYO+)	7.5 $\pm$ 0.1	10.0 $\pm$ 0.1	97.5 $\pm$ 0.1
TGC05 (PYO–)	–	10.0 $\pm$ 0.1	10.0 $\pm$ 0.2
TGC06 (PYO–)	–	22.5 $\pm$ 0.1	25.0 $\pm$ 0.1
TGC08 (PYO–)	2.5 $\pm$ 0.2	10.0 $\pm$ 0.0	5.0 $\pm$ 0.1
TGC09 (PYO–)	–	–	10.0 $\pm$ 0.5

Control (1% SDS) – gasoline (37.5 $\pm$ 0.2), kerosene (10.0 $\pm$ 0.0) and lubricating oil (47.5 $\pm$ 0.2).

Table.5: Removal of anthracene and pyrene after 60 days of bioprocess\*

Strains	Pyocyanin ( $\mu\text{g/mL}$ )	Biodegradation (%)		$\Delta$	CO <sub>2</sub> emission (mg/Kg)
		Anthracene	Pyrene		
TGC07	21.55 $\pm$ 0.92	62.0 $\pm$ 0.1	49.2 $\pm$ 0.1	- 0.25	422.4 $\pm$ 0.5
TGC06	0.36 $\pm$ 0.08	34.1 $\pm$ 0.6	29.8 $\pm$ 2.3	- 0.06	316.8 $\pm$ 0.8

\* Results were calculated with abiotic loss of 15.1 $\pm$ 0,1%

$\Delta$  – ratio of initial and final concentration between anthracene and pyrene