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EARTH SCIENCES

Oil Bioremediation in a Tropical Contaminated Soil Using a Reactor

CATALINA TREJOS-DELGADO, GLORIA E. CADAVID-RESTREPO, ANGELINA HORMAZA-ANAGUANO, EDISON A. AGUDELO, LEONARDO BARRIOS-ZIOLO, JUAN CARLOS LOAIZA-USUGA & SANTIAGO A. CARDONA-GALLO

Abstract: This research was implemented in the Colombian Amazon forest area; to assess the effect of Tween-80® surfactant in the degradation of the Total Petroleum Hydrocarbons (TPH) in bioremediation treatments under aerobic conditions in the laboratory and pilot-scale. One control treatment, Natural Attenuation (AT) and four biostimulation treatments with leonardite with four different dosages of Tween-80® were proposed. The efficacy of organic stimulators and nonionic surfactant in soil microbiota was studied at laboratory and pilot scales, the latter in a passive aeration reactor. The test that presented a better performance was carried out with a Convective Flow Reactor (CFR) at pilot-scale. The results showed that bioremediation strategies improved the natural degradation process; the best outcomes were obtained in a treatment that includes Leonardite and Tween-80® (1.5 g/L) with 52% TPH degradation in 80 days (d). Tween-80® produced an effect in TPH solubility, and increased the production of CO2 in distinctive bioremediation treatments in both periods. The kinetics of CO2 production showed that the system required a periodic addition of a co-substrate as well as an increase of soil microbiota through the addition of compost (pilot scale). In this stage more than 76% of contaminant was degraded in 60d.

Key words: Bioremediation, petroleum hydrocarbon, oil spill degradation, amazon forest.

INTRODUCTION

Attacks on infrastructures, including oil infrastructure, have been adopted as a war tactic by armed groups, especially the National Liberation Army (ELN) and the FARC, with dire environmental consequences (Morales 2017). In Colombia, soil pollution caused by oil is a result of 2500 oil infrastructure attacks from 1980 to 2015 that caused 4.1 million barrels of crude spills into soils and rivers (ECOPETROL 2015). The ecosystems such as the tropical forests of the Amazon play a crucial role in mitigating the effects of climate change (Morales 2017). Soil degradation is currently one of the most serious environmental problems on the planet. During the International Year of Soil held by the FAO (Food and Agriculture Organization of the United Nations) in 2015, it was established that 33% of soils have degraded due to a number of factors such as organic pollutants derived from the oil industry. Crude oil (petroleum) is a highly complex mixture of organic compounds that causes damage to superficial water, human health, plants and animals, reducing growth and interfering with their normal development (Li et al. 2010). The main concern is that 75% of oil is composed of aromatic and aliphatic compounds (Fukui et al. 1999), which have toxins, mutagenic and carcinogenic features

that can be easily transported from the soil matrix to groundwater, damaging the health of populations surrounding the spill area (Halder et al. 1984, Wolicka et al. 2009). In addition, according to the EPA (Environmental Protection Agency), petroleum and its derivatives are classified as priority environmental pollutants, as a result of the adverse effects they cause on human health and the environment (de Souza Pohren et al. 2016, Grace Liu et al. 2011).

To mitigate this problem, the use of physical, chemical and thermal remediation techniques has been implemented, however these processes usually require high energy consumption, specialized machinery, and vast economic resources (Ball et al. 2012). Therefore, the application of biological treatments in the decontamination of soils polluted by oil and its derivatives has been a subject of constant study due to the fact that they are unconventional, environmentally sustainable and cost-effective methods (Franco et al. 2015, Rojas & Hormaza 2015). In this sense, biological stimulation and bioaugmentation technologies stand out among biologic treatments. The first technology is used by adding nutrients or electron receptors in order to increase the native activity of the soil (de Souza Pohren et al. 2016). In the bioaugmentation technology, allochthonous microorganisms are introduced to the contaminated site (Chen et al. 2015). It is important to point out that both technologies work to improve the bioavailability of the contaminant to be processed by soil microbiota, physic and chemical conditions in the affected soil. In the specific case of treatment of soils affected by oil spills, it is known that this type of pollutant, due to its chemical nature, is strongly fixed to the soil matrix, restricting its biodegradation by microorganisms and generating an imbalance in soil physicochemical conditions (Rojas-Avelizapa et al. 2007). Thus, various solutions have been proposed for this phenomenon generated by the hydrophobic characteristic of crude oil. Several studies conclude that the use of non-ionic surfactants such as Tween-80® in biostimulation processes greatly improves the soil condition, (Jayashree & Vasudevan 2007, Pinto et al. 2007) and the addition of soil conditioners such as Leonardite encourages ion exchange of the soil, enabling an improvement in its physical and chemical conditions (Turgay et al. 2010). For the above, it is important to carry out a scaling process technique, guaranteeing that the results obtained in laboratory scale are maintained in a pilot-scale. Among the most suitable alternatives to achieve this purpose are static convective bed reactors, which have been widely used for stabilization of complex organic waste (Young et al. 2016), being highly economical in terms of design and maintenance (Yu et al. 2009). They also generate an effective oxygen transfer in large volumes of soil for aerobic processes. The present study it was evaluated the effectiveness of Tween-80® surfactant under aerobic conditions at laboratory and pilot-scale using organic microbiota stimulators on soil contaminated by an oil spill. In order to develop this purpose, a passive aeration technique was used as a strategy to promote a fast and effective decomposition of oil pollutants.

MATERIALS AND METHODS

Study site, sampling and physicochemical characterization of the soil

The contaminated soil used in the experimental phase was collected close to a crude distribution valve at the Valle del Guamuez municipality, in the Putumayo region, Colombia (South America) near to the Hormiga river (0° 24' 53.75" N and 76° 53' 31.05" W) at 310 meters above sea level. The localization to the sampled site is showed in the figure 1. The mean annual air temperature

was 23°C (±1°C), May was the coldest month with a mean temperature of 24.1 °C and October is the hottest month with a 25.2 °C mean. The annual mean precipitation was 3202 mm which fluctuated between 3000 to 4000 mm over a record year (IGAC 2014). The driest month is March with ± 206 mm and the wettest is May with ± 329 mm. The mean relative humidity was 82%. This region has contrasting soil types ranging from soils with incipient development such as Fluventic Dystrudepts, to soils with a medium-low degree of weathering, such as Oxic Dystrudepts under alluvial and coluvio-alluvial megafan position (IGAC 2014). Soils were well to poorly drained and deep, with high aluminum saturation and low to medium fertility. These soils have high rates of mineralization of organic matter and predominant soil textures are sandy loam and loam.

The soil sample was collected in September 2015; the sampling process was carried out according to the parameters established in the Environmental Protection Agency Manual (USEPA 1991). Surface soil samples were collected after removing the surface vegetation (litter fall) at 20 cm depth from the top. The compose random sample correspond to the most characteristic soil of the study area “Oxic Dystrudepts”, that cover 80% of this zone. The soil samples were dried at room temperature in the laboratory for a week. To ensure homogeneity of pollutants in samples, the soil was crushed and then passed through a 2 mm sieve. Subsequently, the soil was subjected to a physicand chemical analysis in which properties such as soil texture (Bouyoucos 1962), real density, bulk density (Dominguez & Aguilera 1989), pH, water content and total nitrogen (Jackson 1964), porosity, nitrate (Crosby et al. 1968); ammonia nitrogen (Nessler 1999), available phosphorus (Bray & Kurtz 1945), and organic matter (Walkley & Black 1934) were determined. These tests were carried out at the Medellin Campus of the Soil Sciences Laboratory at the Universidad Nacional de Colombia. The physicochemical characteristics of study soils are shown in Table I.

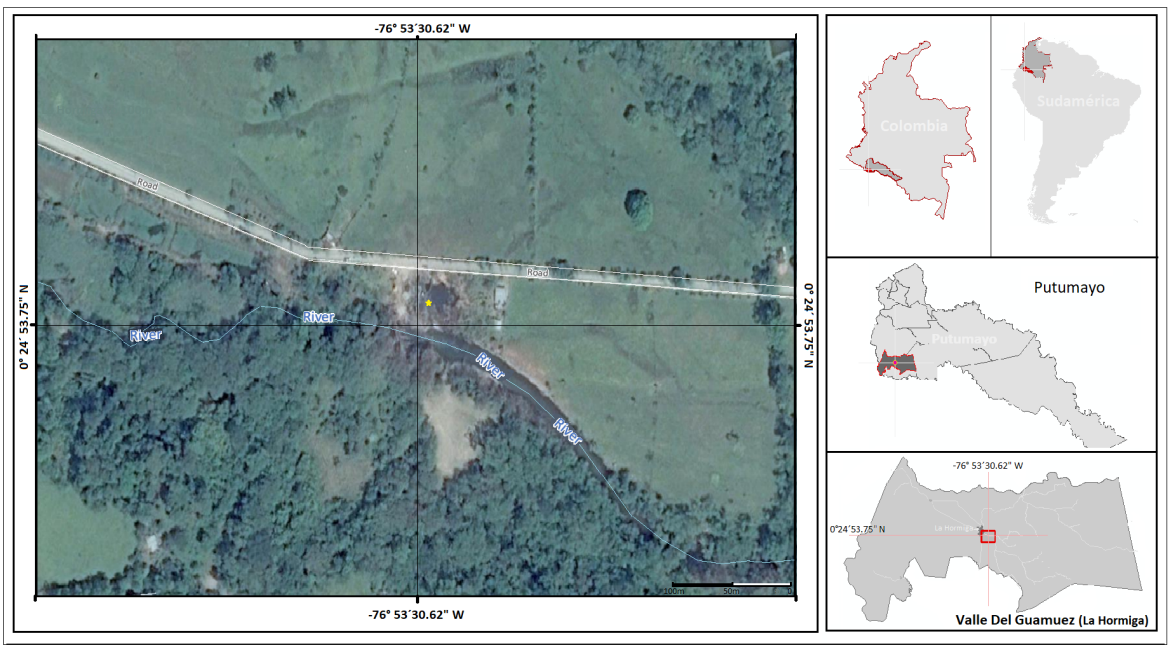

Figure 1. Soil sampled site at the Valle del Guamuez municipality (near to Hormiga River), in the Putumayo region, Colombia (South America).

Design of microcosms at laboratory scale

The efficiency of Tween-80® as a biostimulant agent in the presence of Leonardite was evaluated. This experiment was carried out with 2kg of soil in aluminum trays (25cm x 25cm) in a constant soil moisture content of 30% in all treatments. The dosage of nutrients in biostimulation treatments was calculated by stoichiometric ratios established by the McCarty method, between the concentration of TPHs present in the soil and the amount of nitrogen and phosphorus required to produce biomass and energy (Rittmann & McCarty 2001). Five treatments were replicated three times, the implemented treatments in this phase were: Natural Attenuation (AT), Biostimulation with Leonardite (BS), Biostimulation with Leonardite and 0.015 g/L Tween-80® (BI), Biostimulation with Leonardite and 0.75 g/L Tween-80® (BII), Biostimulation with Leonardite and 1.5 g/L Tween-80® (BIII). Leonardite was added at a constant concentration of 3% (Turgay et al. 2010). A completely randomized statistical design (CRD) was applied to the experiment, with three trials per treatment with the same soil, resulting in a total of 15 trials.

Quantification of TPH

Initial and final TPH concentrations were quantified using the methodology adapted from Standard Methods 5520F, coupled with a chromatographic profile obtained from Gas Chromatography - Mass Spectrometry (GC-MS) using a chromatograph Agilent GC-MS series 6890N. These tests were conducted for samples testing lab scale and pilot scale at the Instrumental Analysis Laboratory of the Medellin Campus at the Universidad Nacional de Colombia.

Table I. Physical and chemical properties of contaminated soil samples from Valle del Guamuez (Putumayo, Colombia).

Parameters	Type of soil		
	(CS)	(LS)	(RS)
Sand (%)	68	84	74
Silt (%)	24	12	20
Clay (%)	8	4	6
Texture	SL	SL	SL
pH	6.5	7.1	7.4
Water Content (%)	1,8	30	30
Real density (g/cm^3)	2.47	2.54	2.1
Bulkdensity (g/cm^3)	1.24	1.24	0.84
Porosity (%)	49	51	60
Organicmatter (%)	9.1	7.3	7.4
Phosphorus (mg/kg)	34	20	177
Nitrate (mg/kg)	1	90	175
Amonianitrogen (mg/kg)	9	180	7

CS: Contaminated Soil, LS: Laboratory Stage, RS: Reactor Stage, SL: Sandy Loam.

Determination of microbial CO_2 production

The production of carbon dioxide was used as an indirect measure of crude oil microbial degradation, and served as a quantitative variable to determine the best treatment. For the carbon dioxide quantification process, the parameters established were followed in each of the treatments (Celis et al. 2009). A 1.0L inverted container was placed on the surface of the soil. Inside each of them was a container with 15 ml of a 1M NaOH solution to absorb the CO_2 produced. Measurements of the amount of remaining (non-neutralized) NaOH were made through the incubation time (every 3 d - 4 d), by titration with 0.1 M HCl, replacing the respective NaOH trap with a new one each time. The results for carbon dioxide concentration for each treatment was extracted from 1.0 ml of NaOH of each treatment, and qualifier added 2.0 ml of 1.0 M to precipitate the inorganic carbon as insoluble. Subsequently, three drops of phenolphthalein were added as a base acid indicator, and the not neutralized NaOH was titrated directly with HCl (Lannotti et al. 2013). The quantity of product of the mixtures was calculated using the Anderson's formula (Anderson et al. 1978). This process was carried out at laboratory and pilot tests scales.

Bacterial culture of heterotrophic and hydrocarbonoclastic bacteria

A nutrient medium (Nutritious Agar, Difco™) was used to isolate the heterotrophic bacteria and a selective medium (Bushnell - Hass mineral medium supplemented with 1% crude oil in hexane as a carbon source) for hydrocarbonoclastic bacteria. In order to estimate the microbial counts, a serial dilution technique was used to determine the growth of heterotrophic and hydrocarbonoclastic bacteria. The incubation period for the nutrient and selective medium was carried out for two and five days, respectively. The equation developed by Madigan et al. (2004) was used to calculate the CFU /g soil.

$$\frac{CFU}{g\ soil} = No\ colonies \times \frac{dilution\ volume}{dilution\ factor} \times \frac{1}{g\ soil}$$

Isolation and molecular identification of bacterial strains

The predominant bacteria were isolated from the implemented microcosms; the bacterial individual with different morphotype in the different medium (color, shape and border of colonies) was selected; and its DNA was extracted using the cell lysis protocol (Silva-Bedoya et al. 2016). To amplify the 16S rRNA gene for isolated identification, 27F and 1492R Espejo et al. (1998) primers were used. All amplification products were purified and sequenced (Macrogen®). The sequences obtained were edited using Chromas Pro and Mega version 7 developed by Kumar et al. (2016). To determine the phylogenetic affiliation of the microbial isolates, similarity searches and phylogenetic analyses were performed using the Basic Local Alignment Search Tool (BLASTN®). The phylogenetic analysis was established with a dendrogram using the Neighbor-Joining method with 1000 repetitions of the Tamura 3 parameter model according to Tamura (1992), taking the Gamma distribution with five category rates into account. This analysis was completed using Molecular Evolutionary Genetics Analysis Software (Mega 6.0 software®); used to analyze DNA and protein sequence data from species and populations (Kumar et al. 2016).

Statistical analysis

The values of triplicate samples of respirometry and the quantification variables of TPH were assessed by an analysis of variance (ANOVA). The significance among the means of different treatments compared whether the applied bioremediation treatments differed significantly

in TPH degradation ($p< 0.05$). Fisher's LSD multiple comparisons test was used to compare the averages. p values less than 0.05 (significant) indicated that surfactant and application of nutrients affected the rate of TPH degradation under different bioremediation treatments. The ANOVA and a multiple comparison tests determined significant differences in order to select the best option for TPH biodegradation.

Convective static bed reactor assembly

In order to scale the best bioremediation treatment obtained in microcosms a convective static bed reactor was used. This model was obtained in a previous study carried out in the laboratory of Bioremediation and Technological Development of the Faculty of Mines at the Medellin Campus of the Universidad Nacional de Colombia. The study was conducted through a statistical design of response surface (2^6) in which relative environmental humidity (A), environment temperature (B), internal reactor humidity (C), internal reactor temperature (D), material height (E), and porosity of the system (F) were analyzed.

The limits of the factors were: A = Lower limit 60% and higher limit 80%; B = Lower limit 8 °C and higher limit 45 °C; C= Lower limit 30% and higher limit 65%; D = Lower limit 20 °C and higher limit 60 °C; E= Lower limit 0.1 m and higher limit 1 m; F= Lower limit 50% and higher limit 70%.

The model obtained could be conceptualized through the polynomial of combination of parameters (or factors under study conditions) that guarantees significant values for operation points within the calculated optimal region. The air flow for specific environmental and internal reactor conditions was established from the following polynomial (R^2 = 0.96). The quantification of dry air available in the porous medium was established according to the value of "adequate precision". The proposed model was able to predict the response according to the adjusted R^2 values 80% of the time.

To obtain the appropriate flux into the system was necessary adjust the factors A, B, C, D, E and F inside the ranges established before, considering the data at laboratory scale. The factors were established at the beginning of the experiment with values of 30% (C), 0.3 m (E) and 60% (F); the other factors (A, B, D) were monitored through the process. Figure 2, shows the design performed to obtain an effective convective flow in the applied bioremediation system. To follow the process in the static bed reactor, variables other than pH, CFU of heterotrophic and hydrocarbonoclastic bacteria, respirometry, and TPH were evaluated. Results obtained by other researchers at the laboratory scale displayed that the reactor system needed a periodic addition of co-substrate. The optimum value for co-substrate addition was 253 g of molasses every 15 d to accelerate microbial metabolism. This addition ensured that hydrocarbon degradation remained constant over the experimental time period.

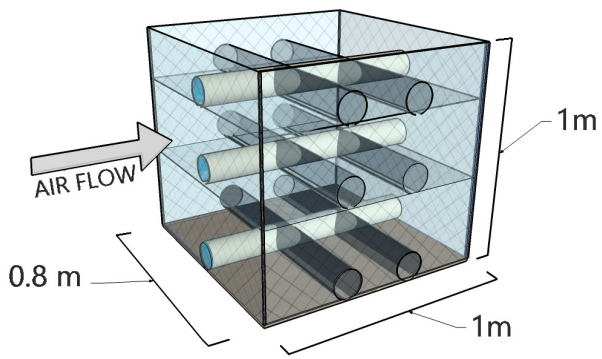

Figure 2. Convective static bed reactor design for bioremediation treatments implementation (Designed by the Authors).

RESULTS AND DISCUSSION

Soil physicochemical characterization

The soil parameters measured in the three treatment stages are shown in Table I. The soil sample was taken at 20 cm depth because previous studies of Amro et al. (2013) in oil spills, reports that soil surface is characterized by high adsorption resulting in very low infiltration depth and fluid migration. According to Takahashi & Shoji (2002) pyroclastic materials are characterized by high adsorption coefficients. The initial value of organic matter in Contaminated Soil (CS) is 9.1%, which is 7.3% high than the values registered for soil in the Laboratory Stage (LS); and 7.4% higher than the pilot Reactor Stage (RS). The Organic Matter (OM) content in LS and RS shows a decrease of approximately 20% more than the value in CS. According to Gonzales-Naranjo et al. (2011) researcher, this response will be attributed to the partial incorporation of carbohydrates to the native OM in the soil from crude oil during the bioremediation process. The application of carbohydrates increased the bioavailability of nitrates, which could be consumed by living soil organisms. The initial value of nitrates in CS (1 ppm) rises to 90 ppm in LS and 175 ppm in RS; this behavior is due to a compensation in the high C: N ratio of the polluted soil. In the case of phosphorus concentration CS showed a value of 34 ppm, this value increased significantly in RS (117 ppm), as a response to the application of composting that favored bacterial growth. In LS phosphorus concentration decreased, probably due to bacteria consumption in the metabolic processes, especially ATP (Adenosine Triphosphate) formation. Besides Leonardite, other phosphorus components were absorbed by the humic substances.

Molecular identification of bacterial isolates

In all treatments except natural attenuation various isolates with different morphological characteristics were selected in both mediums; selective and nutritive mediums. The phylogenetic tree produced by the Neighbor-Joining method according to Tamura (1992) methodology is shown in Figure 3. Four isolates were obtained from the nutrient medium (SecA, SecB, SecC, SecE) and three isolates from the selective medium (SecF, SecG, SecH).

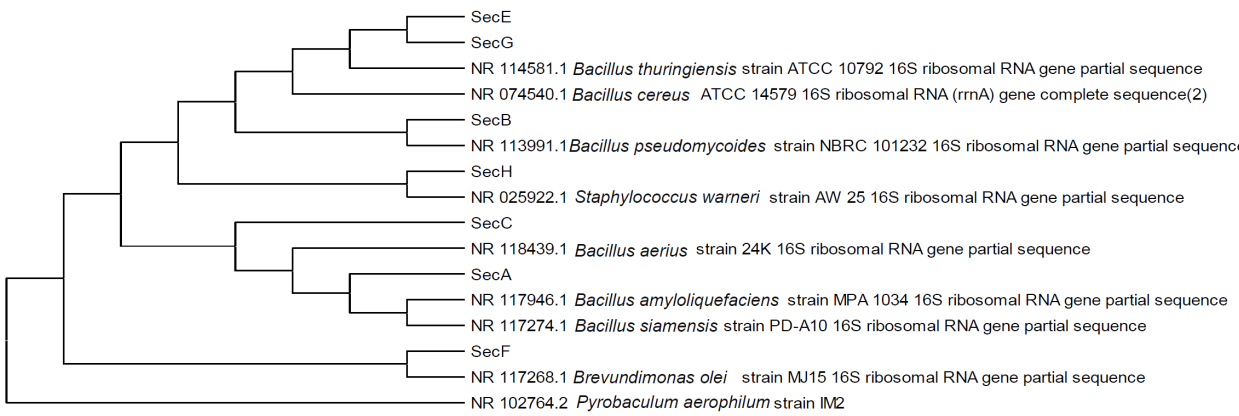

Figure 3. Phylogenetic analysis based on 16S rRNA gene sequences, from soil isolates with reference sequences. The tree was produced by Neighbor-Joining method with 1000 repetitions Tamura 3 model parameters (Tamura 1992) considering Gamma distribution with 5 category rates, using Mega 6.0 software (Kumar et al. 2016). Source: Authors

The processes were performed in 40 d, when the microcosm bioremediation treatment was at the halfway point and bacteria showed stationary behavior. The predominant family in the isolates were *Bacillaceae*; lower amounts of the genera *Brevundimonas* and *Staphylococcus* were observed. Molecular identification of bacteria isolated from the treatments BI, BII, BIII and BS are shown in Table II. Rodriguez et al. (2015) reported that the Bacillus genus produce biosurfactants capable of improving the bioavailability of the contaminant and accelerating the bioremediation process; the genus is able to adapt to many different hydrocarbons as an energy source (Pandey et al. 2016). Inside (SecF) and (SecH) isolates *Brevundimonas olei* and *Staphylococcus warneri* were identified. These species were highly tolerant to environments contaminated with crude oil and biodegradative activity of HAP (Lee et al. 2010, Moscoso et al. 2012). The same authors reported for these bacteria high surviving and adapting capacity to the extreme conditions in a high contaminated soil ecosystem. The study of bacterial composition in soils is important because it allows the establishment a soil bioremediation strategy through degradative routes carried out by the bacteria metabolism. Bacterial identification will be a key factor in the implementation of successful bioaugmentation processes in sites with scarce information about bacterial populations.

Table II. Molecular identification of bacteria isolated from treatment bioremediation soils (BI, BII, BIII y BS) using 16S rRNA gene.

IC	Related organism /Access N° to Gen Bank	S (%)	Ss	Related Activity	Reference
Sec A	*Bacillus siamensis strain PD-A10* /NR_117274.1	96	1380 bp	Production of biosurfactants in soils contaminated with hydrocarbons.	Varadavenkatesan & Murty (2013)
Sec B	*Bacilluspseudomycoides / NR_113991*	96	1467 pb	Strain found in the environment.	Nanclares (2016)
Sec C	*Bacillus aerius strain 24K /NR_118439*	92	1509 pb	Strain isolated from crude oil sediments.	Mansur (2015)
Sec E	*Bacilluscereus ATCC 14579 /NR_074540*	98	1406 bp	Production of biosurfactants and biodegradative activity of hydrocarbons.	Borah & Yadav (2016)
Sec F	*Brevundimonasoleistrain MJ15* /NR_117268	96	1339 pb	Strain isolated in sector contaminated with crude oil.	Lee et al. (2010)
Sec G	*Bacillus thuringiensis strain ATCC 10792/ NR_114581*	97	1417 pb	Potential biodegradation of light crude.	Thamer et al. (2013)
Sec H	*Staphylococcus warneri strain AW 25* /NR_025922	98	1403 bp	Biodegradation of PAH (Polycyclic aromatic hydrocarbons)	Moscoso et al. (2012)

Isolated code: IC, Similarity: S, Sequence size: Ss.

Determination of microbial CO_2 production

The curve slope at the laboratory scale showed a distinctive behavior. The curve was more inclined from 0 d to 16 d and behaving steadily from 17 d to 80 d; this tendency leads an abrupt change of slope in the two periods separated by the vertical red line (Figure 4a). The values for average production of CO_2 (mg/day) for 0 d to 16 d (I) and 17 d to 80 d (II) in the microcosm conditions were: 0.087 (I) – 0.051 (II) for AT; 0.125 (I) – 0.045 (II) in BS; 0.119 (I) – 0.047 (II) to BI; 0.131 (I) - 0.052 (II) for BII and; 0.141 (I) – 0.053 (II) in BIII conditions. These results showed two behaviors for the kinetic production of CO_2. In the first period (0 d to 16 d) the curve slope is approximately two times higher than the second period (17 d to 80 d). This behavior suggests the presence of two reaction systems, order one and order zero mechanisms after 16 d of treatment. Figure 4a showed the results for respirometry in the different treatments under the microcosm conditions. For the first period (0 d to 16 d), it was observed that BII and BIII treatments were similar and differed significantly from AT, BS and BI ($\alpha < 0.05$) according to Table III. In the second period (17 d to 80 d), the BIII treatment was similar from BII and AT and differed significantly from BI and BS ($\alpha < 0.05$) according to Table III.

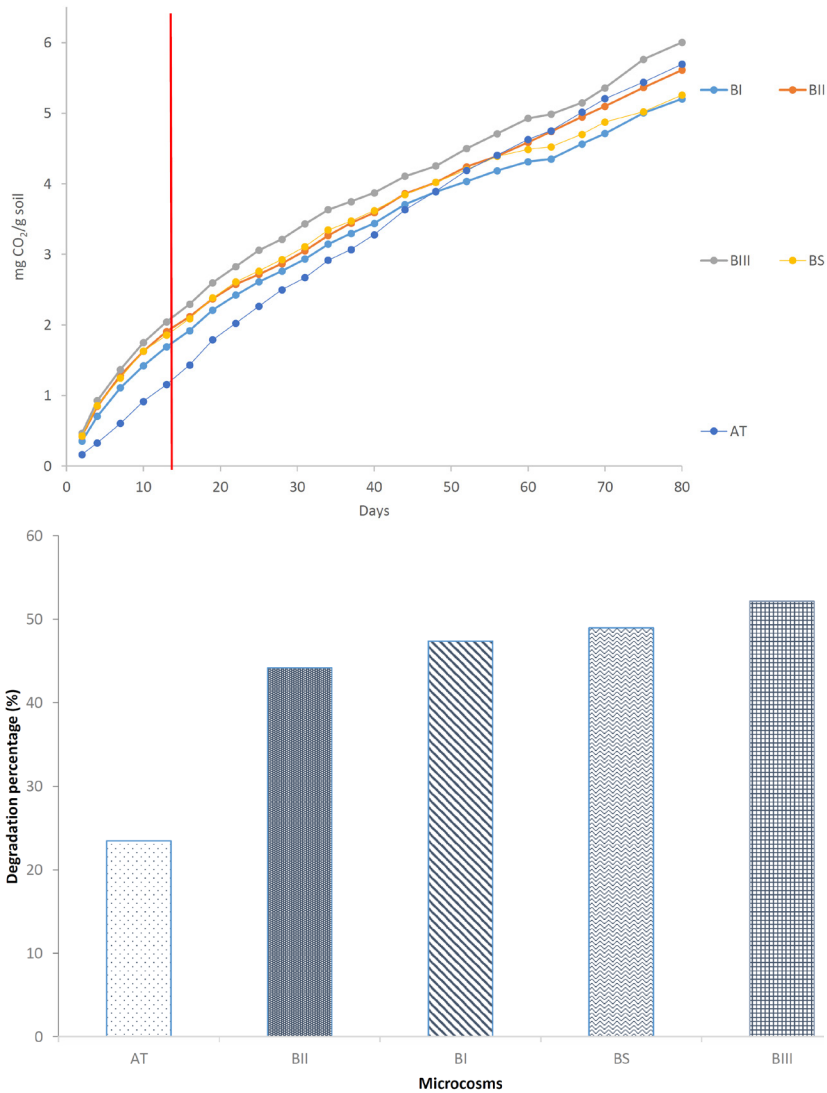

Figure 4. (a) Respirometry results for microcosms essays, (b) The TPH degradation percentage in the microcosms conditions.

In both periods BIII treatment differs to the others treatments due to highest concentration of Tween-80® that increases CO_2 production induced high biodegradation.

For the first period (0 d to 16 d) it was observed that BII and BIII treatments were similar and differed significantly from other treatments; in the case of BIII producing approximately 18% more carbon dioxide than the other treatments. In the second period (17d to 80d), carbon dioxide production of the treatment BIII decreased 62% in respect to the other experimental units. The BIII treatment was similar to BII and AT; it presented significant differences in carbon dioxide production respect to BI and BS. Studies conducted by Tahhan et al. (2011), showed that adding a bacterial consortium with strains of hydrocarbon and nutrients to a soil contaminated with a drilling mud with 82% TPH; the levels of carbon dioxide are low (4 mg / g soil) after 200 d of treatment. Pinto et al. (2007) analyzed the absence and presence of Tween-80® in the production of carbon dioxide in soil contaminated with diesel, finding that the production for the first treatment (Tween-80® absence) was approximately 30% lower than the values obtained in the second treatment (presence Tween-80®). In our research, the highest carbon dioxide production was obtained in the BIII treatment (0.141 and 0.053), with maximum carbon dioxide production of 6 mg / g soil in 80 d. These results are similar to Pinto et al. (2007) studies, that evaluated the application of nitrogen, phosphorus and Tween-80® as stimulating agents in the soil at 80 d period. These authors obtained a 4.84 mg/g of carbon dioxide soil production at 55 d; 3% more than the values found in our study. In the investigation conducted by Pinto et al. (2007) TPH concentration decreased to 5561 ppm, a 25% less than the value of TPH found in this research. According to De Souza Pohren et al. (2016), the application of organic amendments with humic acid substances similar to Leonardite in soil contaminated with PAH, improves the carbon dioxide production after 60 d of treatment by 23%. The cited authors found that the application of humic acid substances showed a significant result with respect to treatments without humic compounds. This behavior could be explained by the high stability induced by humic substances, acting as a surfactant and releasing HAP absorbed in the soil. Conte et al. (2005) and Turgay et al. (2010), found that

Table III. Daily production of carbon dioxide by Fisher's LSD Test and Percentage of biodegradation applying Fisher's LSD test.

Treatments	Day 0-16		Day17-80		% Biodegradation	
	Mean $\left(\frac{mgCO_2}{gsoil * day}\right)$	LSD grouping	Mean $\left(\frac{mgCO_2}{gsoil * day}\right)$	LSD grouping	Mean $\left(\frac{mgCO_2}{gsoil * day}\right)$	LSD grouping
AT	0.087	D	0.05167	A	31.42	C
BS	0.125	BC	0.04583	B	48.96	BC
BI	0.119	C	0.04707	B	47.37	BC
BII	0.131	AB	0.05263	A	44.18	BC
BIII	0.141	A	0.05297	A	52.15	AB

hydrocarbon degradation is largely related to enzymatic production (urease, phosphatase, dehydrogenases); and the application of Leonardite improves enzymatic production in a soil contaminated with hydrocarbons.

The behavior of the curve in the Figure 5 suggests that during the treatment in the pilot scale (microcosm); carbon dioxide production did not maintain a constant kinetic as shown by the changes in different moments of the process. In this case five different reaction mechanisms were determined: V (0 d – 2 d), W (3 d – 21 d), X (22 d – 33 d), Y (34 d – 45 d), Z (46 d – 80 d). These mechanisms explain the behavior of microbial metabolism according to new reactor conditions. The variations of carbon dioxide production throughout the treatment (mg CO_2/g soil x day) were: V 0.72, W 0.23, X 0.19, Y 0.07, Z 0.20. Carbon dioxide production increased the first days of treatment reaching the highest production in V, decreased almost 70% in W changing to constant trend in X and decreasing again in Y. The addition of Tween-80® (1.5 g / L) with molasses achieved desorption and bioavailability of the largest amount of crude oil in the soil matrix; reflected in 60% of CO_2 increase in 46 d; this slope remained for a period of 17 days.

Hydrocarbons degradation

The hydrocarbons found in the soil before and after the treatment were alkanes like Hexadecane, 2,6,10,14-tetramethyl; Heptadecane; Pentadecane, 2,6,10,14-tetramethyl; Nonadecano; Eicosano; Docosano; Heptacosane, 1-chloro. These hydrocarbons showed a decrease in the spectrum of gas chromatography after treatments. The TPH of the soil before starting the bioremediation process was 20900 mg / kg; this value is higher than the values reported by other researchers like Bento et al. (2005), Pinto et al. (2007), Whelan et al. (2015) in other bioremediation processes. The amount of TPH degraded in the natural attenuation treatment had a degradation percentage of 23.4% after 80 d of treatment. Initial TPH values are higher in comparison with the results obtained in treatments with the addition of nutrients and stimulating agents. The mean degradation percentage for the bioremediation treatments was 48%, this result was 50% higher than the values reported for natural attenuation. The

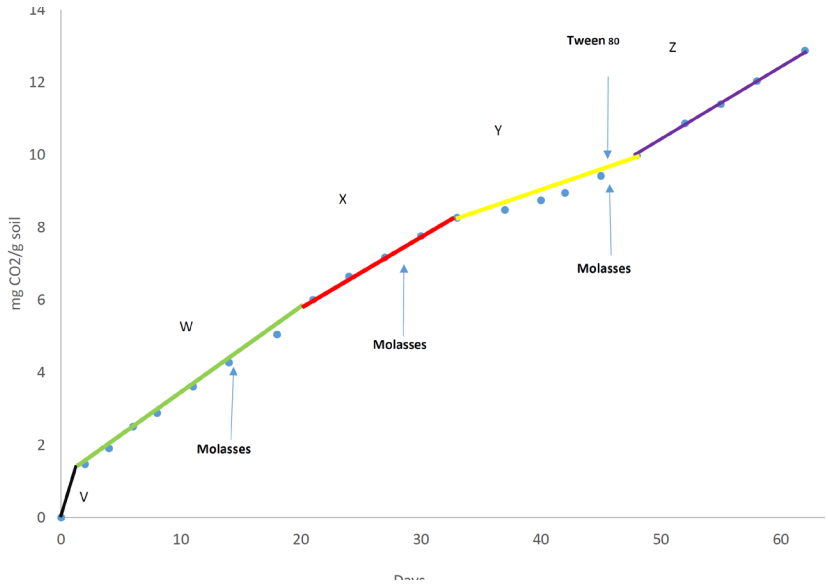

Figure 5. Production of carbon dioxide vs time.

treatments with Tween-80®, nutrients and Leonardite had a similar removal percentage. The BIII treatment showed the highest degradation percentage with values of 52%. The BS treatment (without Tween-80®) exhibited a degradation rate of 48.9%, this value is significantly higher compared to treatments with a surfactant.

The TPH degradation percentage at the microcosm scale is shown in Figure 4b. The statistical analysis showed a significant difference between BS, BI, BII, BIII treatments and Natural Attenuation. The application of biostimulation had a significant effect in TPH removal. BIII differs significantly to the other treatments, in this case the high concentration of Tween-80® (50% to 100% more) increases the production of CO_2 between 9% to 15% which induced biodegradation. The difference in production of CO_2 between AT and more successful treatment (BIII) was 40%. The ANOVA shows difference significance between treatments ($p< 0.0001$), for that reason was necessary to apply Fisher's LSD multiple comparisons test which was used to compare the averages. The results show that the treatments BIII, BS, BI and BII are similar and differed significantly from AT ($\alpha< 0.05$) as is shown in Table III. It evinced that the application of a treatment improves the degradation of TPH in this soil. Besides, TPH degradation in BIII was 12% higher compared with TPH degradation in BI, BS and BII. approx. BIII reported the highest values of TPH removal with 11% more carbon dioxide production per day. Compared to Pinto et al. (2007) studies for treatments with application of nutrients and Tween-80®; our results obtained 12% more TPH degradation than the report of the cited author; and 19% more than Tahhan et al. (2011) research. Considering that Pinto reduces Diesel, a molecule less complex chemically than crude oil (Lin et al. 2010). That confirm the efficiency of the treatment applied in this research. The results showed that the presence of Leonardite increased the biodegradation percentage of the pollutant. According to Loonen et al. (1999), the number of functional groups of a reactive nature such as hydroxyl, carbonyls and carboxyl improve the efficacy of the pollutant biodegradable route. The evaluation of the bioremediation process efficiency at microcosm scale conducted by de Souza Pohren et al. (2016), found highest values of TPH removal in presence of humic substances such as stimulating agents. Results for bioremediation of oil sludge by Tahhan et al. (2011), reported a TPH removal rate of 42% in a period of 80 d for treatments with an application of nutrients and a bacterial consortium.

The TPH removal in the pilot scale was measured at 0 d, 15 d, 30 d, 45 d, and 60 d of treatment. The results for residual degradation kinetics of TPH are showed in Figure 5, when two reaction mechanisms delimited by vertical red lines were observed. The initial amount of TPH at the pilot scale decreased slowly the first 15 d, decreasing until 53% reaching the value obtained in microcosms at 80 d of treatment; at the end of the treatment a 76% of residual TPH was removal. These results were comparable with the study carried out by Sayara et al. (2009), where a high compost stabilization and degradation rates with a removal percentage higher than 92% of PAHs in 30 d was found. Our results showed 76% of degradation with 20% of compost addition in comparison to 50% compost added in Sayara et al. (2009). These differences were related to compost stabilization, in this case the maturing process occurred in more than three months.

To determine the removal rate of TPH it was necessary to calculate the slope with Ln $[C/C_0]$ vs different time intervals in treatment, because two reaction mechanisms were present in the process (see Figure 5). For the first time interval (0 d - 15 d) TPH removal rate in the reactor was

- 0.04 d^{-1}; in the second time interval removal rate was - 0.017 d^{-1}. With these values it was possible to determine the half-life of the pollutant. Therefore, two values were obtained for $t^{1/2}$ 17 d for the first interval and 34 d in the second interval. Pollutant decreases at 17 d falling from 20900 ppm to 11000 ppm, and 11000 ppm to 5000 ppm at 34 d. The values in the second interval are high because of low contaminant bioavailability in this period, preventing easily metabolism by bacteria. The application of Tween-80® increased contaminant bioavailability and improved biodegradation.

Growth of heterotrophic and hydrocarbonoclastic bacteria

After the first week, a considerable growth of heterotrophic bacteria was observed in treatments BI, BII, BIII and BS, starting with 5.8* CFU / g to 1.0* CFU / g. These values remained almost constant (with some slight growth) until day 35, when the cell decline stage was determined. This behavior was similar to the Natural attenuation treatment, although bacterial growth in this condition was 86% lower than the growth evidenced in other treatments. In the case of the hydrocarbonoclastic bacteria, the behavior was different because it is a nutritionally-selective medium for the specific growth of hydrocarbonoclastic bacteria. Therefore, the amount of CFU / g achieved in the first seven days in heterotrophic bacteria was obtained in the selective medium after 40 d of treatment. In general, the behavior of CFU / g in BI, BII, BIII and BS treatments was as follows: exponential growth in the first 20 d, stationary stability between 20 d and 56 d, and declination and death from 56 d to 80 d. Natural attenuation treatment showed an increase in CFU / g at 14 d, and then remained in stationary phase until 63 d, when there was a slight decrease until 80 d.

CFU values obtained in the two culture media are comparable with those found in similar studies by Roy et al. (2014). This bioremediation process was carried out on an oil-contaminated soil through the addition of nutrients and hydrocarbonoclastic bacteria. A maximum $1.2 * 10^{7}$ CFU / g and $5.2 * 10^{10}$ was obtained for heterotrophic and hydrocarbonoclastic bacteria respectively. It was found in this study that the number of heterotrophic bacteria was higher ($3.4 * 10^{7}$), while the number of hydrocarbonoclastic bacteria was lower, than those achieved in the Roy study in approximately three logarithmic units. This increase was caused by the high concentration of bacterial inoculum of hydrocarbonoclastic strains applied to treatments $4.7 * 10^{13}$ (Roy et al. 2014). This bacterial inoculum process was not carried out in the present investigation. The accelerated increase of the heterotrophic and hydrocarbonoclastic bacteria between the initial 14 d and 20 d coincides with the maximum production of carbon dioxide obtained in the range of 0 d to 16 d. The results shown that in this period of time carbon dioxide production increases, in fact bacterial metabolism rises. From 17 d to 80 d the stationary phase begins because bacterial growth remains constant, and therefore the carbon dioxide production rate decreases due to the low presence of soluble carbon of TPH. This phenomenon can be attributed to the bacteria consumed part of the hydrocarbon available, and require additional encouragement (co-substrate) to active their metabolism (Prada et al. 2017, Sayara et al. 2009).

At the pilot scale a high amount of CFU / g was observed; both heterotrophic and hydrocarbonoclastic bacteria, reaching the proposed goal related to the addition of 20% compost; which contributed to the increase in the bacteria amount through treatment. The two types of bacteria identified (see in

molecular identification of bacterial isolates) showed one logarithmic unit increased until 20 d; with a maximum amount of CFU / g in reactor conditions. This value of CFU / g is related to amount of CO_2 production in V and W time intervals, which register the maximum CO_2 production rates. Nevertheless, in the same intervals maximum removal TPH was obtained under reactor conditions.

pH variations

The pH behavior in the laboratory was constant most of the treatment time; except in the initial period of the treatment (0 - 25 days). In the samples without the application of soil treatments, the pH was 6.6. The pH values in most of the treatments fluctuate between 6.0 to 8.0 (neutral to slightly alkaline) ideal for plant growth, these values were stabilizing at the end of treatment after day 50 with values close to 7.0. The first week pH increased in the BS, BI, BII and BIII treatments, probably due to the urea hydrolysis process associated with urease enzymatic action, which generated a basic environment with the consequent increase of pH values, achieving values ≥ 8.0 (Gonzàlez-Osorio et al. 2015). The bioremediation processes in hydrocarbons polluted soils in this research have a similar behavior to Pinto et al. (2007) studies who reports pH values between 6.5 to 7.5 at the end of study. The pH in the substrate play an important role in bioremediation processes with an optimum value between 6.0 to 8.0. According to Pawar (2015), studies in these pH conditions increase bacterial population with high rates of ATP production (adenosine triphosphate). In this study heterotrophic and hydrocarbonoclastic bacteria growth was evaluated, which confirm that these bacteria favor the biodegradation process. The treatment BIII was selected as the treatment to be carried out at pilot scale due to its good performance. Nevertheless, BIII treatment required an additional co-substrate to maintain the biodegradative kinetics at the time and, secure contaminant degradation rates. Several authors attribute degradation rates to a primary encouragement of bacteria from a readily available carbon source to begin their metabolism. The kinetics of carbon dioxide production found in BIII (16d), the slope curve decreased to almost 60% until day 80. This behavior is due to the lack of stimulus generated towards bacteria and the low solubilization of TPH; in order to ensure active bacterial metabolism inside the reactor a molasses co-substrate (source of primary carbon) and Tween-80® (solubilizing agent) was added every 15 d. The molasses quantity was calculated taking into account the amount of contaminant degraded over time; the value was determined in accordance with the slope of carbon dioxide production in the period of 0 d to 16 d.

In an attempt to generate stability in bacterial growth and high porosity (improve air flow) in a pilot scale system a 20% compost was added; this assure a value of $1 * 10^8$ CFU / g that raises degradative kinetics.

At the pilot scale (microcosm) the pH was variable showed an increase in the first days of treatment due to urea hydrolysis; after that it maintained a relatively constant value of 7.5 until the end of treatment. The study carried out by Pawar (2015) reported a mean pH value (basic) was the most suitable for biodegradation of PAHs in a soil contaminated by hydrocarbons. Allowing for the highest amount of bacterial production and contaminant degradation rate, reaching 50% of degradation in 4 d of treatment.

Environmental conditions

The average internal reactor temperature (D) 20 °C was lower than the mean environmental temperature (B) 23 °C. The design of the process favors the temperature increase in the reactor.

The temperature increase was not related to availability of carbon source (organic waste materials) easily assimilated compared with oil carbon. That is the reason why the reactor could not produce a fermentation process inside; these processes will dramatically increase the temperature reaching 60 °C (Barrington et al. 2003, Yu et al. 2009) and affecting the soil decomposition processes. The relative environmental humidity (A) was a high fluctuation ranging from 60% (minimum) to 80% (maximum); this parameter cannot be controlled because it is necessary to isolate conditions under monitored conditions. According to Barrington et al. (2003) and Yu et al. (2009), the reported values guaranteed an effective bioremediation process for the efficient transfer of oxygen in the system.

CONCLUSIONS

The use of Tween-80® in combination to biostimulants and leonardite under aerobic conditions than proposed bioremediation strategy improves the biodegradation of crude oil in the studied soil (Oxic Dystrudepts); showing a high percentage of degradation at laboratory (44% to 52% for BI, BII,BIII in 80 days) and pilot scale (76% in 60 days for BIII). Application of Tween-80® and conditioners such as Leonardite (BIII treatment) improves the degradation of pollutants, increase the CO_2 production even a 40. Tween-80® stimulates the effective recovery of the soil in a short period of time (80 days at the laboratory and 60 days at pilot-scale). Particularly, it was found that BIII treatment produced approximately 18% more carbon dioxide than the other treatments in the first period of observation (0d to 16d). Regarding THP degradation, the BIII treatment showed the highest degradation percentage with a value of 52%. The statistical analysis showed that BIII is the most suitable alternative to implement a pilot scale bioremediation system. In the analysis of carbon dioxide production kinetics for the bioremediation system, it's important to clarify the treatments behavior and requirements; allowing an evaluation of co-substrate application or a microbiota-enhancing agent (composting). Likewise, from the characterization of the native microbiota of impacted soil, it can be interpreted that several species have developed specialized metabolisms to adapt to this type of pollutant and this specific versatility enables a more effective bioremediation process. These organisms will be evaluated and characterized in future research to enhance their use in bioremediation programs. The use of convective static bed reactors in bio-remediate polluted soils was an efficient strategy that provides clean inexpensive technology; easy to use in oil contaminated soils at field scale. A 16S rDNA analysis allowed us to identify predominant bacteria such as*: Brevundimonas olei* and *Staphylococcus warneri* Inside (SecF) and (SecH) isolated; these individual are tolerant to environments contaminated with crude oil. However, the predominant family in the isolates were *Bacillaceae*, the genera *Brevundimonas* and *Staphylococcus* were observed in less quantity.

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CATALINA TREJOS-DELGADO[1]
https://orcid.org/0000-0002-1827-024X

GLORIA E. CADAVID-RESTREPO[2]
https://orcid.org/0000-0003-1026-6358

ANGELINA HORMAZA-ANAGUANO[2]
https://orcid.org/0000-0001-5825-3885

EDISON A. AGUDELO[1]
https://orcid.org/0000-0003-3863-3201

LEONARDO BARRIOS-ZIOLO[1]
https://orcid.org/0000-0002-6476-5366

JUAN CARLOS LOAIZA-USUGA[3]
https://orcid.org/0000-0002-3821-7518

SANTIAGO A. CARDONA-GALLO[1]
https://orcid.org/0000-0002-1875-7330

[1]Universidad Nacional de Colombia, Campus Medellin, Mine Faculty, Geosciences and Environment Department, Bioremediation and Technological Development Group, Carrera 65 N.º 59ª- 110, Medellín, Colombia

[2]Universidad Nacional de Colombia, Campus Medellin, Sciences Faculty, SIRYTCOR (Synthesis, Reactivity and Organic Compounds Transformation Group), Microbiodiversity and Bioprospection Group, Cellular Biology and Molecular Laboratory, Carrera 65 N.º 59ª- 110, Medellín, Colombia

[3]Universidad Nacional de Colombia, Campus Medellin, Mine Faculty, Geosciences and Environment Department, Carrera 65 N.º 59ª- 110, Medellín, Colombia

Correspondence to: **Juan Carlos Loaiza-Usuga**
E-mail: jcloaiza@unal.edu.co

Author contributions

Conceptualization and experiment designs, Trejos-Delgado CATALINA, Cadavid-Restrepo GLORIA ESTER, Hormaza-Anaguano ANGELINA, Agudelo EDISON ALEXANDER, Barrios-Ziolo LEONARDO, Cardona Gallo SANTIAGO ALONSO. Writing-review and editing were conducted by Loaiza-Usuga JUAN CARLOS. Performed the laboratory measurements and data processing Trejos-Delgado CATALINA.