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Bacterial biodiversity analysis in a stream with continuous disposal of hydrocarbon effluents

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Abstract

Since the beginning of its operations, the Urucu petroleum province (Petrobras, Amazonas unit – AM, Brazil) has been continuously discarding industrial wastewater from an effluent dike in a small water stream outside of the perimeter of its operational area. The objective of this research was to perform a study of the bacterial community existent in a small water stream (community 1: prior to effluent disposal, community 2: after effluent disposal) Water samples from the stream were collected to totally extract their genomic DNA and subsequently utilize it as a template in the PCR using specific oligonucleotides of 16S rDNA gene from the Bacteria domain. The PCR product was amplified, and the sequences obtained through a pyrosequencing method were analyzed using the open source software Mothur. The results obtained from the software revealed the Acidobacteria phylum, Deltaproteobacteria class, and the *Gp3* and *Geobacter* genus were all preponderant in community 1. Likewise, in community 2, the Proteobacteria phylum, Betaproteobacteria class, and the *Gp3* and *Geobacter* genus were preponderant. The richness indexes reported a percentage change (both decrease and increase) for both communities, furthermore, these indicated that the diversity was more abundant in community 1. However, no significant variance was observed between both communities ($P>0.001$) and all samples from each community yielded identical genetic structures ($P<0.05$). 25 % of the bacterial genera were cataloged as "unclassified", and approximately 15 % of the genera were cataloged as bio-remediators. The aforementioned data represents a challenge for biotechnological exploration in this environment, with the potential to identifying and classifying new taxonomic groups.

Keywords: Amazon, oil activity, metagenomic study, 16S rDNA gene, richness and diversity indexes.

Biodiversidad bacteriana en un riachuelo con vertido continúo de efluentes de hidrocarburos

Resumen

Desde el inicio de sus operaciones, la provincia petrolera de Urucu (Petrobras unidad Amazonas, Brasil, UN-AM) ha realizado el vertido continuo de efluentes de hidrocarburos en riachuelos alrededor de sus instalaciones. El objetivo de esta investigación fue realizar un análisis de la comunidad bacteriana existente en un riachuelo (comunidad 1: antes del vertido, comunidad 2: después del vertido). Se colectaron muestras de agua en riachuelo para extraer el DNA genómico total y usarlo como molde en la PCR, con oligonucleótidos específicos del gen 16S rDNA para dominio Bacteria. El producto de la PCR fue amplificado, y las secuencias generadas por técnica de pirosecuenciación, fueron analizadas con el programa libre Mothur. Los resultados revelaron que el filo Acidobacteria, la clase Deltaproteobacteria y los géneros Gp3 y Geobacter, mostraron alta presencia en comunidad 1. Asimismo, el filo Proteobacteria fue el más abundante, con la clase Betaproteobacteria y los géneros Geobacter y Gp3, como los predominantes en comunidad 2. Los índices de riqueza presentaron variación porcentual en ambas comunidades (disminución e incremento), siendo la diversidad más abundante en la comunidad 1. Sin

embargo, no se encontró diferencia significativa entre ambas comunidades (p>0,001), y todas muestras de cada comunidad presentaron la misma estructura genética (p<0,05). El 25 % de los géneros bacterianos se consideraron "no clasificados", y aproximadamente el 15 % de los géneros fueron clasificados como biorremediadores. Estos datos representan un desafío para la exploración biotecnológica en este ecosistema, con potencial para identificar y clasificar nuevos grupos taxonómicos.

Palabras clave: actividad petrolera, Amazonia, análisis metagenómico, gen 16S rDNA, índices de riqueza y diversidad.

Introduction

The majority of the diversity in the Amazon ecosystems is represented by fungi and bacteria. In the environmental samples, the structure of the bacterial communities is exceptionally complex and diverse (Torsvik *et al.*, 1990), and the same complexity represents a challenge to biotechnology. Regarding microorganisms that conduct bioremediation in contaminated environments, these can be found widely distributed alongside in the soil and bodies of water, and which communities normally constitute less than 1% of the entirety of the bacterial community. However, when these microorganisms are in conjunction with hydrocarbons, their number is increased 10% (Atlas & Cerniglia, 1995). Established estimations indicate that approximately 99% of microorganisms that exist in various natural habitats are not cultivable through traditional cultivation methods (Amann *et al.*, 1995). The recent advances in molecular biology, particularly in the DNA sequencing technologies, have rendered the exhaustive research in microorganisms in various ecosystems possible (Parmar *et al.*, 2019) and the metagenomic analysis (genomes that are sequenced directly from an environmental sample, with no need for cultivation and isolation of microbes in the laboratory) a valuable asset in determining more realistically the bacterial diversity (Peixoto *et al.*, 2011).

Amidst the Brazilian Amazon region, the Urucu petroleum province represents the only active oil & gas facility from the Brazilian oil company, Petrobras (Amazonas operational unit - AM). Since the beginning of its operations in 1988, Petrobras has been discarding industrial wastewater in a small water stream in the perimeter of its operational area from an effluent dike. The process is preceded by an effluent treatment control in the effluent treatment plant (ETP), thus validating that its physicochemical parameters are within the threshold determined by the environmental guidelines from the Brazilian Environment Council, specifically in resolution nº 357 (March 2005, from CONAMA, 2005).

Despite following the environmental norms for effluent disposal in bodies of water in the Urucu operational unit, the company Petrobras (AM) has not performed an evaluation of the different kinds of microorganisms existent in the ecosystem from the small water stream where it has discarded wastewater since it started operating. The aforementioned evaluation is fundamental for recognizing the ecosystem biodiversity (through a taxonomic analysis) and for guaranteeing its preservation. Taxonomy is a science that is concerned with the identification of a species, which is composed of a group of individuals (specimens) that show to a higher or lesser extent the intrapopulation variance always present (Bicudo, 2004). Among the various technological tools that perform taxonomical analysis, determine the richness and diversity indexes, and calculate the statistical difference between the communities in a study, there exists the free program Mothur which works as a thorough DNA sequence aligner (Schloss, 2009).

The goal of this project was to perform a study of the bacterial community existent in a small water stream (community 1: prior to effluent disposal, community 2: after effluent disposal) where the company Petrobras continuously disposes of its effluents (Amazonas unit – AM) to identify the microbial taxonomy and statistically compared the richness and diversity indexes present in the ecosystem in question.

Experimental

Area of study

This study was performed in the Urucu petroleum province unit, known as the Operational Base Geologist Pedro de Moura (BOGPM) from the company Petrobras, with the following geographical location: 4º30´S/64º30´W; 653 km (405 mi) distant from the city of Manaus (straight line). The operational unit is located in the basin area of Urucu River in the eastern side of the Solimões River in Coari county, state of Amazonas, Brazil.

Sampling and analysis procedure

Following several field visits to the Urucu petroleum province (Petrobras – AM), a collection of samples was carried out for research in September 2011. Eight collection sites were selected (4 of which were upstream from the effluent discharge location and the other 4 downstream from the same location), which added up to a total of 16 l (4.2 gallons) of water that was subsequently classified into two categories: community 1 (water stream prior to effluent disposal) and

community 2 (water stream after the effluent disposal). Furthermore, the collected were deposited in sterilized plastic bottles, 2 l of water in each. Experimental and geographical data of all the samples collection sites can be found in Table 1.

Table 1. Location of the collection sites from the water stream affected by continuous hydrocarbon effluents

N°	Location in the stream	Code	Geographical coordinates	Depth of sample collection (m)
1	Center 50 m upstream	P1	S 04° 51´ 40.4" W 065° 17´ 52.7"	0.2
2	Side 50 m upstream	P2	S 04° 51´ 40.4" W 065° 17´ 52.7"	0.2
3	Center 80 m upstream	P3	S 04° 51´ 40.3" W 065° 17´ 52.8"	0.3
4	Side 80 m upstream	P4	S 04° 51´ 40.3" W 065° 17´ 52.8"	0.2
5	Center 50 m downstream	P5	S 04° 51´ 40.8" W 065° 17´ 50.8"	0.3
6	Side 50 m downstream	P6	S 04° 51´ 40.8" W 065° 17´ 50.8"	0.2
7	Center 80 m downstream	P7	S 04° 51´ 40.9" W 065° 17´ 50.4"	0.2
8	Side 80 m downstream	P8	S 04° 51´ 40.9" W 065° 17´ 50.3"	0.2

In the laboratory, all the samples were centrifuged in 50-ml Falcon™ tubes, then the supernatant water was discarded up until obtaining solid material only (a minimum of 0.25 g required for each DNA extraction). An Eppendorf™ centrifuge model 5810R was utilized for the aforementioned process, operating at a rotation of 4,000 rpm for 10 min. Before performing the metagenomic study of the samples, DNA extraction was conducted following the manufacturer's recommendations from the PowerSoil™ DNA Isolation Kit. (MoBio Laboratories Inc., U.S.).

Furthermore, the collected material was placed in a thermal cycler (thermal cycler model AB Applied Biosystems Veriti™ 96 Well) which was programmed to run the following cycle for a PCR reaction chain initial denaturing at 95°C (203°F) for 4 min, 35 denaturing cycles at 95 °C for 1 min, annealing at 59 °C (138.2 °F) for 1 min and an initial extension at 72 °C (161.6 °F) for 40 s, subsequently followed by a final extension at 72 °C (161.6 °F) for 5 min. Four types of initiator primers were employed: MID1, MID2, MID3, and MID4, each of which was combined with all the samples which were processed afterward in two different platforms, employing a pyrosequencing technique (454 Roche).

To facilitate the upcoming analysis, the samples were grouped in two communities, cataloged as follows:

- Community 1: composed from samples P1, P2, P3, and P4

- Community 2: composed from samples P5, P6, P7, and P8.

The mixture for the PCR was composed of 5 µl of DNA from each sample, 1 µl of initiator primer, 1 µl of reverse primer, Taq DNA polymerase, and Milli-Q™ ultrapure water (deionized water), adding up to a final solution of 25 µl. A quintuplex PCR was used to characterize all eight samples. The combinations of initiators primers with the different samples or libraries are displayed as follows (the initiators' specifications can be found in Table 2):

- Sample 1: P1 with MID1
- Sample 2: P2 with MID2
- Sample 5: P5 with MID1
- Sample 6: P6 with MID2

- Sample 3: P3 with MID3
- Sample 4: P4 with MID4
- Sample 7: P7 with MID3
- Sample 8: P8 with MID4

Table 2. Initiators used in the amplification of DNA fragments for the communities in the water stream affected by continuous hydrocarbon effluents

Type	Specification	Nucleotide sequence (5'-3')
Reverse primer	Lib-L B Key 16S R	CCTATCCCCTGTGTGCCTTGGCAGTCTCAGGGGACTAC CAGGGTATCTAAT
Forward primers	16S-LibL-F-MID1	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACGACGA CTACTCCTACGGRAGGCAGCAG
	16S-LibL-F-MID2	CCATCTCATCCCTGCGTGTTCCGACTCAGACACGTAGTAT ACT CCTACGGRAGGCAGCAG
	16S-LibL-F-MID3	CCATCTCATCCCTGCGTGTCTCCGACTCAGACACTACTCGT ACTCCTACGGRAGGCAGCAG
	16S-LibL-F-MID4	CCATCTCATCCCTGCGTGTCTCCGACTCAGACGA CACGTAT ACTCCTACGGRAGGCAGCAG

The process of DNA purification was performed using the GFX™ PCR DNA and gel band purification kit (from GE Healthcare), following the manufacturer recommendations (the bands were cut in segments of 500 base pairs - bp). To validate the purification of the samples, electrophoresis was employed in 0.8 % agarose gel with a molecular weight of 1 kb (1 kb = 1,000 bp) as a reference, 5 µl of each purified sample, for approximately 1 h at 70 volts.

The final products of each procedure were stored at a cryogenic temperature of -20 ºC (-4 ºF), previous to being sent to the company GenOne (Rio de Janeiro – RJ, Brazil) to apply the pyrosequencing method (Roche 454 platform) and to obtain more comprehensive and high-quality results by using the open source software Mothur (version 1.34.0, February 2014) (Schloss, 2009).

Discussion and Results

As a product of the application of the pyrosequencing method (Roche 454), nucleotide sequences were obtained for all the samples taken into account in this study, all of which were analyzed through the use of the open source software Mothur (Schloss, 2009).

Taxonomic classification

The taxonomic identification and classification for this study were based on the free software Mothur. The taxonomic analysis was carried out as follows: phylum, class, and genus.

Phyla

28 scientifically cataloged Bacteria phyla were detected, in addition to "unclassified" phyla. Figure 1 illustrates the proportion of the preponderant phyla, with relative frequency values >0.1 % in any of the samples.

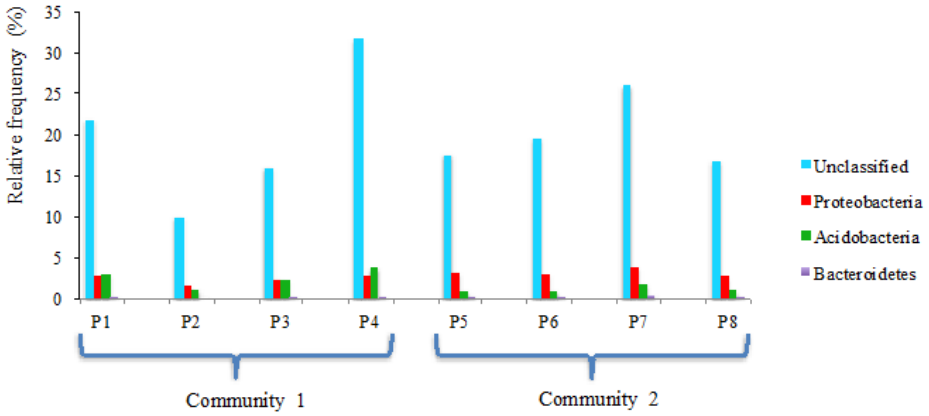

Figure 1. Preponderant bacteria phyla in the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

The preponderant phyla from each of both communities were categorized as "unclassified" (>30 %), followed by the Proteobacteria and Acidobacteria phyla (<5 %). An increment was observed in the Proteobacteria phylum in community 2, which resulted in a maximum value of <4 % for relative frequency; whereas community 1 presented a maximum relative frequency value of <3 %. Regarding to the Acidobacteria phylum, the opposite trend was observed since community 2 suffered a decrement in its relative frequency magnitude (<2%), while in community 1, the maximum relative frequency value reached was <4%.

In a study carried out to analyze the microbial diversity existent in Black River (a tributary of the Amazon River of Amazonas, Brazil), the Proteobacteria phylum was identified as the preponderant phylum (Neves, 2013). Likewise, the Proteobacteria phylum was the most preponderant among other phyla in all the samples collected from rivers and lakes in the Brazilian Amazon region (Peixoto *et al*., 2011; Rodrigues, 2011; Toyama, 2012), and it is characteristic to freshwater environments (Zwart *et al*., 2002).

Classes

40 scientifically cataloged Bacteria phyla were detected, in addition to other unknown phyla (unclassified). The distribution of the predominant classes (Proteobacteria phylum was the most preponderant) in both studied communities is represented in Figure 2.

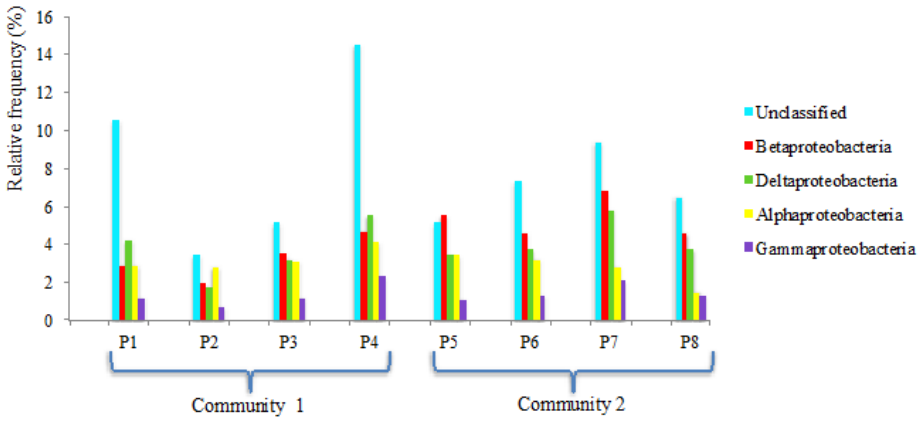

Figure 2. Classes of Proteobacteria phylum (was the most preponderant) in the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

The classes with greater relative frequency in both communities were the ones cataloged as "unclassified" (<15 %). In community 1, the most predominant class was Deltaproteobacteria (<6 %), followed by the Betaproteobacteria class (<5 %). Specifically, in community 2, a predominance in frequency was observed in the Betaproteobacteria class (<7 %), followed by the Deltaproteobacteria class (<6 %) and the Alphaproteobacteria class ranking in third (<4 %). The Alphaproteobacteria class presented no significant variance in both communities (<5%).

Regarding the aforementioned results, Peixoto *et al*. (2011), discovered that the Betaproteobacteria class was the most preponderant in Black River and the Betaproteobacteria class the most preponderant in Solimões River (a tributary of the Amazon River, Brazil). Furthermore, Rodrigues (2011) discovered that the Alphaproteobacteria class was preponderant in the Amazon River's delta.

Genera

267 Bacteria genera were detected, in addition to other unknown genera. The distribution of the predominant genera in both studied communities is represented in Figure 3 (the "unclassified" genera were not included in the figure to facilitate the graphical interpretation).

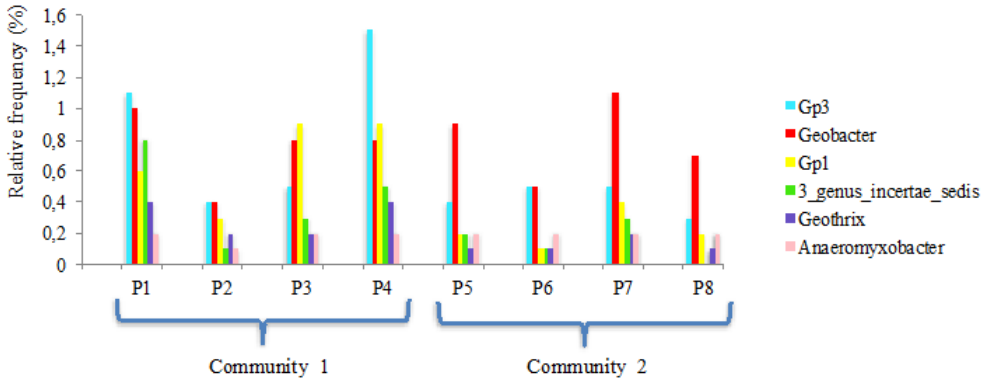

Figure 3. Preponderant Bacteria genera (excluding the "unclassified") in the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

Community 1 exhibited the highest frequency of bacteria genera. The most preponderant genera are listed, as follows: *Gp3* (<1.6 %), *Geobacter* and *Gp1* (both <1 %) and 3*_genus_incertae_sedis* (<0.8 %). Alongside, in a lower proportion, the following genera are listed, as follows: *Geohtrix* and *Anaeromyxobacter* (<0.2 %). Genera with the lowest frequency were not displayed.

In community 2, the genera *Geobacter* (<1.2 %) and *Gp3* (<0.5 %) were the most preponderant, followed by the subsequent genera, as follows: *Gp1* (<0.4 %), *3_genus_incertae_sedis, Anaeromyxobacter* and *Geothrix* (all with <0.3 %).

In Figure 4 only the "unclassified" genera are displayed to improve the visibility of the aforementioned Figure 3. On the one hand, it was observed that the highest proportion of non-cataloged genera is concentrated in community 1 (<31 %). On the other hand, community 2 concentrated ≤25 %.

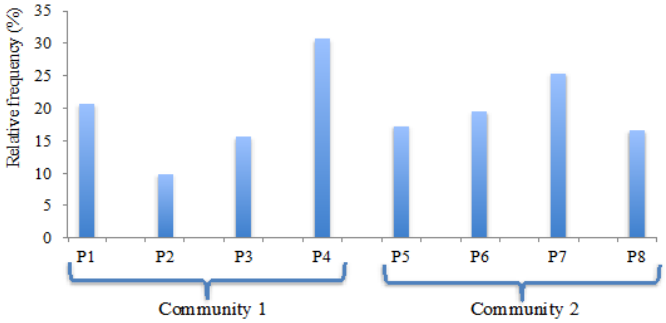

Figure 4. Proportion of "unclassified" Bacteria genera in the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

The genus *Gp3* which belongs to the Acidobacteria phylum and the Acidobacteria_Gp3 class was detected as well in the study carried by Neves (2013) in Black River (<11 %). The *Geobacter* genus, which corresponds to the Proteobacteria phylum and Deltaproteobacteria class, is strictly anaerobic and capable of degrading a diversity of organic compounds and aromatics (Kleinstuber *et al.,* 2012). Moreover, the *Geobacter* genus was identified with a frequency of 0.07 % in Black River (Neves, 2013). Additionally, there is a case study of the microbial community in Grangeiro River in which the *Geobacter* genus yielded an approximate relative frequency of 41 % for zinc-resistant genes (Xavier *et al.,* 2019).

The *3_genus_incertae_sedis* genus belongs to the Verrucomicrobia phylum and *subdivision 3* class. The Verrucomicrobia phylum represents a significant fraction of the freshwater communities (Nishimura & Nagata, 2007), nonetheless, it represents 2-8 % of the bacterial community observed in the rhizosphere soils (Kielak *et al*., 2008). The *Geothrix* genus which belongs to the Acidobacteria phylum and *Holophagae* class is a strictly anaerobic organism capable of reducing iron (Nevin and Lovley, 2002), and nitrate (Jin *et al*., 2015). Cannavan (2007) analyzed the bacterial diversity in soils of the Amazon region, and sequences resulting from the study yielded frequency values lower than 31 % for the *Geothrix* genus.

The *Anaeromyxobacter* genus is defined as a strictly anaerobic organism that is capable of degrading organic substrates (Sun *et al.,* 2012), besides, it was detected in environments with a high content of organic matter and regularly participated in the hydrolysis of complex organic compounds (Hatamono, 2007; Pereyra, 2010). Ramos (2013) identified the aforementioned microorganism, specifically an *Anaeromyxobacter* spp. subspecies, in the characterization of groundwater contaminated with a mixture of diesel and biodiesel with a relative frequency of approximately 18 %. Likewise, Silva *et al.* (2007) identified the *Anaeromyxobacter* genus in samples of mangrove from a petroleum basin (5 %).

Furthermore, in a study carried out in environments with heavy metal contamination, the *Anaeoromyxobacter* genus was preponderant over other genera in Riacho dos Macacos (stream of the monkeys), with a relative frequency of 38 % (Xavier, 2019). The complete genome sequence of *Anaeromyxobacter* sp. Fw109-5 (a metal-reducing bacterium) was isolated from a contaminated medium, moreover, the genus demonstrated great potential for bioremediation processes (Hwang, 2015).

The *Gp1* genus belongs to the Acidobacteria phylum and Acidobacteria_Gp1 class, moreover, and it was identified by Etto (2011) in a community of marshes (plants that belong to the *Gentianaceae* family) and by Ferreira (2011) as well, the latter author revealed the *Gp1* genus as preponderant in the aquatic environment where carnivorous plants develop.

15 % of the total 267 bacterial genera identified were cataloged as bioremediation agents of contaminated environments, according to reports from various authors (Mandri and Lin, 2007; Jacques *et al*., 2007; Alvarado, 2009; Seo *et al*., 2009; Núster *et al*., 2014; Xavier, 2019). Furthermore, these bioremediation microorganisms exhibited an increase in number in the final sequences, and in some specific cases triplicated their number; thus, when in the presence of hydrocarbons, their concentration increases by 10 % from the community as a whole (Atlas and Cerniglia, 1995).

Rarefaction curve and richness and diversity indexes

The group of closely related species or genera is defined as an operational taxonomic unit (OUT), which was used as the base for the calculation of the rarefaction curve, richness, and diversity indexes of the microbial communities included in this study. The number of sequences (from the groups: initial, final, and "unclassified") was displayed in Table 3, as well as the distribution of OTU's among the various samples.

Table 3. Distribution of OTU from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

Community number	Code of sample	N° of initial sequences	N° of final sequences	N° of "unclassified" sequences	N° of OTU's
1	P1	15762	10000	6682	2578
	P2	6969	4000	2691	1109
	P3	18652	13000	4163	1980
	P4	36392	25000	9729	3709
2	P5	28664	22000	3758	1778
	P6	23056	15000	4692	1895
	P7	27311	21000	6153	2626
	P8	16796	12000	4041	1634

OTU: operational taxonomic unit.

Community 2 presented the highest number of sequences, both initial and final, whereas community 1 presented a lower number of both categories. Despite yielding lower numbers in two categories, community 1 exhibited a higher number of non-scientifically-cataloged microorganisms ("unclassified"). Similarly, the number of closely related genera (OUT's) was higher in community 1, even when possessing a lower number of overall sequences when compared to community 2.

Rarefaction curve

A rarefaction curve is used for predicting the expected number of species (or diversity) in each sample, for a standard sample size. These curves allow for the extrapolation between OTU's and the number of sequences; they also indicate the strain resulted from the sequencing process to estimate the highest OTU's values at a phylogenetic level. Due to the obtainment of a curve of this nature, the comparison between samples with different intensities was possible (Ferreira, 2011).

The data used to plot the rarefaction curves of the community in this study was generated from the Mothur software and yielded 97 % of similarity – considering that two sequences belong to the same OTU if they possess a distance "p" of <3 %. The rarefaction curves for both studied communities were displayed in Figure 5. Considering the number of sequences in both communities, it was observed that despite the increase in sequences, the curves tend towards a horizontal alignment.

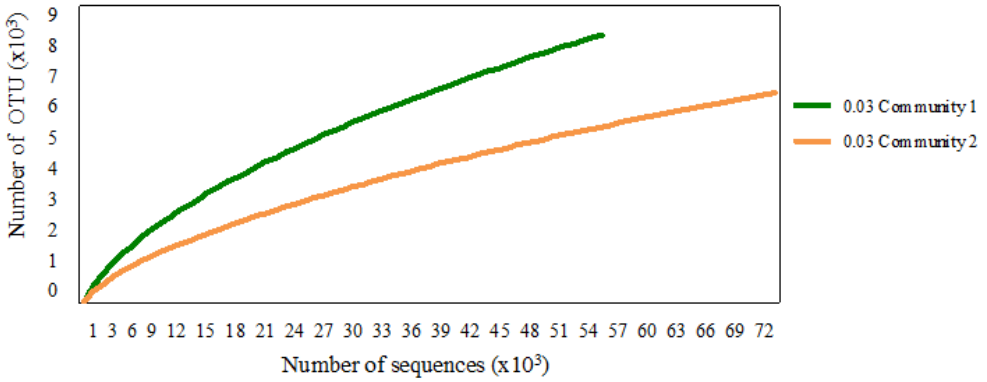

Figure 5. Rarefaction curves of the communities from the water stream affected by continuous hydrocarbon effluents. OTU: operational taxonomic unit**.** Community 1: prior to effluent disposal, community 2: after effluent disposal.

The results exhibited in Figure 5 indicate that the number of sequences generated in each library was relatively sufficient to describe the diversity existent in each of the analyzed communities. Likewise, it was further observed that the diversity was higher in community 1, thus the OTU quantity was higher when compared to community 2.

Richness and diversity indexes

The estimation of the various bacterial richness and diversity indexes was performed through the application of the open source software Mothur (Schloss, 2009). This software keeps the specific indexes of Chao1 and Ace (abundance-based coverage estimator) in consideration to estimate the richness index and the Shannon and Simpson indexes for diversity (97 % of similarity). The values related to the richness and diversity indexes that were based on the OTU's were displayed in Table 4.

The Chao1 richness index is an estimator based on the abundance of OTU's (unique and rare ones), and its function is to estimate the richness of a population of unknown size (Schloss, 2009). The Ace index consists of a non-parametric method that estimates the number of species existent in a microbial community and is defined as the sum of probabilities of the observed species. The Ace method divides the observed frequencies into abundant and rare groups (Kim et al., 2017).

In the results contained in Table 4, specifically concerning the richness indexes, Chao1 and Ace, several values presented a decrease and others presented an increase despite belonging to the same community. Furthermore, there was a contrast between samples from different communities, as discussed previously. Before performing an analysis over the percentage variance of these indexes, a comparison between the sample results in the various sample collection points was carried out (50 m and 80 m upstream and downstream from the effluent discharge location, respectively, both in the center and riverside of the stream). The comparative analysis of the richness indexes percent variance, is exhibited in Table 5.

Table 4. Richness and diversity indexes (97 % of similarity) of the communities from the water stream affected by continuous hydrocarbon effluents. OTU: operational taxonomic unit. Community 1: prior to effluent disposal, community 2: after effluent disposal.

Community number	Code of sample	N° of final sequences	OTU N°	Richness indexes		Diversity indexes	
				Chao1	Ace	Shannon	Simpson
1	P1	10,000	2,578	6,169	9,829	6.62	0.005
	P2	4,000	1,109	3,051	4,823	5.52	0.025
	P3	13,000	1,980	4,353	6,636	5.67	0.016
	P4	25,000	3,709	7,996	12,083	6.01	0.012
2	P5	22,000	1,778	4,390	7,991	3.02	0.363
	P6	15,000	1,895	4,760	7,654	5.44	0.020
	P7	21,000	2,626	5,975	9,407	5.20	0.057
	P8	12,000	1,634	3,790	6,293	4.83	0.083

OTU: operational taxonomic unit.

Table 5. Analysis of the richness indexes percent variance for the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

Location in the stream	Sample collection points in the stream			Richness index Percent variance (%)			
	Community 1	Vs.	Community 2	Chao1		Ace	
Center 50 m both upstream and downstream	P1	….	P5	28.8	↓	18.6	↓
Riverside 50 m both upstream and downstream	P2	….	P6	56.0	↑	58.7	↑
Center 80 m both upstream and downstream	P3	….	P7	37.2	↑	41.7	↑
Riverside 80 m both upstream and downstream	P4	….	P8	52.6	↓	47.9	↓

↓: decrease, ↑: increase.

When comparing the result from sample P1 with the one from sample P5 (Table 5), both points are equidistant (50 m) from the effluent discharge point in the center of the stream, it was observed that there was a decrease in both Chao1 and Ace indexes of 28.8 % and 18.6 %, respectively. A similar pattern was obtained when comparing the samples P4 and P8. Although, among the pairs P2 and P6, and P3 and P7, there was an increase of 56.0 % and 37.2 %, respectively, in the Chao1 indexes; and another increase of 58.7 % and 41.7 %, respectively, in the Ace indexes. Therefore, based on the percent variance (both increase and decrease in percentage) of the Chao1 and Ace indexes, it cannot be determined that the bacterial richness was higher or lower in community 1 over community 2.

Unlike the Chao1 and Ace indexes, the Shannon index measures diversity and is employed in situations where a community as a whole is unable to be inventoried. The calculation of the Shannon index takes into consideration the relative abundances of different species (Rodrigues, 2011). The results of this study (displayed in Table 4) exhibited a decrease in the Shannon index in all the samples from community 2 when compared to the values in community 1, thus indicating that community 1 possessed a higher diversity. In addition to measuring diversity, the Simpson index measures the dominance in a community and the sensitivity to the changes in the abundant species. It is a practical tool used in environmental monitoring and assessment since it quantifies the variance of the most preponderant species when subjected to any perturbances.

The diversity values according to the Simpson index were displayed on a scale from 0 to 1; the higher the number when approaching to 1, which will indicate a higher dominance and subsequently a lower diversity (Ñique, 2010). The results were higher (closer to 1) in community 2, which indicates a higher dominance of species in the second community and hence a decrease in diversity. For the reason aforementioned, community 1 presented a higher diversity than community 2.

Statistical analysis

The AMOVA (analysis of molecular variance) generated from the Mothur software, is a statistical test that is analog

to the ANOVA (analysis of variance) and consists of carrying out the analysis of the molecular variance in samples. This method is widely employed in population genetics to test the hypothesis that genetic diversity within two populations has no significant difference from that which would result from pooling the two populations (Neves, 2013). The AMOVA analysis results generated through the Mothur software are shown in Table 6.

Table 6. Results from the AMOVA (Mothur software) of the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

Variance	**AMOVA**		
Community 1 vs. Community 2	Between groups	Within groups	Total
SS	127.569	138.502	26.607
df	1	6	7
MS	127.569	0.230836	
Fs:	552.637		
p_{value}: 0.023			

SS: sum of squared differences between both communities; df: degrees of freedom; MS: mean squares between both communities; Fs: F value for α=0.01; p_{value}: probability.

As observed in the global comparison between both communities in this study (see Table 6), the results indicate that there is no significant difference between communities 1 and 2 (p_{value}: >0.001). Moreover, the LIBSHUFF analysis (a method used in Mothur software) consists of evaluating whether or not the samples (libraries) in each of the communities in the analysis possess identical genetic structures (Schloss, 2009). The results originated from the LIBSHUFF analysis are shown in Table 7.

Table 7. Results from LIBSHUFF analysis (Mothur software) of the communities from the water stream affected by continuous hydrocarbon effluents. Community 1: prior to effluent disposal, community 2: after effluent disposal.

Community 1		**Community 2**	
Comparison between samples (dCXYScore)	Probability *P* (significance 10 %)	Comparison between samples (dCXYScore)	Probability *P* (significance 10 %)
P1-P2	0.712	P5-P6	0.798
P2-P1	0.712	P6-P5	0.798
P1-P3	0.849	P5-P7	0.520
P3-P1	0.849	P7-P5	0.520
P1-P4	0.790	P5-P8	0.390
P4-P1	0.790	P8-P5	0.390
P2-P3	0.888	P6-P7	0.437
P3-P2	0.888	P7-P6	0.437
P2-P4	0.768	P6-P8	0.551
P4-P2	0.768	P8-P6	0.551
P3-P4	0.854	P7-P8	0.130
P4-P3	0.854	P8-P7	0.130

The results presented prove that there was no significant difference in the genetic structure between the samples from each of the communities in the analysis (p>0.008; the variable p was calculated following the Bonferroni correction, p = significance/number of comparisons, P = 0.1/12).

Conclusions

In community 1, Acidobacteria was the preponderant phylum, and Deltaproteobacteria the preponderant class, furthermore, the genera *Gp3* and *Geobacter* the most preponderant ones in this community in the study. Regarding community 2, Proteobacteria was the preponderant phylum, Betaproteobacteria the preponderant class, followed by *Geobacter* and Gp3 as the most preponderant genera.

A fraction of the microorganisms detected in the study were grouped as "unclassified" (not scientifically cataloged), resulting in approximately 31 % of community 1 and 25 % of community 2. Around 15 % of all the bacterial genera identified in this study were cataloged as bioremediation agents of contaminated environments.

The richness indexes calculated in the study yielded an increment in percent variance as well as a decrement in both communities in consideration, thus indicating that it was not possible to determine the community with a higher richness index. Regarding the diversity index, the results from the study pointed to a higher bacterial diversity in community 1 with respect to 2. However, it was statistically proven that there was no significant difference in the global comparison between both communities (P>0.001). Similarly, the results exhibited similar genetic structures between samples of each of the communities analyzed separately (P>0.008).

The outcomes of this study represent a challenge for the biotechnological exploration and the comprehension of the composition, richness, and diversity of the bacterial community existent in the Amazon rainforest, furthermore, they represent the potential to identifying and classifying new taxonomic groups.

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