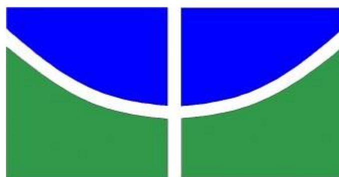

UNIVERSIDADE DE BRASÍLIA – UNB
FACULDADE DE CIÊNCIAS DA SAÚDE – FS

CURSO DE FARMÁCIA

CHEMICAL AND MICROBIOLOGICAL MONITORING OF BIODIESEL BLEND (B7) QUALITY UNDER SIMULATED STORAGE CONDITIONS.

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Trabalho de conclusão de curso apresentado ao Departamento de Farmácia da Faculdade de Ciências da Saúde da Universidade de Brasília, como requisito à conclusão da disciplina Trabalho de Conclusão de Curso (TCC) e obtenção do grau de Bacharel em Farmácia.

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Chemical and microbiological monitoring of biodiesel blend (B7) quality under simulated storage conditions

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Abstract

Biodiesel is an oxygenated biofuel, and it has unsaturated carbon chains. It is more susceptible to oxidation, and because it is more hygroscopic than diesel, an increase in microbial contamination may also be favored. To study the biodiesel/diesel mixture's storage, a commercial sample of diesel B (B7 S500; 7% v / v biodiesel in diesel) was collected in a Center Western region station (Brazil) and stored in tanks. Lab analyses were performed for 90 days, the first time point being zero, and the following time points were taken every 30 days. Chemical analysis was performed using gas chromatography using methyl nonadecanoate as standard. Analysis of variance, with a probability of 95%, indicated that at 60 days of storage, the sample already presented chemical degradation. Microbial monitoring was performed and It was possible to distinguish four bacterial genera (Alkalihalobacillus, Bacillus, Paenibacillus, and Peribacillus) and at least 15 species. Fungi were isolated, and 17 genera were identified (32 species): Aspergillus, Cladosporium, Penicillium, Chaetomium, Curvularia, Epicoccum, Microascus, Paecilomyces, Acanthophysellum, Arthrinium, Bypolaris, Paraconiothyrium, Parengyodontium, Phlebiopsis, Polyporus, Talaromyces, and Xylaria. The isolation results showed a low level of contamination of the as-received B7 blend from the fuel station, which can be considered natural microbial contamination expected in a storage tank. The present study confirmed the contamination of B7 blend storage tanks by various microorganisms that can colonize

and most likely degrade fuels and should be investigated in more detail as possible reference strains in biodeterioration studies.

Keywords: diesel-biodiesel blends; storage stability; microbial contamination; biofuels; fungi; bacteria

1. Introduction

Biodiesel is a biofuel that was inserted in the Brazilian energy matrix through the creation of the National Program of Production and Use of Biodiesel (Programa Nacional de Produção e Uso do Biodiesel - PNPB) in 2004. In 2005, minimum percentages of biodiesel in petroleum diesel fuel were determined [1]. Initially, this incorporation was authoritative at the level of 2% (v/v). As of 2008, mixing has become mandatory throughout the national territory. With the maturation of the market, this percentage has been increased successively, rising to 11% in 2019, with an increase of 1% in the following years, reaching 15% in 2023. Considering the territorial dimensions of Brazil with different biomes and climatic conditions, and the complexity of the logistics of distribution and delivery of biodiesel to the distributors, studies on the quality of the liquid fuels in the fuel chain in the different regions of the country are essential [2].

Biodegradable fuel, derived from renewable sources, formed by long-chain esters (18 carbon atoms on average), biodiesel can be obtained by the transesterification reaction between either a vegetable oil (soy, canola, sunflower, and cotton) or animal fat (bovine, swine, or poultry tallow) and an alcohol, usually methanol or ethanol, in the presence of a catalyst. Biodiesel has similar physical and chemical characteristics to diesel, which consists of approximately: 42% long-chain alkanes, 32% cycloalkanes, 25% aromatics, and 1% other components. Despite the chemical similarity to diesel, the fatty acid profile of the oils and fats used in the manufacture of biodiesel favors the development of the main chemical degradation process that affects it: oxidation. Known as oxidative rancidification, this degenerative action resulting from contact with oxygen in the air, as a rule, acts on biodiesel rich in unsaturated fatty acids, obtained from raw materials that dominate the Brazilian market (soy, cotton, and other materials). This characteristic, associated with the greater hygroscopicity of biodiesel about diesel, tends to cause numerous problems

in the storage and injection systems in diesel-powered vehicles, both chemical and microbial [3].

The chemical degradation of biodiesel and the mixture with diesel can occur by oxidation, photoionization, and thermal and hydrolytic processes. The chemical degradation process begins mainly by the oxidation of the carbons of the double bonds, forming allylic peroxides. As these are unstable, later, other reactions and rearrangements occur that contribute to the formation of smaller chain compounds such as aldehydes and acids; there may still be polymerization reactions [4]. Among the chemical contaminants in biodiesel is water, which is incorporated along the chain, either during transport or supply; mono-, di- and triglycerides, ethanol and methanol, not eliminated in the purification process and steroids and triterpenes, present in the oil [5-8]. Water, besides being a contaminant, promotes hydrolytic degradation, bringing countless consequences. The water dissolved in contact with metals tends to promote the formation of metallic sediments, induce the appearance of free water in the form of microdroplets, or adhered to the walls of the tank, or in the decanted form, culminating in the accumulation of ballast water [2].

Microbial contamination in liquid fuels is most noticeable in storage systems [9]. Historically, microbial contamination during storage has been seen as a problem of diesel [10,11]. Along with the introduction of biodiesel, issues such as microbial contamination, biodegradation, and formation of biomass became more common in diesel/biodiesel samples during storage [11-17]. Water is essential for microbial activity, therefore most recommendations to avoid the presence of or to mitigate microbial activity in fuels include water control [11]. Although high temperatures that occur during the production process of fuels sterilize the material produced [18], depending on transport and storage conditions, water can be adsorbed, therefore allowing microbiological contamination and possibly biodeterioration [19].

For microbial growth, elements as carbon, nitrogen, phosphorus, potassium, and sulfur, can be naturally found in fuels. Calcium, sodium, iron, magnesium, manganese, copper, cobalt, and nickel which are needed in small quantities, can come into the system through dust particles, inorganic salts contained in water, and due to other processes as by adding corrosion inhibitors [13]. The ability to metabolize hydrocarbons (aliphatic and aromatic) of diesel is a determining factor for the growth of microorganisms during storage and transportation [10]. Some fungi and bacteria produce biosurfactants which create favorable conditions for the consumption of

hydrocarbons, as it helps the formation of microemulsions in the aqueous phase, making them more available for microbial consumption [18]. The metabolites generated by hydrocarbon-degrading microorganisms can serve as nutrients for other microbial communities [10]. In the case of biodiesel, its biodegradability is due to the presence of esters of fatty acids, which are easily degraded by different types of microorganisms [12].

Many microbial species have been found in fuels, although not all are capable of metabolizing hydrocarbons. Examples of bacteria capable of using hydrocarbons as a source of carbon are among the genera *Achromobacter*, *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Burkholderia*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*, and *Vibrio* [20-23]. During their review of the groups of bacteria and archaea capable of metabolizing hydrocarbons (aerobically or anaerobically), Prince et al. [24] reported at least 320 different genera of *Eubacteria* (emphasis on the phyla *Actinobacteria*, *Bacterioidetes*, *Firmicutes,* and *Proteobacteria*) and 12 different genera of *Archaea*. Fungi commonly isolated from fuels and capable of metabolizing hydrocarbons include *Alternaria*, *Aspergillus*, *Candida*, *Cladosporium*, *Clonostachys*, *Fusarium*, *Hormoconis*, *Lambertella*, *Paecilomyces*, *Penicillium*, *Pestalotiopsis*, *Pseudallescheria*, *Pseudozyma*, and *Rhodotorula* [13,25-30]. As a consequence of high microbial contamination, clogging of filters, presence of biosediments, corrosion influenced by microorganisms, reduction in chemical stability or increased corrosivity of the fuel can occur [31].

Studies on microbial contamination in liquid fuels are based on cultivation techniques and phenotypic identification [32]. Among the methods available for studying microbial diversity, in addition to the traditional ones, those based on the sequencing of genes encoding ribosomal RNA directly from environmental samples, that is, without the need for cultivation of microorganisms, deserve to be highlighted. Such approaches were developed to complement the traditional culture-dependent approaches, as they consist of the extraction of total DNA from environmental samples, followed by the sequencing of the genes from the ribosomal DNA (rDNA) cluster. Thus, the use of next-generation sequencing (NGS) techniques allows broad coverage of microbial diversity in a given sample.

Microbial diversity studies that use molecular techniques can provide further insight into the efficiency of practices used by industry. Additionally, the correct

identification of contaminants, particularly deterioration indicators (sulfate-reducing bacteria, iron-oxidizing bacteria, and biofilm-forming bacteria), can facilitate interventions and control measures. An increasing number of studies using NGS have been conducted to assess microbial diversity in samples of diesel, biodiesel, and other liquid fuels [14,33-36]. For example, White et al [33] evaluated kerosene, diesel, and biodiesel samples at filling stations using traditional isolation and independent cultivation techniques, such as DGGE (denaturing gradient gel electrophoresis) and 16S ribosomal DNA pyrosequencing. The authors found that bacteria abundant in the culture medium (*Pseudomonas* spp.) were not observed in the same proportion by molecular techniques and that bacteria detected by molecular techniques (*Clostridia*, *Halomonas*, *Deltaproteobacteria*) were not detected in the culture medium, demonstrating the importance of combining different strategies for a better assessment of the composition of microbial communities in samples of liquid fuels.

The use of cultivation-based techniques is a strategy commonly accessible to microbiology laboratories and is recognized as a standard for monitoring liquid fuels [11,18], despite its limitations. Even so, when accompanied by taxonomic identification through DNA analysis, it has contributed to the understanding of the impact of microorganisms on the quality of liquid fuels [37-39]. In this context, this work aimed to carry out chemical and microbiological monitoring of a commercial sample of diesel B S500 (B7) collected at a fuel station in the Midwest region in Brazil and submitted to simulated storage. The results of the periodic analyzes of the chemical characteristics and the composition of the cultivable bacterial and fungal communities during storage are discussed.

2. Materials and Methods

2.1. Fuel sample collection and storage simulation

In August 2015 (dry season), a sample of approximately 40 liters of commercial diesel B7 S500 (blend containing 7% of biodiesel added to diesel S500, 500 ppm of sulfur, which can be considered a high sulfur diesel (HSD) that in Brazil is identified by the addition of a red dye) was collected at a local fuel station in Brasília, Federal District, Brazil (Midwest region of Brazil, one of the main biodiesel producing regions in the country; Cerrado biome; Latitude 15°47’59.46” S, Longitude 47°58’21.92” W).

The fuel was collected from the fuel pump using sterile gallons (four gallons with 10 liters capacity each). Then, the sample was transported to the Embrapa Agroenergia laboratory (Brasília, Federal District, Brazil). The first 10 liters were immediately processed for chemical and microbiological analysis without being stored (zero time), while the other 30 liters were stored in three prototype tanks (10 liters each) in order to simulate the conditions in which it stays at fuel stations. The tanks had a capacity for 20 liters, had a metallic structure (carbon steel and AISI-316 stainless steel), as well as a top opening coupled with a filter and a lateral collector. Each tank had previously been sterilized by being filled up with 20 liters of 70% ethanol for 24 hours before storage of the collected fuel. For safety reasons, the three tanks were kept under environmental conditions in a warehouse dedicated to the storage of flammable products located at Embrapa Agroenergia. For each sample time (30, 60, and 90 days, respectively), samples were collected from the simulated storage tanks and submitted to chemical and microbial monitoring.

2.2. Chemical analysis

For chemical monitoring, samples collected at zero, 30, 60, and 90 days of simulated storage were diluted in a 1.5 mL vial, in the proportion of 25 µL of sample to 975 µL of heptane. For analysis in GC-FID, the internal standard nonadecanoate of methyl at a concentration of 400 $\mu g.mL^{-1}$ was added to the sample. The chromatographic analysis was performed using the following equipment: GC-MS QP 2010 (Shimadzu) and GC-FID 7890A (Agilent). The Rtx -5ms column (30 m x 0.25 mm x 0.25 µm) was used. The analytical conditions of the GC-MS were: injector temperature and interface 250°C, ion source temperature 200°C, helium carrier gas with a linear speed of 38 $cm.s^{-1}$, and injector in split mode (25:1). The column temperature remained at 120°C for 2 minutes isothermally and then increased at 10°C / min to 300°C. This temperature was maintained for 5 minutes isothermally. Due to the complexity of the sample (biodiesel + diesel), the compounds that showed the most intense signal between C15 and C28 were monitored. Thus, the method was optimized in the GC-MS and then adapted for the GC-FID by adjusting the temperature of the FID detector to 320°C.

2.3. Microbiological analysis

2.3.1. Microbial isolation and culture purification

Microbiological analysis was performed following ASTM D6469-16 (*ASTM D6469-16 Standard Practice for Enumeration of Viable Bacteria and Fungi in Liquid Fuels, Filtration, and Culture Procedures*). Firstly, three volumes (2 mL, 20 mL, 200 mL) of each fuel sample (zero, 30, 60, and 90 days) were separately filtered under aseptic conditions using a vacuum pump and a sterile 0.22 μM, 47 mm cellulose ester membrane filter (Millipore®). After the fuel filtration, 10 mL of a sterile TWEEN® 80 0.1% solution was added and filtered, and then 30 mL of Ringer Solution (g/L NaCl 2.25; KCl 0.105; $CaCl_2.2H_2O$ 0.12; $NaHCO_3$ 0.05) was added and filtered. Three membranes were filtered to each volume to each culture medium, resulting in the filtration of 1998 mL (3 x 666 mL) of the fuel at different sample times (0, 30, 60, and 90 days). The washed membranes were transferred to Malt Extract Agar (MEA, Sigma-Aldrich) plates containing the antibiotic chloramphenicol (100 μg/mL) for filamentous fungi isolation, Tryptic Soy Agar (TSA, Sigma-Aldrich) plates containing the fungicide Benomyl (200 μg/mL) for bacterial isolation, and Yeast Extract Peptone Dextrose Agar (YPD Agar, Sigma-Aldrich) plates containing chloramphenicol (100 μg/mL) for yeast isolation. The plates were incubated at 28ºC for up to 15 days.

After incubation, bacterial colonies were collected from the surface of the membranes and inoculated in a new TSA plate. The plates were again incubated at 28°C until the growth of pure cultures. The number of isolated bacteria represents the total number that has been cultivated in the standard culture medium after obtaining pure cultures and not the total number of bacteria present in the filter membranes. Concerning the fungal isolation, all fungal colony growth on the membrane surface were collected and inoculated in a new MEA plate until the growth of pure cultures. After the purification steps, the bacterial and fungal strains were preserved by freezing at −80°C in glycerol solution (30%) as a cryoprotectant agent. The microbial strains were deposited in the "*Collection of Microorganisms and Microalgae Applied to Agroenergy and Biorefineries - CMMAABio*", located at Embrapa Agroenergia, Brasília, Federal District, Brazil. The activity of access to genetic heritage and associated traditional knowledge, in the scientific research category, was registered at the website of the SisGen platform (https://sisgen.gov.br) of the Genetic Heritage Management Council (CGEN) according to the Biodiversity Law from Brazil (registered under number A60E70E).

2.3.2. Nucleic acids purification, PCR amplification, and DNA sequences analysis

The bacterial strains were cultured in 50 mL Falcon tubes containing 10 mL of the Luria-Bertani broth (Sigma-Aldrich) for 16-20 hours with shaking (180 rpm) at a temperature of 28ºC. The cells were harvested by centrifugation of 2 mL of the bacterial culture at 4ºC and 12,000 rpm for 5 minutes. The genomic DNA was purified using the Wizard® Genomic DNA Purification Kit (Promega). The fungal strains were cultured in 250 mL Erlenmeyer flasks containing 50 mL of the complete medium [40] for 7-10 days at 25ºC in the dark without agitation. Then, the mycelial biomasses were collected by filtration and subjected to grinding using a mortar and pestle chilled with liquid nitrogen turning them into dust. Nucleic acid extraction was performed as described by [41].

A partial sequence of the 16S rRNA gene was PCR amplified with the primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1378R (5'-CGGTGTGTACAAGGCCCGGGAACG-3') [42]. PCR was performed in 50 μL reactions containing 20 ng of the bacterial genomic DNA, 1.5 U of GoTaq® DNA Polymerase (Promega) in 1X GoTaq® Reaction Buffer (Promega), 0.2 mM dNTP, 2.5 mM $MgCl_2$, and 0.2 μM of each primer. Initial denaturation was carried out at 94ºC for 4 minutes, followed by 35 cycles of 30 seconds at 94ºC, 1 minute at 67ºC, and 1 minute at 72ºC, with a final extension at 72ºC for 10 minutes.

We used standard PCR procedures for the amplification of the internal transcribed spacer (ITS1-5.8S-ITS2) of the fungal rDNA units. The pair of primers ITS1-F (5'-CTTGGTCATTTAGAGGAAGTAA-3') [43] and ITS-4 (5'-TCCTCCGCTTATTGATATGC-3') [44] were used to amplify the ITS1-5.8S-ITS2 region. PCR was performed in 50 μL reactions containing 20 ng of the fungal genomic DNA, 1.5 U of GoTaq® DNA Polymerase (Promega) in 1X GoTaq® Reaction Buffer (Promega), 0.2 mM dNTP, 3.7 mM $MgCl_2$, and 0.8 μM of each primer. Initial denaturation was carried out at 94ºC for 4 minutes, followed by 35 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, and 72ºC for 30 seconds, with a final extension at 72ºC for 10 minutes.

The amplification products (16S, ITS1-5.8S-ITS2) were checked through electrophoresis on agarose gel (1.0% w/v agarose) and UV visualization of the ethidium bromide-stained gel, after which PCR products were purified (Wizard® SV

Gel and PCR Clean-Up System, Promega). The purified fragments were sequenced in both directions using ABI BigDye terminator chemistry at the Eurofins company (Indaiatuba, São Paulo, Brazil).

The consensus sequences were generated using Geneious R11 (https://www.geneious.com). The resulting sequences were analyzed against the nucleotide collection of the GenBank database (National Center for Biotechnology Information database) using the blastn tool, including the comparison with sequences from type material. Fungal sequences were additionally evaluated using the identification tools of the UNITE (https://unite.ut.ee/) [45] and MycoBank (https://www.mycobank.org/) [46] databases. The results were organized in tables and the DNA sequences were deposited in the GenBank database under accession number X.

3. Results and Discussion

3.1. Chemical monitoring of the commercial B7 biodiesel/diesel blend under simulated storage conditions

Due to the samples' complexity (diesel + biodiesel), the compounds that showed the most intense signal between C15 and C28 were monitored in the chromatographic analyses. The analysis method was optimized in the GC-MS and then adapted to the GC-FID, adjusting the FID detector's temperature to 320°C. **Figure 1** shows the chromatograms obtained on both equipment types for the original B7 S500 blend sample from zero time (without storage).

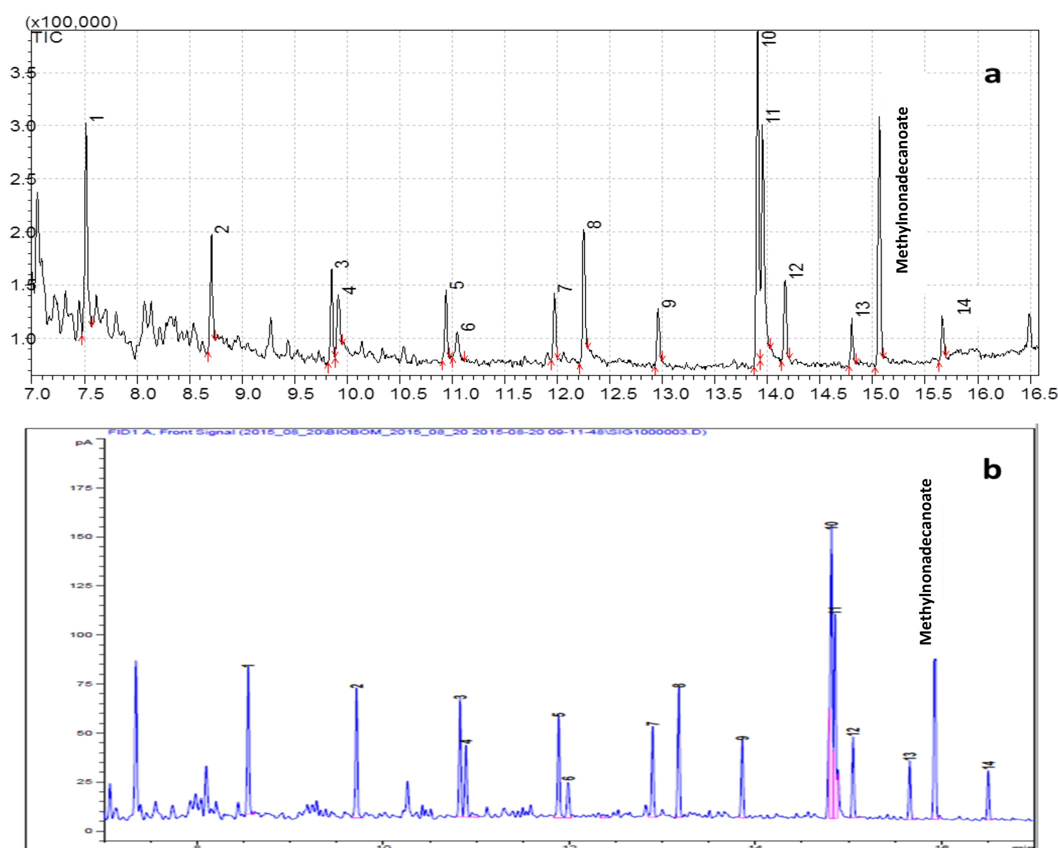

Figure 1. Chromatogram of the B7 S500 sample (zero storage time) by gas chromatography (GC): (a) total ion chromatogram (TIC) and (b) chromatogram by flame ionization detector (FID). 1. Pentadecane, 2. Hexadecane, 3. Heptadecane, 4. 2,6,10,14-tetramethylpentadecane, 5. Octadecane, 6. 2,6,10,14-tetramethylhexadecane, 7. Nonadecane, 8. Methylhexadecanoate, 9. Eicosane, 10. 8,11-Methyloctadecadienoate, 11. 9-Methyloctadecenoate, 12. Methyloctadecanoate, 13. Docosane, 14. Octacosane.

The evaluation of chemical degradation was done by determining the target compounds' peak areas, normalized by the standard compound area, prepared on the same day of the analysis. **Figure 2** presents a comparative graph of the areas of the 14 compounds identified, listed in **Table 1**, during the 90 days of storage. It is observed that the areas of the compounds varied over the 90 days of storage. Given the above, it was necessary to perform the variance analysis followed by the Tukey test, with a significance of 95%, using the Statistica® software. The data with the mean of the chromatographic peak areas are shown in Table 1.

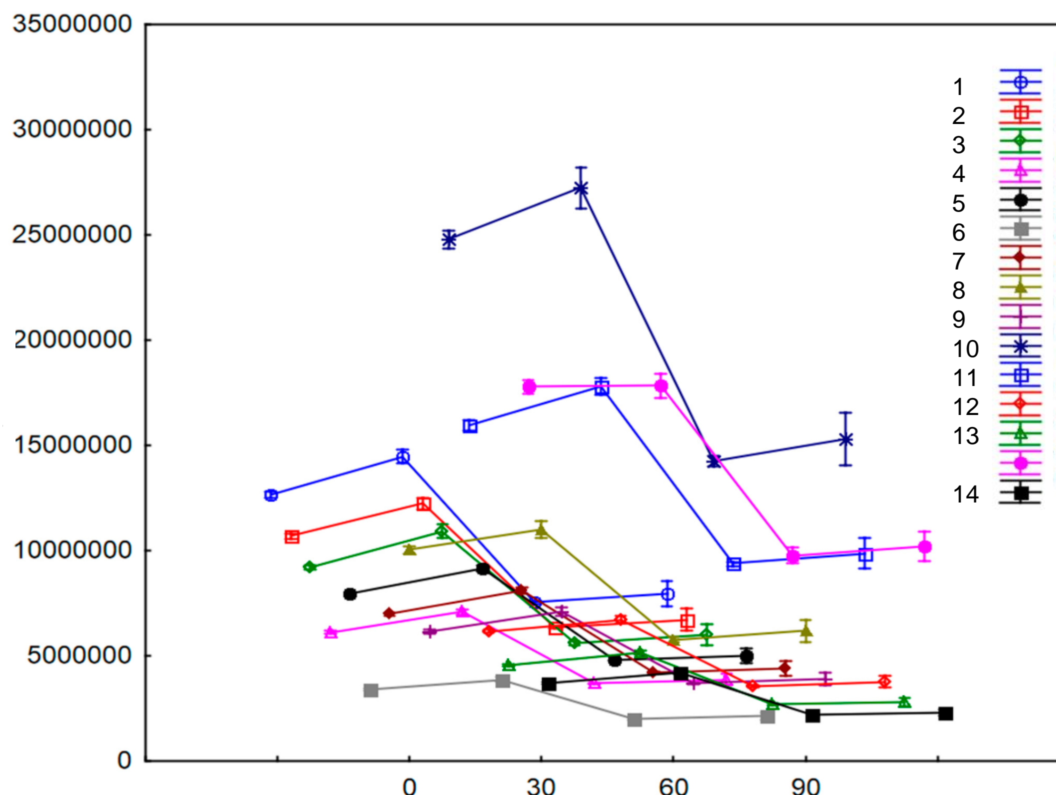

Figure 2. Peak areas of CG-FID of the compounds of interest detected after 0, 30, 60, and 90 days of the B7 biodiesel/diesel blend (B7 S500) simulated storage in replica tanks. 1. Pentadecane, 2. Hexadecane, 3. Heptadecane, 4. 2,6,10,14-tetramethylpentadecane, 5. Octadecane, 6. 2,6,10,14-tetramethylhexadecane, 7. Nonadecane, 8. Methylhexadecanoate, 9. Eicosane, 10. 8,11-Methyloctadecadienoate, 11. 9-Methyloctadecenoate, 12. Methyloctadecanoate, 13. Docosane, 14. Octacosane.

Table 1. Evaluation of the averages of the chromatographic peaks of the compounds monitored in the B7 biodiesel/diesel blend (B7 S500) under simulated storage conditions (variance analysis followed by the Tukey test was performed at 95% probability, using the Statistica® software).

Compounds		Storage time (days)			
		0	30	60	90
1	Pentadecane $C_{15}H_{32}$	12663520 a	14471432 b	7546114 c	7974578 c
2	Hexadecane $C_{16}H_{34}$	10695257 a	12241825 b	6365499 c	6726539 c
3	Heptadecane $C_{17}H_{36}$	9200583 a	10924542 b	5601141 c	5999445 c
4	2,6,10,14-tetramethylpentadecane $C_{19}H_{40}$	6112790 a	7085996 b	3690543 c	3884314 c

5	Octadecane $C_{18}H_{38}$	7966647 a	9156119 b	4782764 c	5006990 c
6	2,6,10,14-tetramethylhexadecane $C_{20}H_{42}$	3418409 a	3871829 b	2013917 c	2134267 c
7	Nonadecane $C_{19}H_{40}$	6987680 a	8113263 b	25323961 c	26446363 c
8	Methylhexadecanoate $C_{17}H_{34}O_2$	10052508 a	11012416 a	5762324 b	6185032 b
9	Eicosane $C_{20}H_{42}$	6153743 a	7100113 b	3686077 c	3891672 c
10	8,11-Methyloctadecadienoate $C_{19}H_{34}O_2$	24791547 a	27249059 a	14258834 b	15298384 b
11	9-Methyloctadecenoate $C_{19}H_{36}O_2$	15961601 a	17807398 b	9381107 c	9879931 c
12	Methyloctadecanoate $C_{19}H_{38}O_2$	6183529 a	6720658 a	3556000 b	3765467 b
13	Docosane $C_{22}H_{46}$	4570349 a	5137574 b	2715177 c	2814453 c
14	Octacosane $C_{28}H_{58}$	3701708 a	4195582 b	2215678 c	2297978 c
15	Methylnonadecanoate (standard) $C_{20}H_{40}O_2$	17798474 a	17837408 a	9771516 b	10226471 b

It can be observed in **Table 1** that, for all compounds, there was an increase in area with 30 days of simulated storage of the B7 biodiesel/diesel blend (B7 S500) compared to zero time. The fact can be explained by the evaporation of volatile diesel compounds, causing the monitored compounds to be concentrated. Statistical analysis revealed that the time of 30 days is different from time zero and that the times 60 and 90 days are statistically equal but different from 30 days for most compounds. These data allow us to conclude that there was chemical degradation of the monitored compounds in 60 days of simulated storage in replica tanks. It is also worth mentioning that, until the end of the 90 days of the simulated storage in tanks, no particulate material or any sludge or biomass, or free water were observed in the monitored samples.

Many studies have been conducted on biodiesel blends' storage stability [34,47-50]. In general, storage conditions, oxygen exposure, temperature, contamination from metals and other radical initiators, water exposure, light exposure, the fatty acid profile of the biodiesel used in blends, and the origin of the fossil diesel

could all contribute to the degradation of fuel quality [34,47,48,50]. In the present work, the storage stability of a Brazilian commercial B7 blend was studied in experiments simulating up to 90 days of storage, and the chemical degradation of the monitored compounds was observed in 60 days of storage in replica tanks.

Bücker et al. [49] monitored the degradation of n-alkanes and methyl fatty acids in pure biodiesel and blends (B5, B10, and B50) using gas chromatography analysis in experiments simulating up to 60 days of storage in glass flasks, after contamination with an uncharacterized inoculum from the biological sludge collected from storage tanks of B100 and B5 biodiesel in Brazil. The authors observed biodegradation of the FAMEs and n-alkanes, the degradation rates of FAMEs were higher in B5 > B10 > B50. The biodegradation of the n-alkanes was higher in B50 and B10 > B5, followed by B0.

Azambuja et al. [34] observed chemical degradation of biodiesel blends under simulated storage conditions in fuel samples with characteristics similar to those surveyed in the present work: blend with up to 10% biodiesel, fossil diesel containing approximately 500 ppm of sulfur (named HSD blend), and a low level of contamination of the fuel as received from the fuel station, which is considered as natural microbial contamination, as expected in a storage tank according to Hill & Hill [51]. The authors evaluated the behavior of the microbial biomass formed in the Brazilian B10 blend containing petroleum diesel with different sulfur contents: ultra-low-sulfur diesel ULSD, high-sulfur diesel (HSD), and ultra-high-sulfur diesel (UHSD), in the condition of natural contamination and with the addition of a microbial inoculum under simulated storage for 40 days. After 40 days of storage, infrared spectroscopy detected structural changes in the various sulfur content B10 blends related to the terminal methyl esters of the biodiesel fatty acids produced by degradation. All treatments underwent molecular alterations, but the natural contamination condition showed a lower degradation tendency than the inoculated treatment. They also observed that the HSD B10 blend was more susceptible to biodegradation due to the reduction of biodiesel terminal methyl and the fraction of aromatic hydrogen from diesel. They also presented pioneering results from metabolomic analysis to elucidate and identify possible metabolites as biomarkers of microbial activities related to biocorrosion and biodeterioration of biodiesel blends.

Any fuel requires storage for some long term for the future use of it [50]. When storing fuel in the long term, a monitoring program is recommended by fuel

specifications [47]. In Brazil, Cavalcanti et al. [2] pointed that the territorial dimensions of Brazil with different biomes and climatic conditions, added to the fact that most Brazilian biodiesel plants are concentrated in the Midwest and Southern parts of the country, presents an extra logistic challenge to keep the quality of the fuel under control over the distribution, commercialization, and retail chains. In this context, studies on the evolution of biodiesel blends' shelf life and storage stability within the country's climatic conditions have fundamental importance [2]. The present work contributes to monitoring the quality of commercial biodiesel blend in the Midwest region, which presents a lack of similar studies compared to other areas in Brazil.

3.2. Microbial monitoring of the commercial B7 biodiesel/diesel blend under simulated storage conditions

Aerobic fungi and bacteria were isolated in the MEA and TSA culture media, respectively, from all sampling times. No yeast was obtained in the YEPD agar culture medium at any of the evaluated times. **Table 2** shows the number of contaminants microorganisms isolated in each sampling time.

Table 2. Microorganisms isolated from a Brazilian commercial B7 blend in experiments simulating 90 days of storage in tanks in the country's Midwest region.

Simulated storage time in tanks	Bacterial strains	Filamentous fungi strains
As received from fuel station (zero time)	53	19
30 days	8	50
60 days	10	9
90 days	35	101
Total =	106	179

The isolation results showed a low level of contamination of the as-received B7 blend from the fuel station, which can be considered natural microbial contamination (~10^3 CFU/L) expected in a storage tank [51]. The microbial population from the as-received fuel did not produce significant or visible biomass during the 90-day storage.

We tried to extract DNA from all samples' filtered membranes (using PowerSoil® DNA Isolation Kit, MO BIO Laboratories Inc.). However, as previously reported [34], we also note that the as-received condition (and the stored samples) did not produce enough biomass for DNA extraction with quality or quantity to be used on next-generation sequencing (not showed).

The limitations of culture-dependent approaches are well known, and < 1% of the estimated microbial diversity is thought to be cultivable under laboratory conditions [52]. Even so, the isolation of microorganisms at all sampling times showed a cultivable microbial community associated with the evaluated commercial fuel. Chemical analysis of the B7 biodiesel/diesel blend (B7 S500) over time revealed the occurrence of chemical degradation of the monitored compounds (**Table 1**). With the low microbial density in the initial sample, it is possible to suggest that the commercial fuel evaluated was not a deteriogenic source during the simulated storage of the B7 blend in up to 90 days of storage. Qualitative indication of microbial growth on B7 blend is evidence but no final proof for hydrocarbon (diesel) and FAME (biodiesel) degrading activity. Therefore, testing for fuel biodegradation is needed to assess the deteriorative potential of the culturable microbial contaminants.

Addressing microbial contamination requires reliable information about microorganisms' identity that cause such problems [53]. In this respect, we identified 99.05% of the bacteria and 69.83% of the contaminating fungi isolated from the B7 blend over time. Supplementary Materials 1 and 2 show the molecular identification results based on the sequence analysis of ribosomal DNA from bacteria (16S rDNA) and fungi (ITS1-5.8S-ITS2 rDNA).

The results showed that the 105 bacterial strains that could be classified belong to the phylum *Firmicutes*. In all, it was possible to distinguish four genera (*Alkalihalobacillus*, *Bacillus*, *Paenibacillus*, and *Peribacillus*) and at least 15 different species. *Bacillus* genus corresponding to 96.22% of the strains. **Table 3** shows the distribution of the 15 commercial B7 blend-contaminating bacterial species in the experiment simulating 90 days of storage in tanks in Brazil's Midwest region.

In general, of the 15 species of bacteria, ten were isolated in only one of the sampled times. *B. subtilis* was isolated at all tested times, followed by *B. australimaris*, obtained at three sampling times. Considering all the microbial monitoring times, the most frequently isolated species were: *B. subtilis*, *B. tequilensis*, *B. siamensis*, *B. australimares*, and *B. amyloliquefaciens* (Supplementary Material 1).

Table 3. Distribution of the commercial B7 blend-contaminating bacterial species in an experiment simulating 90 days of storage in tanks in Brazil's Midwest region.

Bacterial species	Simulated storage time in tanks			
	Zero	**30 days**	**60 days**	**90 days**
Alkalihalobacillus clausii				1
Alkalihalobacillus rhizosphaerae			1	
Bacillus amyloliquefaciens				8
Bacillus australimaris	1	5		7
Bacillus cereus				1
Bacillus licheniformis		1	5	
Bacillus piscis			1	
Bacillus pumilus	1			
Bacillus rugosus	5			
Bacillus safensis	2			1
Bacillus siamensis				15
Bacillus subtilis	23	2	2	1
Bacillus tequilensis	19			1
Paenibacillus provencensis			1	
Peribacillus acanthi	1			
Not determined	1			
Total =	**53**	**8**	**10**	**35**

Regarding the fungi samples, of the 179 sequenced strains, 125 presented sequences from the ITS region with sufficient quality for analysis (Supplementary Material 2). The results showed that, of the 125 strains that could be classified, 3 belong to the phylum Basidiomycota (Class Agaricomycetes) and 122 to the phylum Ascomycota. Among ascomycetes, 67 belong to the Eurotiomycetes class, 46 belong to the Dothideomycetes class, and 9 belong to the Sordariomycetes class. Altogether it was possible to distinguish 17 different genera (and at least 32 species), the most frequent of which were Aspergillus, Cladosporium and Penicillium. Specifically, the 17

genera identified by analyzing the ITS region of the rDNA and their frequency were: 57 *Aspergillus*, 39 *Cladosporium*, 7 *Penicillium*, 4 *Chaetomium*, 3 *Curvularia*, 2 *Epicoccum*, 2 *Microascus*, 2 *Paecilomyces*, 1 *Acanthophysellum*, 1 *Arthrinium*, 1 *Bypolaris*, 1 *Paraconiothyrium*, 1 *Parengyodontium*, 1 *Phlebiopsis*, 1 *Polyporus*, 1 *Talaromyces*, and 1 *Xylaria*. **Table 4** shows the distribution of the commercial B7 blend-contaminating fungal species in the experiment simulating 90 days of storage in tanks.

Table 4. Distribution of the commercial B7 blend-contaminating fungal species in an experiment simulating 90 days of storage in tanks in Brazil's Midwest region.

Fungal species	Simulated storage time in tanks			
	Zero	30 days	60 days	90 days
Acanthophysellum sp.				1
Arthrinium sp.			1	
Aspergillus flavus	2	10		13
Aspergillus fumigatus	3	4		3
Aspergillus niger		4		
Aspergillus sp.				1
Aspergillus tamarii	3	3	2	9
Bipolaris gossypina				1
Chaetomium globosum				2
Chaetomium sp.				2
Cladosporium anthropophilum				2
Cladosporium halotolerans		11		8
Cladosporium sp.		2		8
Cladosporium tenuissimum			1	2
Cladosporium xanthochromaticum				4
Cladosporium xylophilum				1
Curvularia sp.			1	2
Epicoccum nigrum				1

Epicoccum sp.				1
Microascus sp.				2
Paecilomyces saturatus	1	1		
Paraconiothyrium sp.		1		
Parengyodontium album			1	
Penicillium chrysogenum				1
Penicillium citrinum				2
Penicillium glabrum		2		
Penicillium miczynskii		1		
Penicillium simplicissumum				1
Phlebiopsis sp.				1
Polyporus sp.			1	
Talaromyces pinophilus				1
Xylaria sp.				1
Not determined	10	11	2	31
Total =	**19**	**50**	**9**	**101**

Previous studies have also shown fungi in fuel samples, especially in the case of the three genera mentioned above [9,11,25]. Among the identified species of the *Aspergillus* genus, *A*. flavus and *A*. tamarii stand out as having good deterioration capacity in diesel [54]. The second most common genus found in the sample, Cladosporium, is known to produce lipases - enzymes capable of catalyzing a hydrolysis of fats. Gunasekaran et al [55], that *Cladosporium* tenuissimum is capable of producing stable and effective lipase. Silva et al [56] indentified many similar species from samples collected from other places in the Midwest region. In addition, many other species have also been identified, thus showing how varying contamination may be.

4. Conclusion

Chemical analysis of the B7 S500 biodiesel sample showed that it deteriorated after 60 days of simulated storage. As it is considered a sample with low level of contamination, such deterioration must be further investigated in order to determine its origin. A new study is suggested regarding the identification of microbial metabolites to elucidate this issue.

The microbial diversity identified in the sample matches with previous studies results, being the genera Bacillus (bactera) and Aspergillus (fungi) the most commonly found. The identified microorganisms, shall be used as bioremediators in cases of environmental accidents. The statistical evaluation of sample contamination can serve as a basis for establishing microbiological quality control parameters.

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References

[1] BRASIL. Lei nº 11.097, de 13 de janeiro de 2005. Dispõe sobre a introdução do biodiesel na matriz energética brasileira; altera as Leis nº 9.478, de 6 de agosto de 1997, 9.847, de 26 de outubro de 1999 e 10.636, de 30 de dezembro de 2002; e dá outras providências. Diário Oficial da União, 14 jan. 2005.

[2] Cavalcanti E.H.S., Zimmer A.R., Bento F.M., Ferrão M.F. Chemical and microbial storage stability studies and shelf life determinations of commercial Brazilian biodiesels stored in carbon steel containers in subtropical conditions. Fuel. 2019;236:993–1007. doi: 10.1016/j.fuel.2018.09.043.

[3] CAVALCANTI, E. H. de S. Estabilidade do biodiesel e misturas: abrangência, limitações dos métodos de avaliação e alternativas futuras. Revista Biodieselbr, n. 13, p. 71-73, 2009.

[4] KNOTHE, G. Some aspects of biodiesel oxidative stability. Fuel Processing Technology, v. 88, n. 7, p. 669-677, July 2007. DOI: 10.1016/j.fuproc.2007.01.005.

[5] BREWER, M. Identification of precipitate found in depot storage tanks containing Swedish Klass1 B5 fuels. In: INTERNATIONAL CONGRESS ON BIODIESEL, 2007, Vienna, Austria. Proceedings… [Washington]: USDA; [Kuala Lumpur]: AOCS, 2007.

[6] LEE, I.; PFALZGRAF, L. M.; POPPE, G. B.; POWERS, E.; HAINES, T. The role of sterol glucosides on filter plugging. Biodiesel Magazine, v. 4, p. 105-112, Apr 2007.

[7] SELVIDGE, C.; BLUMENSHINE, S.; CAMPBELL, K.; DOWELL, C.; STOLIS, J. Effect of biodiesel impurities on filterability and phase separation from biodiesel and biodiesel blends. In: INTERNATIONAL CONFERENCE ON STABILITY, HANDLING AND USE OF LIQUID FUELS, 10., 2007, Tucson, Arizona. Proceedings... [S.l.]: IASH, 2009.

[8] PFALTZGRAF, L.; LEE, I.; FOSTER J.; POPPE, G. Effect of minor components on the cloud point and filterability. Biodiesel Magazine, Nov 2007.

[9] GAYLARDE, C. C.; BENTO, F. M.; KELLEY, J. Microbial contamination of stored hydrocarbon fuels and its control. Revista de Microbiologia, v. 30, n. 1, p. 1-10, 1999. DOI: 10.1590/S0001-37141999000100001.

[10] YEMASHOVA, N. A.; MURYGINA, V. P.; ZHUKOV, D. V.; ZAKHARYANTZ, A. A.; GLADCHENKO, M. A.; APPANNA, V.; KALYUZHNYI, S. V. Biodeterioration of crude oil and oil derived products: a review. Reviews in Environmental Science and Bio/Technology, v. 6, n. 4, p. 315-337, Jan 2007. DOI: 10.1007/s11157-006-9118-8.

[11] PASSMAN, F. J. Microbial contamination control in fuels and fuel systems since 1980 – areview. International Biodeterioration & Biodegradation, v. 81, p. 88-104, July 2013. DOI: 10.1016/j.ibiod.2012.08.002.

[12] SCHLEICHER, T.; WERKMEISTER, R.; RUSS, W.; MEYER-PITTROFF, R. Microbiologicalstability of biodiesel-diesel-mixtures. Bioresource Technology, v. 100, n. 2, p. 724-730, Jan. 2009. DOI: 10.1016/j.biortech.2008.07.029.

[13] BÜCKER, F.; SANTESTEVAN, N. A.; ROESCH, L. F.; JACQUES, R. J. S.; PERALBA, M. do C. R.; CAMARGO, F. A. de O.; BENTO, F. M. Impact of biodiesel on biodeterioration of stored Brazilian diesel oil. International Biodeterioration & Biodegradation, v. 65, p. 172-178, 2011. DOI: 10.1016/j.ibiod.2010.09.008.

[14] BÜCKER, F.; BARBOSA, C. S.; QUADROS, P. D.; BUENO, M. K.; FIORI, P.; TE HUANG, C.; FRAZZON, A. P. G.; FERRãO, M. F.; CAMARGO, F. A. de O.; BENTO, F. M. Fuel biodegradation and molecular characterization of microbial biofilms in stored diesel/biodiesel blend b10 and the effect of biocide. International Biodeterioration & Biodegradation, v. 95, part B, p. 346-355, Nov. 2014. DOI: 10.1016/j.ibiod.2014.05.030.

[15] SØRENSEN, G.; PEDERSEN, D. V.; NØRGAARD, A. K.; SØRENSEN, K. B.; NYGAARD, S. D. Microbial growth studies in biodiesel blends. Bioresource Technology, v. 102, n. 8, p. 5259- -5264, Apr. 2011. DOI: 10.1016/j.biortech.2011.02.017.

[16] DODOS, G.; ZANNIKOS, F. Microbiological growth study of biodiesel fuel. SAE International Journal of Fuels and Lubricants, v. 6, n. 2, p. 419-429, 2013. DOI: 10.4271/2013-01-1148.

[17] SORIANO, A. U.; MARTINS, L. F.; VENTURA, E. S. de A.; LANDA, F. H. T. G. de; VALONI, É. De A.; FARIA, F. R. D.; FERREIRA, R. F.; FALLER, M. C. K.; VALÉRIO, R. R.; LEITE, D. C. de A.; CARMO, F. L. do; PEIXOTO, R. S. Microbiological aspects of biodiesel and biodiesel/diesel blends biodeterioration. International Biodeterioration & Biodegradation, v. 99, p. 102-114, Apr. 2015. DOI: 10.1016/j.ibiod.2014.11.014.

[18] ASSOCIAÇÃO BRASILEIRA DE NORMAS TÉCNICAS. NBR 16732: combustíveis líquidos e sistemas associados: requisitos para prevenção, monitoramento e controle de contaminação microbiana. Rio de Janeiro, 2019.

[19] BENTO, F. M. Até que ponto a contaminação microbiana pode comprometer a qualidade da mistura estocada diesel-biodiesel? In: WORKSHOP CONTROLE PLENO DA QUALIDADE DO BIODIESEL E MISTURA COM DIESEL, 2012, Brasília, DF. Anais... Brasília, DF: Embrapa Agroenergia, 2014. p. 9-18. (Embrapa Agroenergia. Documentos, 17).

[20] BASTIAENS, L.; SPRINGAEL, D.; WATTIAU, P.; HARMS, H.; RUPERT deWACHTER, R.; VERACHTERT, H.; DIELS, L. Isolation of adherent polycyclic aromatic hydrocarbon (PAH)-degrading bacteria using PAH-sorbing carriers. Applied and Environmental Microbiology, v. 66, p. 1834-1843, 2000. DOI: 10.1128/AEM.66.5.1834-1843.2000.

[21] BOONCHAN, S.; BRITZ, M. L.; STANLEY, G. A. Degradation and mineralization of high- -molecular-weight polycyclic aromatic hydrocarbons by defined fungal-bacterial cocultures. Applied and Environmental Microbiology, v. 66, p. 1007-1019, 2000. DOI: 10.1128/ AEM.66.3.1007-1019.2000.

[22] DAS, N.; CHANDRAN, P. Microbial degradation of petroleum hydrocarbon contaminants: an overview. Biotechnology Research International, v. 11, article ID 941810, 2011. 13 p. DOI: 10.4061/2011/941810.

[23] BRZESZCZ, J.; KASZYCKI, P. Aerobic bacteria degrading both n-alkanes and aromatic hydrocarbons: an undervalued strategy for metabolic diversity and flexibility. Biodegradation, v. 29, n. 4, p. 359-407, 2018. DOI: 10.1007/s10532-018-9837-x.

[24] PRINCE, R. C.; AMANDE, T. J.; MCGENITY, T. J. Prokaryotic hydrocarbon degraders. In: McGENITY, T. (Ed.). Taxonomy, genomics and ecophysiology of hydrocarbon-degrading microbes. Springer, Cham, 2018. p. 1-41. (Handbook of hydrocarbon and lipid microbiology). DOI: 10.1007/978-3-319-60053-6_15-1.

[25] BENTO, F. M.; GAYLARDE, C. C. Microbial contamination of stored diesel oil. Brazilian Journal of Microbiology, v. 27, p. 71-75, 1996.

[26] CHAILLAN, F.; LE FLÈCHE, A.; BURY, E.; PHANTAVONG, Y.-H.; GRIMONT, P.; SALIOT, A.; OUDOT, J. Identification and biodegradation potential of tropical aerobic hydrocarbon-degrading microorganisms. Research in Microbiology, v. 155, n. 7, p. 587-595, Sept. 2004. DOI: 10.1016/j.resmic.2004.04.006.

[27] BENTO, F. M.; BEECH, I. B.; GAYLARDE, C. C.; ENGLERT, G. E.; MULLER, I. L. Degradation and corrosive activities of fungi in a diesel-mild steel-aqueous system. World Journal of Microbiology and Biotechnology, v. 21, p. 135-142, 2005. DOI: 10.1007/s11274-004-3042-2.

[28] BOELTER, G.; CAZAROLLI, J. C.; BEKER, S. A.; QUADROS, P. D. de; CORREA, C.; FERRÃO, M. F.; GALEAZZI, C. F.; PIZZOLATO, T. M.; BENTO, F. M. Pseudallescheria boydii and Meyerozyma guilliermondii: behavior of deteriogenic fungi during simulated storage of diesel, biodiesel, and B10 blend in Brazil. Environmental Science and Pollution Research, v. 25, n. 30, p. 30410-30424, 2018. DOI: 10.1007/s11356-018-3015-x.

[29] MARTIN-SANCHEZ, P. M.; BECKER, R.; GORBUSHINA, A. A.; TOEPEL, J. An improved test for the evaluation of hydrocarbon degradation capacities of diesel-contaminating microorganisms. International Biodeterioration & Biodegradation, v. 129, p. 89-94, Apr. 2018. DOI: 10.1016/j.ibiod.2018.01.009.

[30] BECARELLI, S.; CHICCA, I.; SIRACUSA, G.; LA CHINA, S.; GENTINI, A.; LORENZI, R.; MUNZ, G.; PETRONI, G.; LEVIN, D. B.; DI GREGORIO, S. Hydrocarbonoclastic Ascomycetes to enhance co-composting of total petroleum hydrocarbon (TPH) contaminated dredged sediments and lignocellulosic matrices. New Biotechnology, v. 50, p. 27-36, May 2019. DOI: 10.1016/j.nbt.2019.01.006.

[31] ALLSOPP, D.; SEAL, K.; GAYLARDE, C. C. Introduction to biodeterioration. 2nd ed. Cambridge: University Press, 2004. DOI: 10.1017/CBO9780511617065.

[32] GUIDELINES for the investigation of the microbial content of petroleum fuels and for the implementation of avoidance and remedial strategy. 2nd ed. London: The Energy Institute, 2007.

[33] WHITE, J.; GILBERT, J.; HILL, G.; HILL, E.; HUSE, S. M.; WEIGHTMAN, A. J.; MAHENTHIRALINGAM, E. Culture-independent analysis of bacterial fuel contamination provides insight into the level of concordance with the standard industry practice of aerobic cultivation. Applied and Environmental Microbiology, v. 77, p. 4527-4538, 2011. DOI: 10.1128/AEM.02317-10.

[34] AZAMBUJA, A. O.; BÜCKER, F.; QUADROS, P. D.; ZHALNINA, K.; DIAS, R.; VACARO, B. B.; CORREA, C.; FERRÃO, M. F.; CAMARGO, F. A. de O.; TRIPLETT, E.; BENTO, F. M. Microbial community composition in Brazilian stored diesel fuel of varying sulfur content, using high- -throughput sequencing. Fuel, v. 189, p. 340-349, Feb. 2017. DOI: 10.1016/j.fuel.2016.10.108.

[35] FONSECA, M. M. B.; MINNICELLI, C. F.; SILVA-PORTELA, R. C. B.; FARIAS, M. F.; SANTOS, P. R. S.; FERNANDES, G. J. T.; AGNEZ-LIMA, L. F. Unlocking and functional profiling of the bacterial communities in diesel tanks upon additive treatment. Fuel, v. 236, p. 1311-1320, 2019. DOI: 10.1016/j.fuel.2018.09.107.

[36] HU, D.; LIN, W.; ZENG, J.; WU, P.; ZHANG, M.; GUO, L.; YE, C.; WAN, K.; YU, X. Profiling the microbial contamination in aviation fuel from an airport. Biofouling: the journal of bioadhesion and biofilm research, v. 35, n. 8, p. 856-869, Oct 2019a. DOI: 10.1080/08927014.2019.1671977.

[37] RESTREPO-FLÓREZ, J.-M.; BASSI, A.; REHMANN, L.; THOMPSON, M. R. Effect of biodiesel addition on microbial community structure in a simulated fuel storage system. Bioresource Technology, v. 147, p. 456-463, Nov 2013. DOI: 10.1016/j.biortech.2013.08.068.

[38] CAZAROLLI, J. C.; BOELTER, G.; LIMA, A. M. d. l. de; HENGLES, T.; CORREA, C.; PERALBA, M. C.; FERRÃO, M. F.; CAVALCANTI, E. H. S.; BISOL, M. V.; BEZERRA, K. S.; ANTONIOSI FILHO, N. R.; BENTO, M. V. Nature of insoluble material found in the bottom of soybean biodiesel storage tank: chemical and microbiological approach. Journal of the Brazilian Chemical Society, v. 29, v. 10, p. 2034-2045, 2018. DOI: 10.21577/0103-5053.20180078.

[39] HU, D.; ZENG, J.; WU, S.; LI, X.; YE, C.; LIN, W.; YU, X. A survey of microbial contamination in aviation fuel from aircraft fuel tanks. Folia Microbiologica, v. 65, p. 371-380, 2019b. DOI: 10.1007/s12223-019-00744-w.

[40] Pontecorvo, G.; Roper, J.A.; Hemons, L.M.; McDonald, K.D.; Bufton,A.W.J. 1953. The gene- tics of Aspergillus nidulans. Adv. Genet, 5:141-238.

[41] Raeder, U. and Broda, P. (1985), Rapid preparation of DNA from filamentous fungi. Letters in Applied *Microbiology*, 1**,** 17-20.

[42] Lane D.J. - 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M, editors. Nucleic acid techniques in bacterial systematics. New York, NY: John Wiley & Sons, Inc. pp. 115-175, 1991.

[43] White TJ, Bruns T, Lee S, Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR Protocols: a guide to methods and applications. (Innis MA, Gelfand DH, Sninsky JJ, White TJ, eds). Academic Press, New York, USA: 315–322.

[44] Gardes M, Bruns TD: ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. Mol Ecol. 1993, 2: 113-118.

[45] Nilsson RH, Larsson KH, Taylor AFS, et al. The UNITE database for molecular identification of fungi: handling dark taxa and parallel taxonomic classifications. *Nucleic Acids Res*. 2019;47(D1):D259-D264. doi:10.1093/nar/gky1022.

[46] Robert V, Vu D, Amor AB, et al. MycoBank gearing up for new horizons. *IMA Fungus*. 2013;4(2):371-379. doi:10.5598/imafungus.2013.04.02.16

[47] Christensen, Earl & McCormick, Robert. (2014). Long-term storage stability of biodiesel and biodiesel blends. Fuel Processing Technology. 128. 339-348. 10.1016/j.fuproc.2014.07.045.
[48] Rashed, M.M. & Kalam, M. A. & Masjuki, H.H. & Rashedul, H.K. & Alam, Ashraful & Shancita, I. & Ruhul, A.. (2015). Stability of biodiesel, its improvement and the effect of antioxidant treated blends on engine performance and emission. RSC Adv.. 5. 36240-36261. 10.1039/C4RA14977G.

[49] Bücker, Francielle & De Moura, Tiane & da Cunha, Michele & Quadros, Patrícia & Beker, Sabrina & Cazarolli, Juciana & Caramão, Elina & Frazzon, Ana & Bento, Fatima. (2018). Evaluation of the deteriogenic microbial community using qPCR, n-alkanes and FAMEs biodegradation in diesel, biodiesel and blends (B5, B10, and B50) during storage. Fuel. 233. 10.1016/j.fuel.2017.11.076.

[50] Reddy, Yanala & Reddy, Chittepu & Subhadra, Maringanti & Rajagopal, Kurapati. (2020). Long – term storage effect on molecular interactions of biodiesels and blends. Energy Sources Part A Recovery Utilization and Environmental Effects. 1-15. 10.1080/15567036.2020.1776798.

[51] Hill, Edward & Hill, Graham. (2008). Microbial Contamination and Associated Corrosion in Fuels, during Storage, Distribution and Use. Advanced Materials Research. 38. 257-268. 10.4028/www.scientific.net/AMR.38.257.

[52] Amann RI, Ludwig W, Schleifer KH. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol Rev*. 1995;59(1):143-169.

[53] Martin-Sanchez, Pedro & Gorbushina, Anna & Toepel, Jörg. (2016). Quantification of microbial load in diesel storage tanks using culture- and qPCR-based approaches. International Biodeterioration & Biodegradation. 126. 10.1016/j.ibiod.2016.04.009.

[54] LIRA D.D., Caracterização de Aspergillus sp. Quanto a capacidade de degradação de óleo diesel. Fev2014- https://repositorio.ufpe.br/handle/123456789/11900

[55] Gunasekaran, Saranya & Ramachandra, T V. (2020). Novel Biocatalyst for Optimal Biodiesel Production from Diatoms. Renewable Energy. 10.1016/j.renene.2020.02.053.

[56] SILVA, Tamyris Borges. Contaminação microbiológica de diesel comercial no Distrito Federal. 2015. xiv, 68f. Universidade de Brasília, Brasília, 2015. http://dx.doi.org/10.26512/2015.07.D.20062