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# São Paulo Zoo composting as a source of bacteria with bioremediation potential

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**As the world population increases, the need for energy resources increases and for decades, petroleum has sustained this demand. Oil spill during petroleum extraction and processing has a negative impact on the environment. Methods that can decrease the impact of xenobiotic compounds on the environment have been developed. Among many bioremediation methods described, microbial remediation is of great importance. Our previous work demonstrated that Organic Waste Composting Unit (OWCU) located at the São Paulo Zoo Foundation (FPZSP) had a great diversity of microorganism. In the present work, we viewed this environment searching for xenobiotic degrading microorganisms by sampling the composting at various stages of the process with the aim of isolating the bacteria that would break down n-hexadecane, a model compound for hydrocarbon degradation. Two bacterial collections were assembled and tested in a 96-well plate model using n-hexadecane as a sole carbon source. Among the 418 isolates screened, eight were selected based on their ability to assimilate n-hexadecane. Molecular identification revealed their genus and species which are associated with xenobiotic degradation activities in different microbial consortia. However, these microorganisms have not being isolated from the same transforming process. Future studies with these isolates may shed light on the bacteria hydrocarbon degradation mechanism.**

**Key words:** Composting, crude oil degrading microorganism, bioremediation, n-hexadecane degrading bacteria.

## INTRODUCTION

The increase in population and consumption has led to large amounts of organic waste accumulation, which has been a serious problem to manage in both urban and rural areas. Composting is an alternative technique for disposing organic waste and avoiding its accumulation. The end product generated is a natural fertilizer, which can be used in agriculture closing a self-sustainable cycle. The São Paulo Zoo (São Paulo, Brazil) is a good example where its Organic Composting Production Unit (OWCU) transforms about 2000-2500 kg/day of organic material into fertilizer within a ninety-day period.

Composting is an ancient biological process carried out by a microorganism consortium. The microbial metabolism changes the substrate composition over time, which in turn reflects on microbial population structure. In this process, the interaction between biotic and non-biotic factors leads to constant transformation of the complex microbial community over time, which is the mechanism underlining organic matter transformation (Schulze, 1962; Waker et al., 1999; Dees and Ghiose, 2001; Hua et al., 2010). Therefore, composting is a rich source for microbial diversity studies suitable for investigation with the aim of getting a generation of biotechnological products such as microorganisms that are capable of degrading xenobiotic contaminants in soil.

In the past years, the world has witnessed many oil spill

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disasters that contaminated water and soil. Among several means for soil and water decontamination, bioremediation has gained a lot of attention because living organisms convert contaminant into chemical compounds more friendly to the environment (Rojo, 2009; Zhang et al., 2010; Nagata et al., 2010).

Crude oil is a complex mixture of hydrocarbons and other organic compounds (Zhang et al., 2010). Saturated hydrocarbon alkanes are the major fraction constitutes of crude oil and depending on their oil source, they can reach up to 50% of its constitution. These molecules are chemically inert and can be used by microorganisms as carbon source. Therefore, researchers have used alkanes as single carbon source to isolate microorganisms with bioremediation potential (Rojo, 2009).

Our previous work (Bitencourt et al., 2010; Pascon et al., 2011; Farage-Martins et al., 2013) has shown the potential of the OWCU compost as a source of interest and useful microorganisms. Therefore, the aim of the present work was to explore the microbial diversity present in the zoo composting mixture in order to select and characterize those that can use the model compound n-hexadecane (n-alkane) as sole carbon source. We expected that these microbes would have a potential for bioremediation and will be used in further degradation studies.

## MATERIALS AND METHODS

### Microorganism collection

Two microorganism collections were screened in this work. CL001 collection was generated previously as described by Bitencourt et al. (2010) and Pascon et al. (2011), whereas CL002 collection was generated for this work and comprised samples taken at a defined time points from a single compost cell. Each composting cell unit has eight cubic meters and it is assembled with organic waste collected from the São Paulo Zoo (Bitencourt et al., 2010), which is converted into fertilizer after approximately 90 days. During this process, the temperature can rise up to seventy degrees Celsius and water sprinkling is used to bring the temperature down. When the temperature drops below fifty-five degrees Celsius, the cell is revolved to recover its aerobic conditions and humidity in the range of 40-60%.

The whole process is finalized when the cell temperature becomes stabilized at about fifty degree Celsius for several days. The time points chosen aimed to cover the three stages of composting described are as follows: 1) soon after the composting cell unit was assembled at time zero ($T_0$), 2) 82 days after $T_0$ during the compost mixing and 3) 99 days after $T_0$ when the process was finished and the temperature was below fifty degrees Celsius. The sampling at each time point was performed as described by Bitencourt et al. (2010).

### Microorganism isolation

Two isolation protocols were employed in this work. The first one was described by Bitencourt et al. (2010) and was isolated in rich medium without selecting xenobiotic degradation bacteria. This isolation method was used to assemble collection CL001. The second microbial collection (CL002) comprised bacterial isolates that were obtained by direct isolation as in CL001 and microorganisms capable of growing in n-hexadecane enriched medium as described in the study of Mrozik and Piotrowska-Seget (2010) and Vasconcellos et al. (2010). In brief, five grams of compost was added to 10 mL of saline (0.9% NaCl); it was mixed and after 1 h at room temperature, an aliquot of 5 mL was used to inoculate 500 mL of minimal medium M9 (41) containing 1% (w/v) of glucose and 0.25% (v/v) of n-hexadecane (Sigma, cat.# H6703). The culture was incubated at 30°C for 72 h with 150 rpm. The cells were collected by centrifugation at 3220 g for 2 min (Eppendorf 5810R with *swing* rotor A-4-62) and added to a fresh 500 mL of minimal medium M9 supplemented with 0.5% (v/v) of n-hexadecane. The culture was incubated for 48 h at 30°C with 150 rpm.

Again, the cells were recovered as described above and brought to suspension and inoculated in 500 mL of minimal medium M9 supplemented with 1% (v/v) of n-hexadecane. The culture was incubated for 48 h at 30°C with 150 rpm. The growth of the cultures was checked daily in a spectrophotometer (Bel photononic UV2000) at 630 nanometers. Isolation of microorganisms was performed as described in the study of Bitencourt et al. (2010) in nutrient agar for each step of the enrichment procedure at three time points: at time point zero ($T_0$) when the culture was set up, at time point one ($T_1$) after 24 h of growth and at time point two ($T_2$) after 48 h of growth. A $T_3$ time point was taken only for the first step of enrichment since this culture has grown for 72 h. All the experiments were made in triplicate.

### n-Hexadecane as sole carbon source

From a freshly grown bacterium culture, a small inoculum was removed and added to 5 mL of LB broth. This culture was grown for 12-16 h at 30°C with 150 rpm. Then a 1:10 culture dilution was made and its optical density was determined in a spectrophotometer at 630 nm (Bel photononic UV2000). The optical density was adjusted to 0.26. An aliquot of 15 μL was added to wells containing 135 μL of minimum medium M9 with 1% of n-hexadecane in triplicate. The culture was incubated for 48 h at 30°C with 150 rpm. Then the cell density was analyzed in a spectrophotometer (Bel photononic UV2000) at 630 nanometers of optical density. The cell activity was determined by adding 20 μL of a 1 mg/mL solution of Thiazolyl Blue Tetrazolium Bromide MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide, Merck® cat. # 1117140001] and incubating at room temperature with 150 rpm for one hour. The wells that yielded purple color were scored as positive for cell growth, whereas no cell activity was indicated by a yellow color (French et al., 1998).

### Bacteria genomic DNA isolation

Bacterial genomic DNA isolation was performed using the QIAamp DNA Mini Kit (Qiagen cat. #51304). The DNA integrity was determined in a 0.8% agarose gel in a 1X TAE (Tris, Acetate and EDTA) buffer as described by Sambrook et al. (1989).

### Amplification of the ribosomal small subunit (16S) by PCR

The ribosomal small subunit (16S) was amplified by PCR using the pair of primers forward (5' GTGCCAGCMGCCGCGG 3') and was reversed (5' ACGGGCGGTGTGTRC 3'); the reaction conditions were described by Lane (1991) and Borneman and Hartin (2000). The PCR product yielded was checked in 0.8% agarose gel in a 1X TAE (Tris, Acetate and EDTA) buffer as described by Sambrook et al. (1989). Prior to BigDye sequencing (BigDye® Terminator v3.1 Cycle Sequencing Kit, Applied Biosystems), the PCR product was purified by QIAquick PCR Purification Kit (Qiagen, cat. #28106).

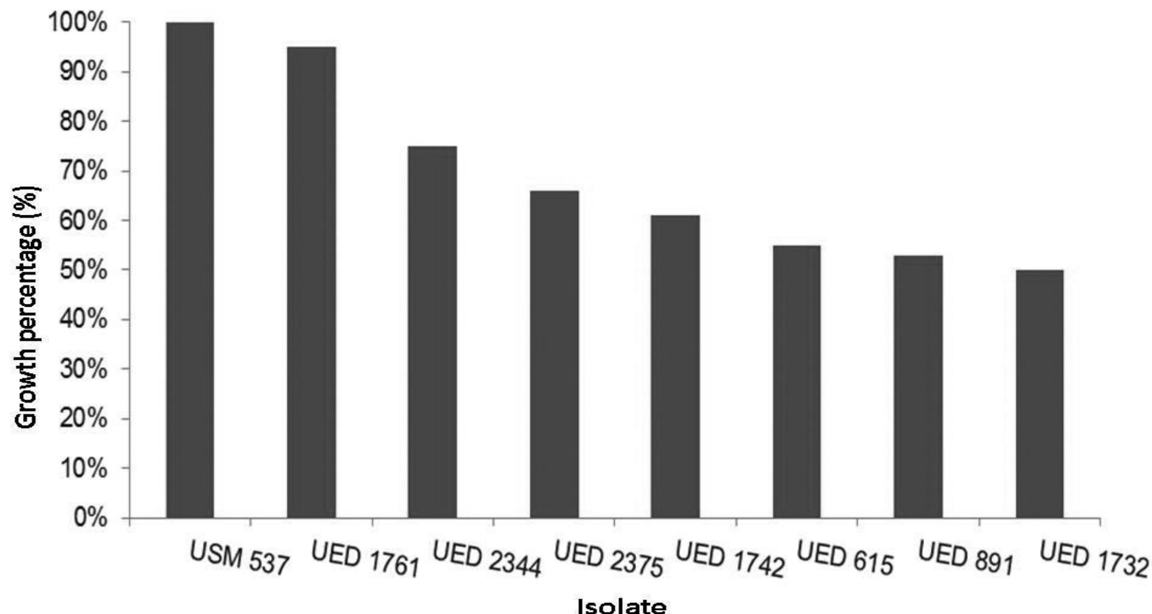


**Figure 1.** Growth rate of eight isolates in minimal medium M9 supplemented with n-hexadecane on a 96 well plate assay. Isolate USM537 had the best growth rate under this condition when when compared with others.

The sequences yielded by the BigDye sequencing were blasted against the following databases: NCBI (National Center for Biotechnology Information) and the Ribosomal Database Project - Release 10 (http://rdp.cme.msu.edu/).

## RESULTS AND DISCUSSION

### Micro-organisms collections and the 96 well plate assay for n-hexadecane degradation

Two collections (CL001 and CL002) of isolated microorganisms from composting were subjected to n-hexadecane assimilation as carbon source. CL001 yielded 259 bacteria as described by Pascon et al. (2011). On the other hand, CL002 collection was obtained by sampling a single compost cell unit at three different time points that covered three important stages during the composting process as follows: at time point zero ($T_0$) where the temperature was 60.4°C with pH 6.0; at time point one ($T_1$) it was sampled 64 days after the initiation of the process ($T_0$) and soon after a compost cell unit mechanical aeration where temperature was 56.6°C with pH 7.0. The time point two ($T_2$) was taken 99 days after $T_0$ where the temperature was about 49.6°C with pH 6.5. Even though the composting material presented high temperature (60.4 to 49.6°C) at different collection points, the bacteria isolation for CL002 was performed at 30°C as previously described in our laboratory (Pascon et al., 2011). And also, this temperature was employed because our aim was to isolate microorganisms capable of degrading substances that contaminate the environment, therefore thermopiles were not the subject for this purpose. The CL002 collection comprised 159 bacteria, which were obtained by direct isolation as described for CL001 (Pascon et al., 2011) and by the enrichment methodology described previously by Mrozik and Piotrowska-Seget (2010) and Vasconcellos et al. (2010). Therefore, a total of 418 isolates were tested for their ability to grow on n-hexadecane, an n-alkane, as a sole carbon source. Among all the bacteria tested, 79 (18.9%) were able to degrade n-hexadecane to obtain energy. The cell growth was confirmed by the addition of MTT [3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide]. The USM537 isolate had the best growth pattern under these conditions and therefore was considered as the reference strain for comparing the others. Figure 1 shows seven isolates that were able to grow at least 50% of the USM537 on n-hexadecane containing minimal medium M9 on a 96 well plate assay. Among these eight isolates (including USM537), two are from CL001 collection and six are from CL002 (Table 1).

### Molecular identification

In order to uncover the genus and/or species of these bacteria, molecular identification was performed. A PCR fragment comprising the ribosomal small subunit (16S) DNA was amplified and sequenced. These sequences were used to conduct similarity searches at the GeneBank (National Center for Biotechnology Information) and Ribosomal Database Project - Release 10 (http://rdp.cme.msu.edu/) using BLAST. The results are presented in Table 1.

As seen in Table 1, we identified eight isolates as good

**Table 1.** Molecular identification of eight isolates that are able to grow on minimum medium M9 supplemented with n-hexadecane as sole carbon source.

| Isolate | 16S | Collection | Max. ID (%) |
|---|---|---|---|
| USM 537 | *Klebsiella oxytoca* | CL001 | 99 |
| UED 615 | *Enterobacter cloacae* | CL001 | 99 |
| UED 891 | *Klebsiella sp.* | CL002 | 100 |
| UED 1732 | *Sphingobacteria sp.* | CL002 | 96 |
| UED 1742 | *Isoptericola sp.* | CL002 | 99 |
| UED 1761 | *Isoptericola variabilis* | CL002 | 99 |
| UED 2344 | *Microbacterium sp.* | CL002 | 99 |
| UED 2375 | *Bacillus licheniformis* | CL002 | 99 |

candidates for bioremediation of crude oil and its derivatives from contaminated locations employing n-hexadecane (n-alkane) as an indicator. According to many publications, these genera and species have been reported to be involved in many bioremediation processes.

The genus, *Klebsiella* has ubiquitous distribution in terms of its habitat like sewage, drinking water, soils, surface waters, industrial effluents and vegetation. Also, this bacterium has been described as resident or transient in the gastrointestinal tract. Thus, *Klebsiella* is also known as common opportunistic pathogen for humans and other animals. Six genera of *Klebsiella* are associated with several environments as follows: *Klebsiella pneumonia* found in humans, animals, sewage and polluted waters and soils; *Klebsiella oxytoca* is often associated with most habitats; *Klebsiella terrigena* found in unpolluted soils and surface waters, drinking water and vegetation; *Klebsiella planticola* is associated with sewage, polluted surface waters, soils and vegetation; and *Klebsiella ozaenae*/ *Klebsiella rhinoscleromatis* is rarely detected; however is primarily associated with humans (Bagley, 1985).

Moreover, the genus, *Klebesiella* is known to be involved in bioremediation of several pollutants. Li and Gu (2007) have isolated *Kleibesiella oxytoca* from mangrove and they showed that this strain in consortium with *Methylobacterium mesophilicum* can rapidly metabolize dimethyl isophthalate (DMI) as sole carbon source. DMI is used in the sodium dimethyl isophthalate-5-sulfonate (SIPM) production, which is used to enhance the chromaticity of polyethylene terephthalate (PET). Khalid et al. (2009) have isolated *K. oxytoca* and two other microorganisms (*Acinetobacter* sp. and *Citrobacter freundii*) that were able to grow on 4-nitroaniline from textile dye wastewater. This microorganism consortium was able to remove 100% of 4-nitroaniline in 72 h. Kim et al. (2009) have isolated *K. oxytoca* from phenanthrene (PHE) which is a polycyclic aromatic hydrocarbon composed of three fused benzene rings. Together with *K. oxytoca*, they also isolated *Acinetobacter baumannii* and *Stenotrophomonas maltophilia*. Used alone, *K. oxytona* was able to remove only 11% of the phenantrhere contaminant, whereas in a consortium, the three microorganisms degraded 80% PHE in 360 h. It is interesting to note that *K. oxytoca* is reported by Khalid et al. (2009) and Kim et al. (2009) as having the ability to degrade compounds like PHE and 4-nitroaniline which have cyclic closed carbon rings. In the present report, this microorganism was isolated in an enrichment process with a linear hydrocarbon molecule. However, other researchers have reported the isolation and ability of this microorganism to grow and degrade crude oils, which are rich in alkanes (Zhang et al., 2010). Rajasekar et al. (2010) reported the isolation of *K. oxytoca* in diesel and naphtha pipelines in the Northwest and Southwest regions in India. They have also isolated other 10 bacteria (*Serratia marcescens* ACE2, *Bacillus subtilis* AR12, *Bacillus cereus* ACE4, *Pseudomonas aeruginosa* AI1, *Pseudomonas stutzeri* AP2, *Bacillus litoralis* AN1, *Bacillus* sp., *Bacillus pumilus* AR2, *Bacillus carboniphilus* AR3 and *Bacillus megaterium* AR4). Chamkha et al. (2011) have isolated *K. oxytoca* from Tunisian offshore oil field. Their isolate was capable of degrading a wide range of aliphatic hydrocarbons from C13 to C30. In 45 days, *K. oxytoca* was able to degrade 75% of n-alkanes; however with the addition of a surfactant (Tween 80), the degradation rate reached 98%. Also, they showed that this isolate was able to completely metabolize aromatic compounds within 24 h. Interestingly, our isolate failed to grow on phenol containing medium as sole carbon source (data not shown).

The species, *Enterobacter cloacae* has the ability to degrade various xenobiotic compounds. It has been reported that it can also accumulate heavy metal (Fang et al., 2010; Kanaly et al., 2000; Turgay et al., 2012; Rajasekar et al., 2010), grow on explosives such as pentaerythritol tetranitrate (PETN) and 2,4,6-trinitrotoluene (TNT) as sole nitrogen source (Binks et al., 1996; French et al., 1998) and it can decontaminate water from herbicides (Chena et al., 2009; Sakultantimetha et al., 2001; Ngigi et al., 2012). Also, this microorganism has been isolated from materials contaminated with crude oil. Saadoun (2002) isolated six

different species of bacteria growing on soil contaminated with crude oil and among them he reported that *E. cloacae* was able to degrade diesel as well. Zhang et al. (2009) reported that a consortium between *E. cloacae* and *Cunninghamella echinulata* elevated the biodegradation of total petroleum hydrocarbon (TPH) from 29.2 to 48.0% after about 20 days. They added wheat straw to the contaminated soil and after 45 days, the biodegradation ratio of TPH reached 75%. Wu et al. (2011) reported that applying wheat straw and a different consortium (*E. cloace*, *Pseudomonas* sp. and *Rhodothermus* sp.) showed an increase in the overall degradation ratio from 44 to 56% after 56 days of treatment.

*Sphingobacterium* is known by its ability to degrade pollutants such as pesticides, textile dyes and petroleum and its derivatives (Kanaly et al., 2000; Carvalho et al., 2002; Macbeth et al., 2004; Fang et al., 2010; Nagata et al., 2010; Tamboli et al., 2010; Janbandhu and Fuleka, 2011). Polycyclic aromatic hydrocarbons (PHA) can be found in oil, coal and tar deposits, and they are also produced as byproducts of fuel burning. These compounds are found in contaminated soils and sediments and as pollutants; the concerns fall on the fact that they can be carcinogenic, mutagenic and teratogenic (Wong et al., 2004).

Bacteria from the genus, *Sphingobaterium* can degrade PHA. Janbandhu and Fulekar (2011), using an enrichment protocol, have identified *Sphingobacterium* sp., *Bacillus cereus* and a novel bacterium, *Achromobacter insolitus* (MHF ENV) within a consortium which was able to degrade phenanthrene from a three decade old petrochemical refinery. In their experimental conditions, the consortium was able to mineralize 100% of phenanthrene at 100 mg/L in 14 days.

We have also identified *Isoptericola variabilis* (formely know as *Cellulosimicrobium variabile* (Stackebrandt et al., 2004) and the isolate UED1742 (*Isoptericola* sp.) as microorganisms that can use n-hexadecane as a sole carbon source. Radwan et al. (2010) studied crude oil-coated gravel particles covered with blue-green biofilms in the Arabian Gulf Coast in autumn, winter and spring. They have isolated and characterized many bacteria that were able to grow on crude oil supplemented or not with a nitrogen source. Among the many microorganisms isolated they found *Isoptericola* sp. that was able to degrade hydrocarbon as sole carbon source in the presence and absence of nitrogen with the following hydrocarbon attenuation percentages: 22.4±0.1 and 27.1±2.0%, respectively.

*Microbacterium* species have been isolated as part of a microbial consortium from soil contaminated with xenobiotics such as dyes, heavy metals, pesticides and crude oil and its derivatives (Lal et al., 2010; Waranusantigul et al., 2011; Chanthamalee and Luepromchai, 2012; Juárez-Ramirez et al., 2012; Turgay et al., 2012; Shin et al., 2012; Simarro et al., 2013). Lal et al. (2010) have isolated a micro-bacterium from a microorganism consortium growing on hexachlorocyclohexane-contaminated soil.

Waranusantigul et al. (2011) and Turgay et al. (2012) have isolated from plant rhizosphere, *Microbacterium* species that were able to promote heavy metal accumulation and plant growth. They concluded that the rhizosphere bacteria have the potential to improve the efficiency of phytoremediation of plumb and nickel-contaminated sites. Shin et al. (2012) have isolated thirty-seven carbofuran-degrading bacteria through enrichment processes from a variety of rice paddy soils and among them they found a species of *Microbacterium* (*M. oxydans*) that was able to degrade this pesticide. *Microbacterium* was isolated from a biofilm capable of degrading 4-aminonaphthalene-1-sulfonic acid used in the process of decolorization of azo dyes (Juárez-Ramirez et al., 2012). Also, *Microbacterium* species were isolated from different environments and were shown to be able to degrade polycyclic aromatic hydrocarbons (PAHs), crude oil, hydrocarbon used as boat lubricant and as single carbon source (Chanthamalee and Luepromchai, 2012; Hassanshahian et al., 2012; Simarro et al., 2013).

Among the microorganisms that are able to grow on n-hexadecane as sole carbon source, we have identified *Bacillus licheniformis*. This *Bacillus* species is known to participate on organic matter conversion (composting) as reported by Haruta et al. (2002). This microorganism can produce bioemulsifier that helps to enhance crude oil recovery (Suthar et al., 2008), biodegrade textile dyes (Lu et al., 2012) and crude oil and its derivatives (da Cunha et al., 2006; Itah et al., 2009). However, its ability to degrade crude oil and its derivatives (such as jet fuel) is shown when *Bacillus licheniformis* grows within a microbial consortium with other microorganisms.

The present work used enrichment protocol to increase the amount and/or diversity of microorganisms and to obtain results as those achieved and described by many authors. It was found that the genus diversity for n-hexadecane degrading microorganisms CL002 was higher as compared to CL001 (data not shown).

The degradation of all the organic matter present in the zoo composting cells is not done by a single microorganism but by the synergistic action of several of them. Therefore, our findings are in agreement with several publications that report that microorganism consortium is capable of transforming the environment where they are placed and/or found (Binks et al., 1996; Kanaly et al., 2000; Carvalho et al., 2002; Haruta et al., 2002; Li and Gu, 2007; Itah et al., 2009; Khalid et al., 2009; Kim et al., 2009; Fang et al., 2010; Hua et al., 2010; Radwan et al., 2010; Rajasekar et al., 2010; Jandandhu and Fulekar, 2011; Sakultantimetha et al., 2011; Waranusantigul et al., 2011; Chanthamalee and Luepromchai, 2012; Juárez-Ramirez et al., 2012; Turgay et al., 2012; Shin et al., 2012; Simarro et al., 2013).

The results indicate that the microbial consortium has a promising application in bioremediation of xenobiotic con-

taminated environments. Thus, the eight isolates that are object of this work should in a future study be tested altogether in a more challenging environment for degradation of crude oil and its derivatives.

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