Bacterial Identification and Assessment of Treatments to Avoid Microbial Growth in Diesel Fuel Storage Tanks

Armando Salmerón
Repsol Technology Centre. E-28935 Móstoles, Madrid, Spain
E-mail: asalmeronc@repsol.com

Yolanda Murillo
Repsol Technology Centre. E-28935 Móstoles, Madrid, Spain

Luis Fernando Bautista
Department of Chemical and Environmental Technology, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain

Natalia González
Department of Biology and Geology, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain

María Carmen Molina
Department of Biology and Geology, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain

Carolina Vargas
Department of Chemical and Environmental Technology, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain

Raquel Simarro
Department of Biology and Geology, Universidad Rey Juan Carlos. E-28933 Móstoles, Madrid, Spain

Summary

The use of petroleum fractions by some microorganisms as a food source has been known for years. This property has been used for the bioremediation of hydrocarbon polluted soils. Nevertheless, microbial growth in hydrocarbons has become a serious problem for the oil industry since it has been observed inside fuel storage tanks. This causes problems such as corrosion, filter plugging or blockage of pipes. Although this process is given in several oil fractions, it is more frequent in crude oil, diesel fuel and kerosene. In the particular case of diesel fuel, both progressive reduction of sulphur content and increasing amount of biodiesel have significantly improved the conditions for microbial growth. This is a major concern for oil companies worldwide due to the lack of an ultimate solution. In the present work, the research aimed to perform the biological characterization of the microbial contamination and, simultaneously, to assess the effectiveness of different treatment strategies to reduce and prevent microbial growth inside diesel fuel storage tanks is presented.

1 Introduction

The presence of microorganisms in storage tanks is becoming a serious concern for the oil industry in the last decades, since it causes problems such as corrosion, filter plugging or blockage of pipes [1]. Petroleum fractions can be used as carbon source by some microorganisms. Hydrocarbons with 10 to 18 carbon chains, like diesel fuel and kerosene, are more easily metabolized. General conditions of oil storage tanks such as pH or temperature are favourable for microbial growth. In addition, the presence of some additives in the product such as antiknock agents, corrosion inhibitors, lubricity modifiers, cetane number and cold flow diesel fuel improvers, detergents, dispersants, metal deactivators, antifoam or antioxidants for the elimination of free radicals, can be a nutrient source for microorganisms promoting microbial growth in the aque-

ous phase and sludge formation [2].

Diesel fuel storage tanks of petrol stations are mostly underground. In this way, microorganisms can access inside of the tanks through faulty seals or cracks or during the filling operations and ventilation holes or with the water used for washing [3].

The presence of water in diesel fuel storage tanks allows microbial develop within them. Only 1 % (v/v) content of water is enough for microbial growth [4]. The regulations in the European Union allow maximum water content in diesel fuel of 200 mg/kg (EN 590 European Standard). In the case of biodiesel (neat FAME), this limit is 500 mg/kg, according to EN 14214 European Standard. Possible reasons for water presence in tanks are water content allowed by regulations, condensation of the air humidity entering inside the tanks through the vents, faults during tank filling or draining operations and water formation as the final product of cellular respiration as a consequence of microorganisms metabolic activity [2]. In addition, the growth of microorganisms in the oil storage tanks is also affected by factors such as the presence of oxygen generating oxygenated compounds which are easily metabolized by microorganisms [2]. Moreover, both pH and temperature conditions of oil storage tanks are two other factors that favour microbial proliferation because the proper values are within the optimal range of growth for many microbial species [4].

The problem of microbial growth inside the storage tanks has increased in recent years as a result of the new regulations applied to automotive diesel fuels. Some of the specifications of the new EN 590 revisions allow the addition of products derived from vegetable oils such as biodiesel (FAME) [4] or reduce the sulphur content of diesel fuel, favouring the growth of microorganisms. Although the presence of sulphate reducing bacteria in oil storage tanks has been widely described [5] there are more bacteria genera able to growth in diesel fuel.

The best way to avoid microbial contamination inside hydrocarbon storage tanks and their proliferation is prevention. Maintaining internal cleaning, sealing systems involved, the use of suitable coatings and aqueous phase drainage are key factors. Thus, the incidence of this problem would be significantly reduced. However, the development in practice of preventive actions is difficult to maintain. It is not usual the emptying of the tank for an extensive cleaning (removal of sludge and biofilms).

Different physicochemical treatments, such as ultraviolet radiation, sonication or the use of hydrogen peroxide and biocides can be alternatives to avoid and remove microbial contamination [6]. The ultraviolet radiation causes photobiochemical reactions altering the structure of the DNA of microorganisms. In a previous study [7], carried out with *Escherichia coli*, a direct relation between radiation intensity and decrease in microbial population was observed. However, a concomitant increase in medium temperature was achieved due to the UV radiation favouring, in turn, the proliferation of microbial colonies which overshadow each other blocking the radiation and decreasing the effectiveness of disinfection [8]. Sonication is used as an alternative for microbial disinfection. The application of this technique generates air bubbles by acoustic cavitation in the medium which release energy when they collapse destroying the cellular structure of the microorganisms. Moreover highly reactive radicals are also formed at the same time contributing to cell death [9]. The use of low frequency (typically 20 KHz), combined with a high intensity can produce enough energy to destroy the microorganisms present in the medium.

In this work we use both sonication and the addition of hydrogen peroxide and biocides to compare their effect on the control of microbial growth in diesel fuel from different storage tanks of petrol stations in Spain. Hydrogen peroxide and biocides are chemical compounds with a high disinfecting power. They are lethal components for microbes due to the formation of free radicals and toxic compounds affecting microorganisms vital functions and also causing damage to the DNA structure. In treatments with biocides, factors such as toxicity, solubility in water and diesel and their compatibility with fuel and its additives are important in order to select them to avoid the microbial growth inside the tanks [10].

2 Experimental

2.1 Reagents

Reagents and solvents for physicochemical analyses (pH, conductivity, chloride and nutrients concentration, organic and inorganic carbon and metals) were purchased from Panreac (Barcelona, Spain). Luria-Bertani culture medium (LB), Phosphate Buffer Saline (PBS) and other reagent for biological characterization such as formamide and *bis*-acrilamide were purchased from Sigma-Aldrich (Steinheim, Germany).

2.2 Sample Preparation

Samples from diesel fuel storage tanks were obtained from different petrol stations in Spain. Samples showed both a sludge-water phase and a diesel (organic) phase. They were centrifuged to allow phase separation. Physicochemical and biological analyses were performed in the water phase since most of the microorganisms were located in this phase.

2.3 Experimental Treatments

Application time, volumetric power and number of applications were the factors selected to study the efficiency of ultrasonic treatment. A Misonix sonicator 3000 equipment was used with a 13 cm diameter titanium probe and a working frequency of 20 kHz. The probe

was submerged in a container with the contaminated samples [11].

In treatments with hydrogen peroxide and organic compounds used as biocide agents, the effect of concentration and time were studied. Ten different biocides with different functional groups (isothiazolone, oxazolidine, thiocyanate, morpholine and thiocarbamate) were used in order to study their effect on microbial growth in the diesel fuel samples.

2.4 Biological and Molecular Characterization

In order to estimate the effectiveness of the treatments, the number of microorganisms growing in water and diesel phases of the samples, before and after applying the treatments, was estimated by a miniaturized most probably number technique (MPN) in 96-well microtiter plates with eight replicates per dilution. Total heterotrophic microbial population was estimated in 180 µL of LB medium supplemented with glucose (15 $g \cdot L^{-1}$) and 20 µl of the sample. Previously, a battery of dilutions was prepared from a mixture of the sample and PBS. Finally, the number of microorganisms present in each sample was estimated by the MPN calculator program, taking into account the initial dilution concentration, the number of replicates per dilution and the number of dilutions. In addition, cultivable bacteria and fungi contamination level were determined using Desinfectest Mix dipslides (Microkit Laboratories. Madrid, Spain).

Total bacterial community present in the aqueous phase of the samples was characterized by a non-culture-dependent molecular technique, i.e. denaturing gradient gel electrophoresis (DGGE). DGGE was performed from total DNA extracted from 6 ml of the water phase using a Microbial DNA Isolation Kit (MoBio Laboratories. Solano Beach, CA, USA) and amplified using the primers set 16S 338F-GC and 16S 518R, according to ExTaq HS DNA polymerase protocol (Promega Corp. Madison, WI, USA). Primers 338-GC included a GC clamp at the 5´end (5-CGC CCG´CCGCGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G-30). DGGE conditions were as described by Molina et al. [12] and González et al. [13]. Some DGGE bands were cloned and molecularly identified according to Simarro et al. [14]. DGGEs were performed to analyze total bacterial community of all samples and change in bacterial communities after treatment.

3 Results and Discussion

3.1 Physicochemical Properties

Conductivity, pH, presence of different anions and elements were determined and inorganic and organic carbon in the samples were calculated. Physicochemical characterization was previously determined in order to study the *in-situ* conditions for microbial growth inside storage tanks. In addition, most probable number (MPN) was determined to quantify the presence of microorganisms.

As shown in Table 1, conductivity and pH values were similar for most samples. The highest conductivity found for sample 23 was in agreement with the amount of anions, being especially notable Cl^- and NO_2^-. All these parameters depended on the different environmental conditions of the diesel fuel storage tanks. In this way the samples showed no comparable amounts of some metals such as silicon and calcium. The different values determined depended on soil conditions where diesel fuel storage tanks were located. Moreover, total carbon concentration in all samples was within the range of 8000-19000 mg/L. Organic carbon determined in aqueous samples is due to the solubility of diesel fuel in water. Additives and in some cases part of the biodiesel (FAME) present in the tanks enhance diesel solubility [15]. This carbon is the growth source for microorganisms in the aqueous phase forming sludge and generating more organic and inorganic carbon as a result of their metabolic activity.

Table 1. Physicochemical properties of five diesel fuel samples.

Sample	4	18	19	21	23
Conductivity (µS/cm)	9070	9800	4000	4000	10000
pH	5.59	5.59	5.41	5.61	8.61
Cl^- (mg/L)	189.67	107.11	52.53	117.12	593.33
SO_4^{2-} (mg/L)	0.68	31.35	8.03	4.46	257.45
NO_3^- (mg/L)	1.91	4.96	1.77	0.17	2.51
PO_4^{3-} (mg/L)	0.14	0.14	107.26	6.18	190.26
NO_2^- (mg/L)	96.90	76.55	29.95	54.40	731.74
C_{total} (mg/L)	9298	19383	13720	11677	8264
$C_{organic}$ (mg/L)	9247	19340	13677	11630	81680
$C_{inorganic}$ (mg/L)	50.82	40.81	42.21	44.94	961.37
Al (mg/L)	0.21	4.18	0.26	2.37	8.94
Ca (mg/L)	52.88	471.53	241.93	nd	43.92
Fe (mg/L)	95.60	197.14	3.34	109.14	nd
K (mg/L)	nd	92.55	15.96	41.09	21.09
Mg (mg/L)	17.73	48.15	21.39	189.97	5.25
Mn (mg/L)	32.66	7.13	0.81	11.80	17.84
Na (mg/L)	191.58	nd	51.95	320.26	128.63
S (mg/L)	99.36	98.23	36.44	234.43	67.80
Si (mg/L)	226.24	420.12	56.42	240.72	232.62
MPN (cell/mL)	7321	29523093	277316	1412639	1009

3.2 Sonication Treatment

Application time, volumetric power and number of applications were the variables selected to assess the sonication treatment where a 20 kHz probe and a nominal power of 100 W were used. A series of experiments were undertaken to sonicate the contaminated samples, with exposition times and number of applications ranging from 1-10 min and 1-3, respectively (Table 2). Fig. 1 shows MPN variation during 1, 15 and 45 days in the seven sonication experiments developed. The number of microorganisms (measured as MPN) at the end of each sonication treatment was the same as that of the control in most treatments except for the experiment 4 performed in the following conditions: maximum sonication power, 2 applications for 5 min each. These results are consistent with ultrasound treatments applied in bacterial suspensions [16, 17]. Nevertheless, the above results showed that scaling-up the process to treat contaminated diesel fuel storage

tanks in petrol stations (usually ranging from 20 to 50 m^3) is not sustainable due to the high energy required.

Table 2. Sonication tests.

Test	Time (t)	Volumetric power (P)	Number of applications (n)
1	t_1	P_2	n_2
2	t_2	P_2	n_2
3	t_3	P_2	n_2
4	t_2	P_1	n_2
5	t_2	P_3	n_2
6	t_2	P_2	n_1
7	t_2	P_2	n_3

t_1, t_2, t_3: 1, 5 and 10 min; P_1, P_2, P_3: 100 % in 100, 200 and 500 mL; n_1, n_2, n_3: 1, 2 and 3

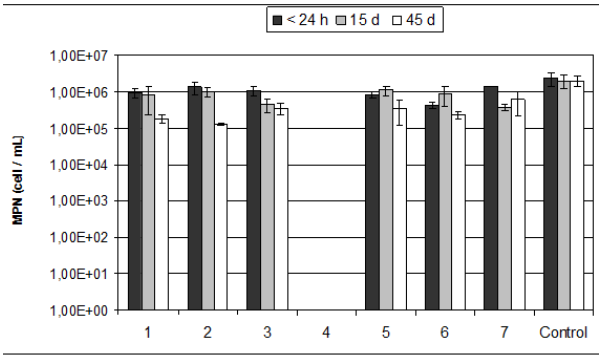

Fig. 1. Influence of sonication on the evolution of MPN of sample 18.

3.3 Biocide Treatments

Presence of microorganisms in the samples in both phases was determined by using dipslides (Fig. 2) and MPN measurements.

As it is shown, the abundance of microorganisms in the aqueous phase (Fig. 2A) was significantly higher than that in the diesel fuel phase (Fig. 2B). In addition, initial number of microorganisms in the aqueous phase of the samples was within the range 10^3 to 10^7 cell/mL.

Hydrogen peroxide and organic biocides with different functional groups were used as chemical agents to control the microbial growth and assess their disinfection effectiveness. Biocides with oxazolidine, thiocyanate and thiocarbamate groups are soluble in water and biocides with isothiazolone, morpholine and oxiborinane groups are partitioned between diesel fuel and water phases.

In general, water-soluble biocides showed a higher reduction of microorganisms, compared to H_2O_2 and biocides soluble in both phases, where no reduction was found in most cases. 80% of MPN reduction was observed after 15 days when biocides with oxazolidine (B2, B4 and B8) and thiocarbamate (B9) groups were used, reaching total disinfection after 45 days (Fig. 3).

On the other hand, no reduction in the number of microorganisms along time was found when biocides soluble in both phases were used. Only in the case of biocide with oxiborinane (B7) as functional group, a complete reduction of MPN in samples after 45 days was achieved.

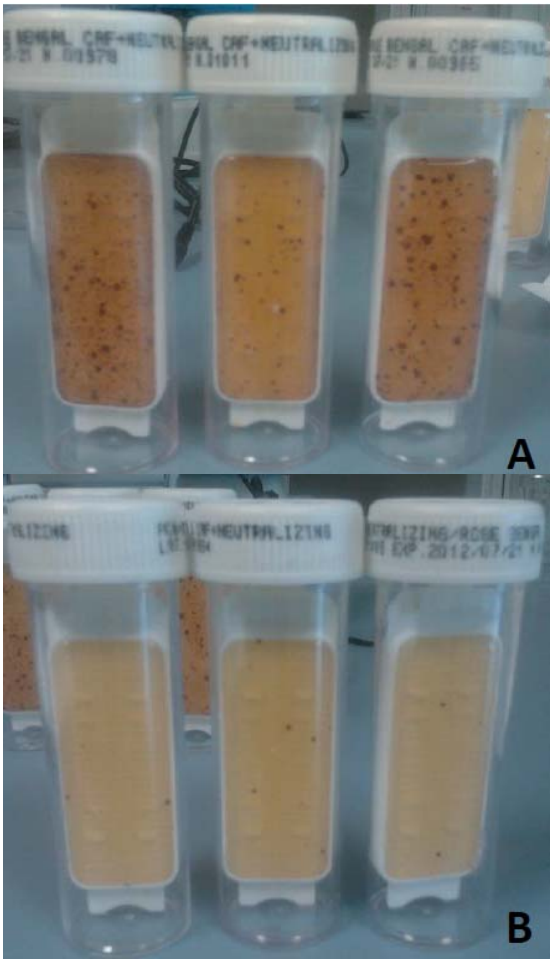

Fig. 2. Dipslides of aqueous (A) and diesel fuel (B) phases in three samples before treatment.

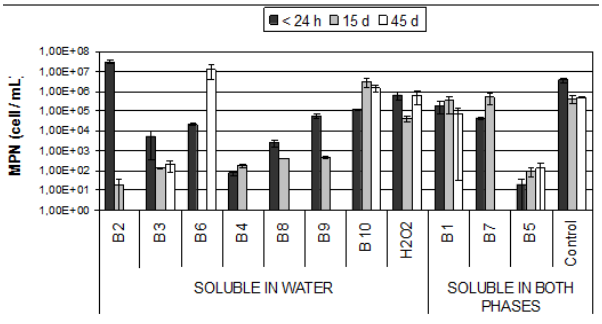

Fig. 3. Evolution of MPN at 1, 15 and 45 days in sample 18 after treatment with different biocides.

Two effective water soluble biocides with oxazolidine (B2) and thiocarbamate (B9) groups were selected in order to study the microbial growth in another sample. After 45 days of treatment, biocide with oxazolidine group produced greater disinfection in water phase

than biocide with thiocarbamate group since lower MPN values were obtained (Fig. 4).

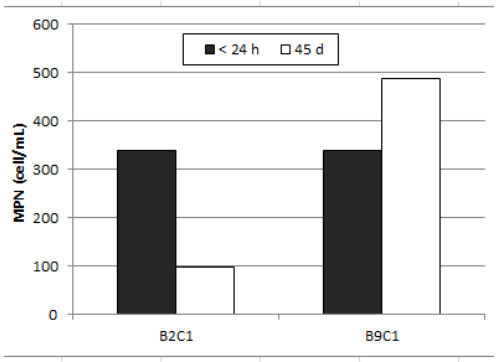

Fig. 4. Evolution of MPN with biocides 2 y 9 in the aqueous phase.

Analysis of the bacterial community composition by DGGE did not show a similar pattern between samples (Fig. 5). Although most of the samples showed high diversity in the number and intensity of the bands, some samples showed almost no visualized bands. Molecular identification of the DGGE bands provided rybotipes of degrading uncultured bacteria belonged to five different phyla: *Bacteroidetes*, *Spirochaete*, *Firmicutes*, *Proteobacteria* and *Thermotogae*.

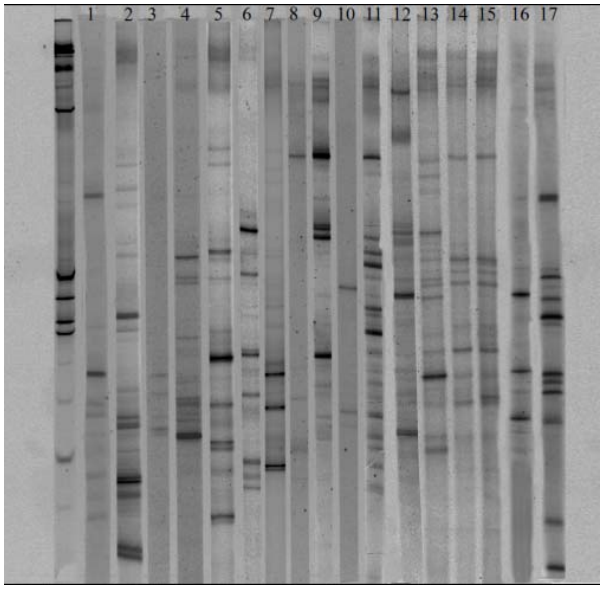

Fig. 5. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA gene fragments corresponding to samples from different petrol stations (lanes numbered 1 to 17). The left lane shows molecular weight markers.

Changes in bacterial community in samples treated with biocides with oxazolidine group and with thiocarbamate group after 45 days of the application are shown in Figs. 6 and 7, respectively. In both cases, bacterial community was significantly reduced and the band pattern changed, resulting in a new pattern of community with lower number of species and lower number of individuals thereof, i.e., less biodiversity. In addition, it is important to highlight that although initial communities of the samples did not show similar patterns, after 45 days from treatment, the communities were remarkably similar in both cases.

Fig. 6. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA gene fragments from the samples (2, 8, 9, 11 and 17) after 45 days of treatments with biocides with oxazolidine group. Lane (A) shows molecular weight markers.

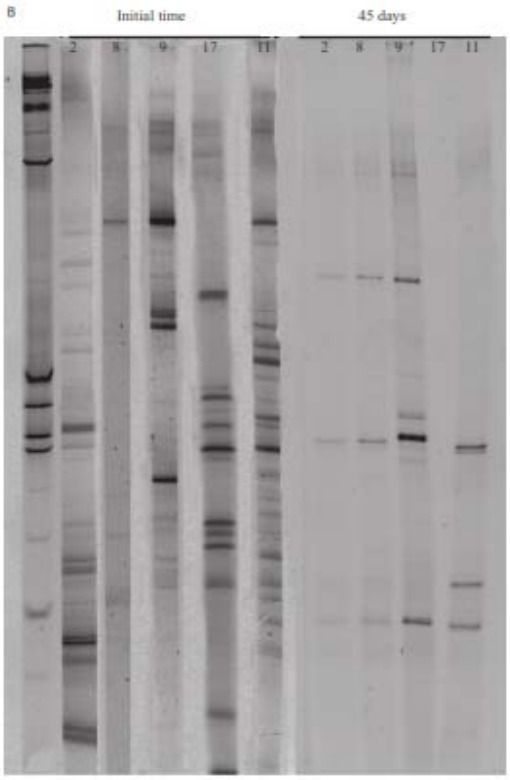

Fig. 7. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA gene fragments from the samples (2, 8, 9, 11 and 17) after 45 days of

treatments with biocides with thiocarbamate group. Lane (B) shows molecular weight markers.

4 Conclusions

Regarding the bacterial community of diesel fuel samples, different ribotypes belonged to phyla *Firmicutes*, *Disgonomonas*, *Flavobacterium*, *Ptoteobacteria* and *Bacteroidetes* were identified in samples from diesel fuel storage tanks. Several uncultured microorganisms were also found in diesel fuel samples, although some of them have not been previously associated with diesel fuel degradation. They can be defined as potential hydrocarbon-degrading microorganisms, so they could be useful for bioremediation of diesel-polluted environments. In addition, appearance and abundance of the different microorganisms in each tank showed a stochastic distribution, that is, none of studied parameters could explain their presence or absence in the samples. Consequently, it is difficult to carry out general purpose treatments for microbial growth control in diesel fuel storage tanks, so that prevention is a key action to perform in order to avoid biological contamination problems.

In this work we have shown that a non-culture-dependent molecular technique such as denaturing gradient gel electrophoresis (DGGE) and a miniaturized most probably number (MPN) technique are very useful to characterize and estimate total bacterial community and the effectiveness of different microbial reducing treatments.

Sonication treatment can be effective to prevent the growth of microorganisms in diesel fuel. However, it has been demonstrated that high volumetric power were needed for effective and persistent microbial disinfection, so that the large energy demand make sonication not economically viable.

However, when biocides were used for this purpose good results were observed by using low concentrations. Although satisfactory microbial disinfection was achieved by using these chemicals it will be necessary searching for new alternatives in order to solve the problem. Thus, it is very important using chemicals in both an appropriate and friendly way to prevent environmental damage. Moreover, a study based on microbial disinfection efficiency to find the optimum biocide for each particular case could decrease both treatment costs and environmental risk.

Nevertheless, the best solution to this problem is prevention which consists, essentially, in searching a proper design of the storage tanks to minimize the possible cracking and water intrusion, with a periodic draining system of water in the bottom to prevent microbial growth in case of accidental water inlets.

5 Acknowledgements

Authors wish to thank financial support from Repsol, S.A.

References

[1] Bento, F. M., Gaylarde, C. C.: Biodeterioration of Stored Diesel Oil: Studies in Brazil. Int. Biodeter. Biodegrad. 47 (2001) 107-112

[2] Gaylarde, C. C., Bento, F. M., Kelley, J.: Microbial Contamination of Stored Hydrocarbon Fuels and its Control. Rev. Microbiol. 30 (1999) 1-10

[3] Rodríguez-Rodríguez, C. E., Rodríguez-Cavallini, E., Blanco, R.: Bacterial Contamination of Automotive Fuels in a Tropical Region: the Case of Costa Rica. Rev. Biol. Trop. 57 (2009) 489-504

[4] Klofutar, B., Golob, J.: Microorganisms in Diesel and in Biodiesel Fuels. Acta Chim. Slov. 54 (2007) 744-748

[5] Rajasekar, A., Maruthamuthu, S., Palaniswamy, N., Rajendran, A.: Biodegradation of Corrosion Inhibitors and their Influence on Petroleum Product Pipeline. Microbiol. Res. 162 (2007) 355-368

[6] Bautista, L. F., Vargas, C., González, N., Molina, M. C., Simarro, R., Salmerón, A., Murillo, Y.: Physical and Chemical Treatments to Prevent the Growth of Microorganisms in Diesel Fuel Storage Tanks. Chim. Oggi-Chem. Today 32 (2014) 56-61

[7] Wang, T., MacGregor, S. J., Anderson, J. G., Woolsey, G. A.: Pulsed Ultra-Violet Inactivation Spectrum of *Escherichia coli*. Water Res. 39 (2005) 2921-2925

[8] Gómez-López, V. M., Ragaert, P., Debevere, J., Devlieghere, F.: Pulsed Light for Food Decontamination: a Review. Trends Food Sci. Technol. 18 (2007) 464-473

[9] Al Bsoul, A., Magnin, J. P., Commenges-Bernole, N., Gondrexon, N., Willison, J., Petrier, C.: Effectiveness of Ultrasound for the Destruction of *Mycobacterium* sp. Strain (6PY1). Ultrason. Sonochem. 17 (2010) 106-110

[10] Muthukumar, N., Maruthamuthu, S., Palaniswamy, N.: Role of Cationic and Nonionic Surfactants on Biocidal Efficiency in Diesel-Water Interface. Coll. Surf. B: Biointerf. 57 (2007) 152-160

[11] Segura, Y., Molina, R., Martínez, F., Melero, J. A.: Integrated Heterogeneous Sono-Photo Fenton Processes for the Degradation of Phenolic Aqueous Solutions. Ultrason. Sonochem. 16 (2009) 417-424

[12] Molina, M. C, González, N., Bautista, L. F., Sanz, R., Simarro, R., Sánchez, I., Sanz, J. L.: Isolation and Genetic Identification of PAHs Degrading Microorganisms. PAHs Degradation and Toxicity from a Microbial Consortium. Biodegradation, 20 (2009) 789-800

[13] González, N., Molina, M. C., Bautista, L. F., Delgado, L., Simarro, R., Villa, J. A.: Effect of Surfactants on PAH Biodegradation by a Bacterial consortium and on the Dynamics of the Bacterial Community during theProcess. Biores. Technol. 102 (2012) 9438-9446

[14] Simarro, R., González, N., Molina, M. C., Bautista L. F.: High Molecular Weight PAH Biodegradation by a Wood Degrading Bacterial Consortium at Low Temperatures. FEMS Microbial Ecol. 83 (2012) 438-449

[15] Jiménez Islas, D., Medina Moreno, S. A., Gracida Rodríguez, J. N.: Propiedades, Aplicaciones y Producción de Biotensoactivos: una Revisión. Rev. Int. Contam. Ambient., 26 (2010) 65-84

[16] Joyce, E., Phull, S. S., Lorime, J. P., Mason, T. J.: The Development and Evaluation of Ultrasound for the Treatment of Bacterial Suspensions. A Study of Frequency, Power and Sonication Time on Cultured *Bacillus* Species. Ultrason. Sonochem. 10 (2003) 315-318

[17] Foladori, P., Laura, B., Gianni, A., Giuliano, Z.: Effects of Sonication on Bacteria Viability in Wastewater Treatment Plants Evaluated by Flow Cytometry-Fecal Indicators, Wastewater and Activated Sludge. Water Res. 41 (2007) 235-243