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Physical and chemical treatments
to prevent the growth of microorganisms in diesel fuel storage tanks

KEYWORDS: microorganisms, diesel, fuel, tanks, biocides, sonication

Abstract The use of petroleum fractions by some microorganisms as a food source has been known for years. This property has been applied mainly in the bioremediation of soils contaminated with hydrocarbons. Nevertheless, this natural degradation of hydrocarbons by microorganisms, has become a serious problem for the oil industry, since it has been observed that microbial growth occurs inside the fuel storage tanks. This fact is causing problems such as corrosion, filter plugging or blockage of pipes. Ultraviolet radiation, sonication and biocides treatments are alternatives to solve the problem.

INTRODUCTION

The use of petroleum fractions by some microorganisms as a food source has been known for years. This property has been applied mainly in the bioremediation of soils contaminated with hydrocarbons. Nevertheless, this natural degradation of hydrocarbons by microorganisms, has become a serious problem for the oil industry, since it has been observed that microbial growth occurs inside the fuel storage tanks. This fact promotes problems such as corrosion, filter plugging or blockage of pipes (1). However, the presence of microorganisms in the storage tanks causes slight changes in the physicochemical properties of fuels (2).

FUEL CHARACTERISTICS

Table 1 shows the fractions of fuels derived from petroleum. Properties of different types of fuels are controlled in terms of density, distillation, viscosity, lubricity, volatility, octane number, cetane number or stability, among others (3). In this sense additives are needed such as antiknock agents, corrosion inhibitors, lubricity modifiers, cetane number and cold flow diesel fuel improvers, detergents, dispersants, metal deactivators, anti-foam or antioxidants for the elimination of free radicals. The presence of some additives is a nutrient resource for microorganisms promoting microbial growth in the amount of water and sludge formation. The growth of microorganisms in petroleum fractions storage tanks has been described and studied previously. Although this process is generated in various types of hydrocarbon fractions of petroleum, crude oil, diesel fuel and kerosene are those where microbial growth occurs with greater intensity and frequency. In general, hydrocarbons more easily metabolized by microorganisms are those having a chain of 10 to 18 carbon atoms (C10-C18). Lower molecular weight hydrocarbons inhibit the growth. Therefore, diesel fuel (C16-C22) and kerosene (C9-C15) are the fractions where microbial growth is more significant. However, there is an optimum chain length for the metabolism of each microorganism species.

Fraction	Gas	Gasoline	Kerosene	Diesel
Number of C atoms	1-4	5-8	9-15	16-22

Table 1. Composition of petroleum fractions related to the number of Carbon atoms.

FACTORS THAT ENHANCE THE MICROBIAL GROWTH

The fuel storage tanks are usually buried, especially in petrol stations, being exposed to microbial contamination due to limitations in the design and maintenance. Moreover, serious pollution problems derived from fuel contamination can be generated because of corrosion and possible damage of tanks. Therefore, in some areas, underground tanks are being replaced by aboveground tanks where the microbiological contamination is usually reduced. Nevertheless, other dangers are associated to outdoor tanks such as an increase of risk of fire. Microorganisms can access the inside of the storage tanks through one of the following pathways (4):

- From the ground (buried tanks), through faulty seals or cracks in the tank.
- From the air, during loading operations and through the vents.
- Introduced along with the water during the washings of tanks.
- Through contaminated piping, filling openings or other access to the interior of the tanks.
- Residual microorganisms from previous washing operations.

Once the microorganisms are in the tank, they stay adhering to the walls or settling in the fluid phase, especially in the fuel-water interface at the bottom of the tank. Presence of water inside the tank is a limiting factor for microbial growth. Water can access inside storage tanks from different ways:

- The water dissolved in product (within specifications) can condense on the tank walls.
- Condensing humidity. Air can enter through tank lock, floating roofs or through the vents.
- In some distribution operations it can be entered as ballast water (boats) or through purging pipes.
- By filtration through cracks in old tanks and other sealing defects.
- The metabolic activity of microorganisms generates more water as a final product.

As soon as the microbial contamination has occurred inside the tank, the presence of a small amount of water allows the biological activity to start with further growth and multiplication of microorganisms. In addition, cell metabolism generates more water, contributing to the cycle amplification. Underground tanks are more easily contaminated because their designs present drainage problems of the aqueous phase. It has been described that water content in the fuel of 500 mg/L is enough to trigger the initial microbial growth (3). As an example, in the European Union, water content in diesel fuel is regulated to a maximum value of 200 mg/kg (5). Moreover, water content in biodiesel (neat FAME) is regulated to a maximum value of 500 mg/kg (6).

The oxygen, typically recharged with each filling of the tank, is necessary for aerobic metabolism of microorganisms, and it can also be dissolved in significant quantities in both the aqueous and the organic phases. However, although the medium becomes low in oxygen or even anaerobic, the biological activity of both facultative (such as *Bacillus* sp.) and anaerobic microorganisms (such as sulphate reducing bacteria, SRB) continue under these conditions.

The presence of electron acceptor species (oxidants) which allow maintaining oxidative metabolism of hydrocarbons located in the aqueous phase or in the oil-water interphase is needed. These oxidizing compounds are mainly sulphates (SO_4^{2-}) and nitrates (NO_3^-).

It is well known that water, organic matter and oxygen or, alternatively, another oxidizing chemical species in nature are needed to stimulate growth of microorganisms. Moreover, other mineral salts acting as nutrients are required. We underline the importance of phosphorus, nitrogen and metals such as iron, among others, present in small amounts. Phosphorus is generally the element which can cause growth restriction in a diesel system.

Fuels have been subjected to a progressive reduction in the sulphur content due to stringent environmental regulations which facilitates microbial activity. Furthermore, significant percentages of products derived from vegetable oils such as biodiesel, can be added to the formulation of fuels (especially diesel). This blending has also worsen the situation because these compounds are more easily biodegraded by microorganisms than petroleum hydrocarbons.

In addition, some additives to fuels (aliphatic amines, chelating agents, detergents, corrosion inhibitors…) may also be used as nutrients for microorganisms, promoting their growth.

On the other hand, temperature is also important to favour the microorganisms proliferation. Although there are bacteria that can grow in extreme values of temperature (7), the value should be within the physiological range of microorganisms to grow. Finally, the pH value is also important in the development of microbiological communities. Although the optimum pH is close to neutrality, a large variety of bacteria are capable to grow at acid pH.

MAIN MICROORGANISMS DESCRIBED INSIDE THE FUEL STORAGE TANKS

The fuel stored remains short periods of time depending on the location of the container and the season. However, this time may be enough for these containers to be contaminated by microorganisms passing within the water or the air inlet or filtered through the walls of tank (8). Water accumulates at the bottom of the tanks due to its higher density, creating a favourable environment for the growth of microorganisms. Only 0.1 percent of water is required to develop optimally microbial activity (9). Dead microorganisms fall down to the bottom forming sediment with live microorganisms and water contributing to the so called "sludge". This sludge may affect the quality of both the fuel and infrastructures: wear and corrosion of pipes and pumps, filter blocking effect, etc.

Conditions generated in storage containers mainly favour those microorganisms capable to metabolize hydrocarbons under anaerobic conditions. Many species of microorganisms growing in the presence of hydrocarbons have been identified. Some of them are shown in Table 2 (10,11).

Sulphate-reducing bacteria (SBR) are an example of these microorganisms (Table 2). *Desulfovibrio* sp., *Desulfobaculata toluolica* or *Desulfobacterium* are an important group of microorganisms which commonly proliferate in oil tanks (12-14). SRB are usually mesophilic and halotolerant or moderately halophilic δ-proteobacteria producing spores. This group typically proliferate when water is rich in sulphate, as for example seawater. SRB oxidize the organic compounds reducing sulphate to hydrogen sulphide causing significant corrosion damage in both pipes and storage containers.

Another important group of microorganisms are denitrifying bacteria such as the group of β-proteobacteria (*Thauera aromatica* and *Azoarcus tolulyticus*, among others) that mainly oxidize alkylbenzenes, reducing nitrate to nitrogen. Apart from the oxidation of organic compounds, nitrate reduction can be made from inorganic compounds such

Bacteria	Fungi	Yeast
Actinetobacter, Bacillus sp., *Clostridium sporogenes, Flavofacterium diffusum, Micrococcus* sp., *Pseudomonas* sp., *Pseudomonas aeruginosa, SRB**, etc.	*Acremonium* sp., *Aspergillus* sp., *Aspergillus fumigatus, Cladosporium* sp., *Fusarium oxysporum, Penicillium* sp., *Penicillium citrinum, Penicillium funiculosm, Trichiderma* sp., etc.	*Candida* sp., *Candida famata, Candida lypolytica, Candida silvicola, Candida tropicalis, Rhodotorula* sp., *Saccharomyces* sp., etc.

SRB: Sulphate-reducing bacteria

Table 2. Microorganisms identified in fuel storage tanks.

as hydrogen sulphide, producing elemental sulphur and nitrogen. This group of microorganisms can sometimes balance the negative effects of the SRB since they oxidize corrosive H_2S to elemental sulphur.
Another important microbial consortium for hydrocarbon degradation under anaerobic conditions is methanogenic archaea (15) that are able of degrading toluene and xylene to produce methane.

ALTERNATIVES TO PREVENT MICROBIAL GROWTH

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) and a subsequent use of biocides to prevent the growth after each refilling. Alternatives to the above actions are the following:

Ultraviolet radiation (UV)

UV radiation is used as a disinfectant technique due to its ability to produce photobiochemical reactions. DNA can absorb ultraviolet radiation, producing crosslinks in structure, forming both thymine and pyrimidine dimers. These structural modifications prevent DNA replication and cell division accordingly. The region of the electromagnetic spectrum causing these reactions is mainly 200-280 nm (named UV-C region) (16,17).
In a previous study (16) on *Escherichia coli* using a xenon lamp as a UV light source, it was observed that the removal of microorganisms was directly proportional to the energy dose received, with a maximum action at around 270 nm. There is no agreement about the most effective method of applying light. Some authors suggest a continuous way (16) while others have found that pulsing irradiation is more effective (17).

Sonication

Sonication applied to a liquid medium causes a cavitation phenomenon in which the huge pressure caused by the waves leads to bubbles formation that collapse and release energy. The bubbles radius and therefore the released energy are inversely proportional to the sonication frequency. Thus, the use of low frequency sonication (typically 20 kHz), combined with a high intensity can produce enough energy to destroy the microorganisms present in the medium, breaking their cell structures (18,19).
It has been observed that the application of low frequency and high intensity sonication produces a constant decrease in the concentration of microorganisms, and that this removal increases with the intensity and exposure time to sonication (20).

Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a lethal component for bacterial cell through the production of free radicals which attack a wide variety of organic compounds including proteins and lipids that are present in cell membranes of microorganisms and affecting bacterial vital functions such as transport of nutrients, elimination of waste substances, osmotic and membrane potential control, etc. It can also cause permanent damage to the DNA, preventing replication and promoting cellular aging. This compound is used as a powerful bactericide against anaerobic microorganisms with the absence of the enzymes capable of neutralizing it.

Microbiocides

Microbiocides are chemicals that destroy viable forms of microorganisms (spores and vegetative forms). These chemicals are highly toxic molecules and therefore it is absolutely necessary a responsible use of them. Microbiocides are used in the removal of microorganisms located in a medium due to its disinfectant power. Their effects and mechanisms of action in bacterial populations are very variable (21), altering vulnerable structures such as the cell wall, plasma membrane, structural proteins or DNA.
The presence of microorganisms in hydrocarbons storage tanks is a common case where growth takes place in the lower aqueous phase, instead of in the organic phase where a small fraction of water is dissolved. Table 3 shows families of biocides soluble in water hydrocarbons (3).

Hydrocarbon soluble	Water soluble
Isothiazolones	Morpholines
Organoboranes	Oxazolidines
Pyridinthiones	Halides
Hexahydrotriazines	Aldehydes
Imidazole carbamates	Phenols

Table 3. Biocides used for controlling microbial growth in hydrocarbons.

Regarding the solubility characteristics of biocides, there are four categories described according to their use in hydrocarbon/water systems (22).

a) Products which are dispersed in the organic phase and are completely soluble in water. For treatment of the lower aqueous phase.
b) Products soluble in the organic phase spread over partially in the aqueous phase. For fuel treatment in which the water content is very low.
c) Mixtures soluble in the organic phase with two components, one active in water and the other in the hydrocarbon. To prevent growth in both the aqueous phase and the water/hydrocarbon interphase.
d) Products soluble in the aqueous phase. For the lower aqueous phase of tanks.

In general, it has been observed that the addition of biocides can produce a reduction in the population of bacteria, and particularly on the SRB, which produce hydrogen sulphide. As a drawback, repeated addition of the same biocide may cause the emergence of strains with intrinsic or acquired resistance. Bacterial resistance to biocides was first described in the 1950s. Since then, the list of these compounds has been growing. Chemical compounds such as formaldehyde, biguanides or iodophors, cause selection and persistence of bacteria in contaminated media (23).
Sometimes the use of biocides leads to problems such as high cost and safety to human health and the environment. Therefore, in the case of SRB bacteria, an alternative proposal is the addition of nitrate to encourage competition with nitrate-reducing bacteria and prevent production of sulphide (24). It has been demonstrated that by adding a

concentration between 5 and 10 mM of nitrate, the sulphide presence is reduced to negligible levels but the presence of nitrate-reducing bacteria is increased (25).
With the aim of studying the most appropriate mechanisms to prevent the growth of microorganisms in diesel storage tanks (prevention) or eliminate (treatment), it is necessary to identify them. From this information, and also with the physicochemical characterization of the test samples, it is possible to find the most effective treatments. Moreover, taking into account the results obtained, it is possible to develop a pattern of growth of microorganisms in the petrol stations.

Identification and biologic characterization of the microbial population

There are different methods in order to assess the extent of the microbial contamination in diesel storage tanks.

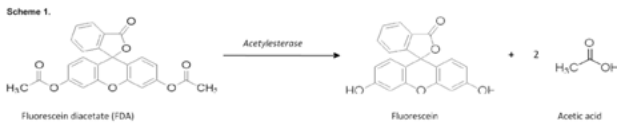
Estimation of the number of microorganisms

MPN (Most Probably Number)

The number of total microorganisms able to growth in diesel fuel can be estimated by a miniaturized most probable number (MPN) technique in 96-well microtitre plates with eight replicate wells per dilution in 180 µL of Bushnell Haas Broth medium (BHB) with surfactant Tween-80 (1 percent v/v), 10 µL of diesel fuel as only carbon source and 10 µL of the microorganisms, contained in the water phase of the samples. The microtitre plates are incubated at the natural growth temperature of the samples (17°C in storage tanks). Growth is determined by the presence or absence of turbidity and subsequent extrapolation to number of cells per unit volume (cells/mL), as a function of the dilutions compared to the initial sample.

FDA (fluorescein diacetate)

The number of dead or degraded cells can be determined by the detection of cells producing fluorescein in a fluorescence spectrophotometer. To this, certain amount of fluorescein diacetate is added to medium containing microorganisms which when die, release the intracellular enzyme actylesterase that hydrolyzes fluorescein diacetate producing fluorescein, according to the reaction depicted in Scheme 1. The production rate of fluorescein is proportional to the amount of acetyl esterase released and, therefore, the number of cells died or degraded.



Scheme 1. Reaction between FDA and acetylesterase.

Bioluminescence

Bioluminescence is a technique base in ATP (adenosine triphosphate) detection. The ATP is the energy carrier molecule of all live organisms and so, it is an indicator of the active microorganisms present in the sample. For this, the sample is filtered and microorganisms are retained. By the addition of certain reagents, breakdown of the cell wall and subsequently release of the ATP to the medium occur. The ATP present in the sample reacts with a specific chemical reagent, producing a bioluminescence (detected in a spectrophotometer) that is proportional to the amount of ATP.

Isolation of the colony forming units (CFU)

It is so important to mention that less than 1 percent of the microorganisms are cultivable, i.e., capable to form colonies in a culture medium and so, they can be isolated and identified by traditional culture methods. The vast majority are non-cultured microorganisms that can be isolated by other techniques such as DGGE (denaturing gradient gel electrophoresis), explained later.
The laminocultures are interesting and useful since they allow optimizing time, cost and yields. This technique consists in the isolation of bacteria and fungi by growing in two different types of specific media in the same device: colourless media is composed by PCA, TTC and a specific neutralizer for the bacterial growth; rose media is composed by rose bengale medium and a neutralizing that only allow fungus and yeast to growth. It is a simple methodology that consists of contacting both side of the device with the contaminated media and its incubation in culture chambers at ordinary temperature.

Molecular identification of the culture microorganisms

Bacterial DNA from the isolated colonies can be extracted and, subsequently, amplified by PCR (polymerase chain reaction) with the polymerase enzyme and a pair of specific primers that sequence informative and knowledge regions of the genetic material. The nucleotide sequences obtained are compared with the more similar sequence of a data base (NCBI-Genbank, DDBI, EMBL) to obtain the taxonomy classification.

Molecular identification of the total microorganisms

DGGE (Denaturing gradient gel electrophoresis)

DGGE (Figure 1) allows separating the DNAs of the total population of the environmental sample through a denaturing agent (urea-formamide) that acts as a function of the number of base pairs and composition in cytosine guanine (C-G). The horizontal bands represent different ribotypes. Predominant bands in DGGE are excised, amplified by PCR and sequenced to identify the specie. In some cases, it is not possible to re-amplify bands due to DNA degradation by the exposition to UV light or due to the overlapping of different bands. In these cases, bands are amplify by bacterial cloning in *Escherichia coli* inserting each DNA segment of different ribotypes mixed in a band, in different DNA vectors. Their later multiplication in the *E. coli* following the normal cycle of multiplication of the bacteria, generate multiple vectors that contain the DNA segment of interest that later are extracted with specific reagent kits.

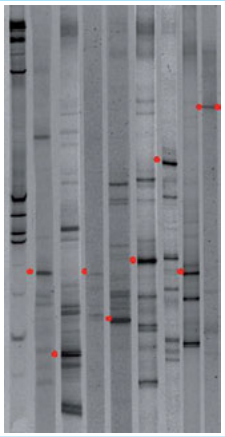


Figure 1. DGGE. Vertical lines are the total DNA of each sample. Horizontal lines are the DNA of the different ribotypes of the samples. Red points represent the more significant bands regarding abundance, presence or absence in other bands.

FISH (Fluorescence in situ hybridization)

FISH is an additional technique that can contribute with interest physiologic information by using molecular marker. In the process, nucleic acids probes marked with fluorescein are used to join to specific DNA or RNA region of microorganisms, allowing the identification and quantification by fluorescence microscopy, remarking functional differentiation (i.e., ability to reduce sulphates) in front to genetic variability (26).

Physicochemical characterization

A physicochemical characterization of the samples is very important in order to study the conditions in which microorganisms grow within the tanks. The metal ions content (Cl^-, SO_4^{2-}, NO_3^-, etc.), total carbon (organic and inorganic), or properties such as conductivity or pH, are crucial parameters in order to develop a microorganisms growth pattern in the sampled stations. Thereby it is possible a physicochemical characterization of diesel fuel or kerosene by using techniques of atomic emission spectroscopy inductively coupled plasma, or ion chromatography using selective electrodes, or analysis of catalytic combustion and oxidation by no dispersive infrared detection for the determination of carbon in the samples.

Sonication

In order to evaluate the effectiveness of treatment in removing microorganisms in samples of gas oil from storage tanks, the sensitivity of the cell membranes against the action of ultrasound is studied. The treatment is performed by generating ultrasonic with fixed frequency of 20 kHz and a nominal power of 100 W. In Table 4, experiments are shown with sonication under different conditions.

Figure 2 shows the results of cell density (MPN) after sonication with a sample contaminated with microorganisms. Three replicates have been done per experiment at times of <24 h, 15 and 60 days.

Country	Netherlands	Japan	Singapore
current daily salt intake	8.7 g	10.6 g	8.3 g
of which 80% out of processed food	7.0 g	8.5 g	6.6 g
recommendation for 2015	6.0 g	8.8 g	5.0 g
gap: current - recommended salt intake	2.7 g	1.8 g	3.3 g
current feasible reduction	32%	34%	35%
feasible reduction by processed food	2.2 g	2.9 g	2.3 g

Table 4. (T) Application time: 1 min (T1); 5 min (T2); 10 min (T3) (P) Volumetric power: 1.0 W/cm3 (P1); 0.5 W/cm3 (P2); 0.2 W/cm3 (P3) (N) Number of applications: 1 (N1); 2 (N2); 3 (N3)

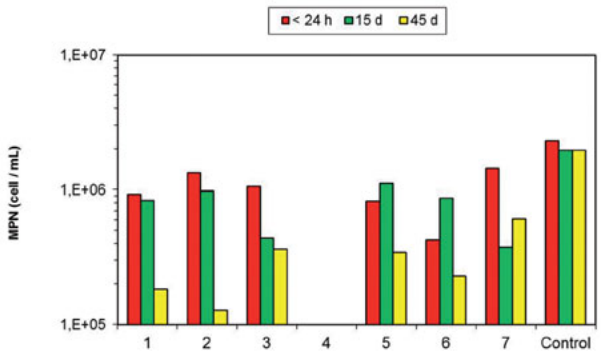


Figure 2. Cell density of treatments after sonication.

It is observed that the number of microorganisms after application of treatment 4 (T = 5 min, P = 1 W/cm^3 and N = 2) is negligible, and remained so for 60 days. For the other treatments the reduction is moderate, less than one order of magnitude. The prominent microbial density reduction achieved with treatment 4 was obtained with the highest volumetric power studied (1 W/cm^3).

Use of biocides

In addition we have studied the effectiveness of various biocide active products, including H_2O_2, to incorporate a certain amount of dissolved oxygen to the aqueous medium. Hydrogen peroxide releases free radicals which act on living cells (27). It was also assessed the effect of five commercial biocides with different functional groups. Three concentrations were tested for each of them.

By determination of MPN, cell density is measured in the samples after treatment with different biocides concentrations and after incubation periods noted above (<24 hours, 15 days and 60 days). Results are shown in Figure 3, 4 and 5, respectively.

As a result of the data of cell density is found that, immediately, biocides 2, 3, 4 and 5 reduce the microbial population of the sample 4-5 orders of magnitude (except concentration C1 of biocide 3). Biocide efficacy of 1 and hydrogen peroxide is much more limited because the reduction is 1-2 orders of magnitude.

After 15 days, the samples treated with both hydrogen peroxide and biocide 1 (except those at the highest concentration) have cell densities similar to control samples (no treatment). However, treatments with biocides 2, 3, 4 and 5 have retained control of the microbial population.

Within 60 days, the presence of biocides 3 and 5 in the samples

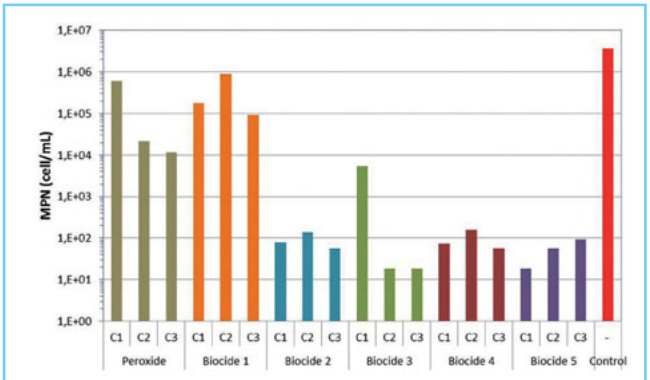


Figure 3. Cell density (MPN) for biocides treatment. Immediate answer (<24 h).

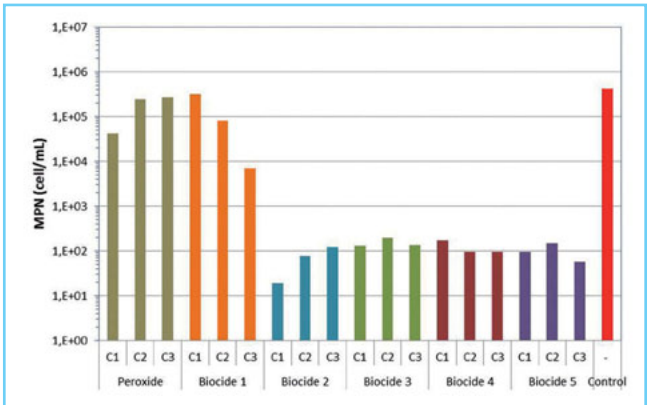


Figure 4. Cell density (MPN) for biocides treatment. Answer: 15 days.

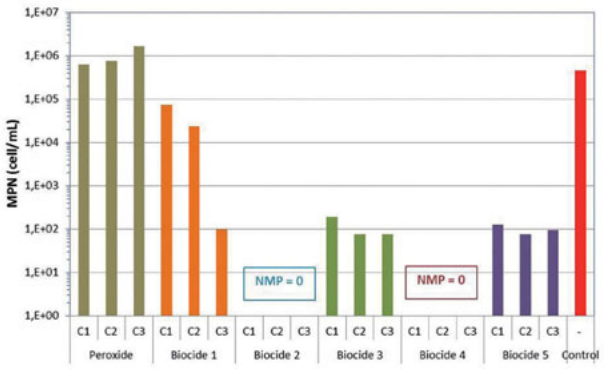


Figure 5. Cell density (MPN) for biocides treatment. Answer: 60 days.

show the same cell density levels that have been observed throughout the entire study. The biocide 1 treatment performed with the highest concentration (C3) has a significant long-term effect because in these conditions microbial population has been reduced to levels similar to those obtained with biocides 3 and 5. After 60 days, the most remarkable result is that the presence of biocides 2 and 4 in the samples has completely reduced the population of microorganisms due to continued contact with these products. Efficacy of both biocides is based on the formaldehyde release to the medium.

CONCLUSIONS

Considering sonication experiments, the MPN of treatment 4 which corresponds to samples subjected to higher volumetric power, has been shown the best results, eliminating all microorganisms present in the sample and avoiding possible re-growth at least 45 days.

In this work we have demonstrated that biocides treatment is more effective to prevent the growth of microorganisms than sonication. Chemicals have to be use in both an appropriate and friendly way to prevent environmental damage. Despite the good results that have been obtained, it is necessary to continue studying and searching alternatives for the most suitable one to solve the problem. It is clear that the correct choice of the biocide needs to be done through experimental assessment since the effect of each biocide in reducing microbial contamination can be variable. Thus, it could decrease the cost of the treatment and the environmental risk.

However, the best solution is always prevention: a suitable design of the storage tanks where the possible formation of cracks and entrance of water is minimized and a periodic drainage of the water at the bottom where microbial growth proliferates, if despite all it enters the tank.

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