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Effects of Biodiesel Concentration on Microbial Deterioration of Polyethylene in a Simulated Fuel Storage Tank

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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

EFFECT OF BIODIESEL CONCENTRATION ON MICROBIAL DETERIORATION OF POLYETHYLENE IN A SIMULATED FUEL STORAGE TANK

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by

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Abstract

In this study a simulated fuel storage tank was used to investigate the effect of biodiesel concentration on biodegradation of polyethylene. This research is relevant in the field of fuel storage. The simulated storage system consisted of a number of identical conical flasks. Each flask was comprised of two layers, an upper one consisting of a fuel blend of diesel with biodiesel in concentrations ranging from 0 to 100% of biodiesel and the bottom layer containing an aqueous mineral media inoculated with a community obtained from a real fuel storage facility. Polyethylene slabs cut to a specific size were immersed in the aqueous layer and were aged for 200 days, the system was kept at environmental temperature of approximate 25°. The microbial composition of the aqueous layer, biofilm development on polyethylene slabs and changes in polymer surface were studied. The results in this study confirm that biodiesel in a mixture of diesel-biodiesel can affect both the composition and metabolic capabilities of microbial communities in diesel storage tanks. Biodiesel can also affect the biofilm community structure and the biodegradation of polyethylene. However, microorganisms induced only surface damage and it is unlikely that in the short term it represents a risk for the infrastructure.

Keywords

Biodiesel, polyethylene, biofilm, biodegradation, fuel storage

Co-Authorship Statement

In the development of this work five papers were written and coauthored, the extent of the collaboration of the co-authors is stated below.

Chapter 1

Article title	Effect of biodiesel addition on microbial population in diesel storage tanks
Current status	Published, NACE international corrosion conference and expo, (2013).
J-M Restrepo-Flórez performed the experimental design, the lab work and wrote the paper, A Bassi and M. Thompson were the technical and theoretical support both for the analysis and experimental design, they also review and corrected the multiple drafts of the paper.	

Chapter 2

Article title	Degradation and deterioration of polyethyelene by the action of microorganisms-a review
Current Status	Submitted, International biodegradation and biodeterioration
J-M Restrepo-Flórez wrote the paper, A. Bassi and M. Thompson were the technical and theoretical support. They reviewed, edited and corrected multiple drafts of the paper	

Chapter 3

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Article title	Investigation of Biofilm Formation on Polyethylene in a Diesel/Biodiesel Fuel Storage Environment
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Chapter 5

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Current status	Submitted, Polymer Degradation and Stability
J-M Restrepo-Flórez performed the experimental design, the lab work and wrote the paper. J-A Wood, L. Rehmann, A. Bassi and M. Thompson were the technical and theoretical support both for the analysis and experimental design. They also reviewed and corrected multiple drafts of the paper.	

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Dedication

To my grandfather Pastor Restrepo who keeps teaching me what a good man is

(a mi abuelo Pastor Restrepo que sigue enseñandome lo que és un buen hombre)

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List of Abbreviations

ANOVA: Analysis of variance

ATR: Attenuated total reflectance

AWCD: Average well color development

BIC: Bayesian information criteria

B0: Pure diesel

B25: Blend of diesel with 25% biodiesel

B50: Blend of diesel with 50% biodiesel

B75: Blend of diesel with 75% biodiesel

B100: Pure biodiesel

CFU: Colony forming units

CLPP: Community level physiological profiling

DGGE: Denaturing gradient gel electrophoresis

DSC: Differential scanning calorimetry

FTIR: Fourier Transformed infrared spectroscopy

HDPE: High density polyethylene

HT-GPC: High temperature gel-permeation chromatography

HT-SEC: High temperature size exclusion chromatography

LDPE: Low density polyethylene

LLDPE: Linear low density polyethylene

M_n: Number average molecular weight

PA: Polyamide-11

PCA: Principal component analysis

PCR: Polymerase chain reaction

rDNA: ribosomal deoxyribonucleic acid

SEM: Scanning electron microscopy

UMTS: Universal mechanical testing system

UV: Ultraviolet light

XDLVO: Extended Derjaguin, Landau, Verwey, Overbeek theory

XLPE: Cross-linked polyethylene

XRD: X-ray diffraction

16s RNA: 16s Ribosomal nucleic acid

Nomenclature

γ_l^+: Acid component of a liquid energy of adhesion (mJ/m^2)

γ_l^-: Basic component of a liquid energy of adhesion (mJ/m^2)

γ_l^{LW}: Van der Waals component of a liquid energy of adhesion (mJ/m^2)

γ_s^+: Acid component of a solid energy of adhesion (mJ/m^2)

γ_s^-: Basic component of a solid energy of adhesion (mJ/m^2)

γ_s^{LW}: Van der Waals component of a solid energy of adhesion (mJ/m^2)

Chapter 1

1 Introduction

Biodiesel has been gaining an important place in the fuels market as a replacement for regular diesel. Concerns regarding both the sustainability and stability of the supply chain of regular diesel have led to a growing biodiesel industry. The addition biodiesel to diesel can have an impact on the corrosive properties of the fuel; this is a potential danger for the contacting infrastructure. For this reason, some previous research has focused on biodiesel damage of metal surfaces (1-4). However, an important fraction of the infrastructure (mainly storage tanks) is made of polyethylene, a polymer thought to be chemically and biologically inert. Currently, there are only few studies that explore the possible effects of biodiesel compatibility with polyethylene (5–7).

Blending biodiesel with diesel may result in changes in the properties of polyethylene due to two different mechanisms: on the one hand there is a possible chemical interaction between the fuel and the polymer that might lead to deterioration of its mechanical properties (5–7) and on the other, it is possible that biodiesel favors the development of microorganisms able to use the polymer as carbon source. The scope of this study was to explore the second hypothesis.

The biodeterioration hypothesis mentioned above is supported by some evidence, which shows that deterioration of the mechanical properties of polyethylene can be boosted in the presence of microorganisms (8–16). Although the biochemical metabolic pathway/s for biodegradation of polyethylene is/are not completely understood, it is clear that some biochemical utilization of the polymer is possible and that external factors such as UV irradiation can influence the bioavailability of the polymer (17).

It is known that diesel and biodiesel storage tanks usually have a water layer produced by condensation of environmental moisture (18); this water layer constitutes a perfect place for the development of microorganisms that can use the fuel as carbon source. A number of studies have been conducted to study the biodiversity present in this kind of environment (19–21). Given that the chemical nature of biodiesel is different from the

chemical nature of regular diesel, it is reasonable to think that addition of biodiesel to diesel can lead to changes in the microbial community present within a fuel tank.

These changes in the microbial composition may or may not have an effect on the way the microorganisms interact with the surface of containers. However, it must be highlighted that there is important evidence showing how environmental conditions can affect the ability of microorganisms to interact with surfaces (22). Microbial interactions with materials are usually mediated by biofilm formation, a kind of structure that is formed on the surface of solid materials in a liquid environment that binds the microorganisms to the surface. Formation of biofilms is especially important in the degradation of materials that are insoluble in water, such as polyethylene (9,16). The organisms use the material as a support and can use it as well as a source of nutrients to maintain their metabolism. Bio-corrosion processes are therefore usually very dependent on whether or not biofilm formation occurs on the surface of a material.

Even when it is generally accepted that biofilm formation is a crucial step in polyethylene biodegradation, it is not possible to conclude that microorganisms able to form biofilms on a polymer surface can also metabolically degrade the polymer. For this reason before stating conclusions about the biodegradation rate of the material it is necessary to analyze the polymer properties to detect signs of the deterioration process.

In conclusion, it can be said that in order to study the effect of biodiesel on the biodegradation of polyethylene in a fuel storage tank, three different questions have to be answered: (i) is the microbial community changing due to the presence of biodiesel? (ii) Is the biofilm formation capability of this community changing because of the presence of biodiesel? (iii) Are these changes boosting the degradation of the polymer?

To solve the first question and track the composition of a microbial community, there are basically three different approaches.

-The most classical one is based on the platting in selective culture media for different microorganisms, counting the number of colonies after a period of time gives an approximation of the composition of a community. The big disadvantage

of this approach is that most microorganisms are not able to grow in synthetic media, in such a way that the composition of the community obtained in this way is skewed (23).

-The second approach used to track communities is based on the isolation and sequencing of 16S rDNA. This is a culture independent strategy and gives an idea of the true composition of the community; the drawbacks or this technique are that that it is subjected to the biases typical of PCR and that the rDNA of dead microorganisms can also be identified (24).

-The final strategy used to track microbial composition is based on the metabolic capabilities of the community, it is called community level physiological profiling (CLPP) and it is based on the rate of consumption of different carbon sources by a microbial community. This technique is very useful to identify changes in a microbial community over time, but does not give information about the composition of the community (25–27).

To answer the second question regarding to the effect of biodiesel on biofilm formation capabilities of a community, at least three questions are relevant and should be answered in order to outline proper conclusions:

- Are cells growing on the surface?

- Are these cells metabolically active?

-Does biodiesel have any effect on the 3D structure of this biofilm?

Finally, to determine if a biofilm is causing polyethylene deterioration it is possible to follow 4 different groups of properties on the polymer:

-The topography of the samples, usually evaluated by Scanning Electron Microscopy (SEM) or Atomic Force Microscopy (AFM).

-The chemical characteristics of the surface, commonly analyzed by FTIR and contact angle determination.

-The mechanical properties of the material, analyzed with an Instron instrument.

-The structure of the polymer, which refers mainly to the percentage of crystallinity and molecular weight distribution.

1.1 Research objectives

Based on what has been stated, this thesis has three main research objectives:

(1) To study the effect of biodiesel concentration on the microbial community established in the water layer in the bottom of a diesel storage tank. In this section the effect of biodiesel addition on the composition of the suspended microbial community was assessed in three ways: by platting in selective media for anaerobes and bacteria, by analysis of 16s rDNA libraries constructed with samples before and after biodiesel was added and finally by CLPP analysis of the samples to identify changes in the metabolic profiles of the communities associated with biodiesel addition.

(2) To study the effect of biodiesel concentration and polymer type on the amount, structure and composition of biofilms developed on the walls of a simulated fuel storage tank. In this part of the work, biofilm forming capabilities were explored in different polymers (linear low density polyethylene, cross-linked polyethylene and linear low density polyethylene half coated with polyamide-66) for communities of microorganisms growing under different biodiesel concentrations. In all cases viability, growth, composition and structure of these communities were determined.

(3) To study the effect of biodiesel concentration on the biodeterioration of polyethylene. In this section, the effect of microorganisms on the chemistry, topography and crystallinity of polyethylene were studied under different biodiesel concentrations.

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Chapter 2

2 Literature Review

As was mentioned earlier, in this study the intent was to explore the effects of microbial activity of a complex community in a diesel storage tank on polyethylene biodegradation and how the addition of biodiesel in this system affects the polymer degradation. The literature review below is structured as follows: first, some fundamental concepts on the materials used are reviewed. Secondly, the microbiology of diesel storage tanks is discussed and finally, the known effects of microorganisms on polyethylene are presented.

2.1 Materials used in this study

2.1.1 Polyethylene

Polyethylene is the polymer of ethylene, chemically, a long backbone of covalently linked carbon atoms (Figure 2-1) (1-2). Polyethylene does not present a unique molecular weight, rather it has a molecular weight distribution, with molecules ranging from 1400 to around 250.000 Da. Although polyethylene is mainly a linear molecule it gets branching, the higher the branching of a polyethylene sample the lower the density (2).

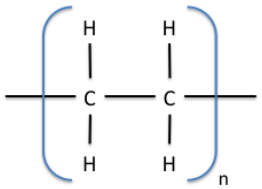

Figure 2-1. Chemical structure of polyethylene

Besides the chemical arrangement described above, polyethylene also has a three dimensional structure that can be described as semi-crystalline, defined as a system that consist of two or more solid phases with at least one highly organized fraction (crystalline portion) and the other phase(s) in a disorganized disposition (2). The polyethylene three-dimensional structure has been described using a three-phase model, one crystalline

phase that is surrounded by non-crystalline regions (amorphous), and between these two phases there is an interfacial region with a moderate degree of order (2).

Based on the final density that is obtained there are different kinds of polyethylene that are available in the market. Differences in density are obtained mainly by generating changes in the degree of branching of the molecules. Different kinds of polyethylene differ both in their structure as well as in their physical and mechanical properties such as density, degree of crystallinity and melting point (2). Although there are many kinds of polyethylene based on the differences mentioned before, the most important ones are high-density polyethylene (HDPE), low density polyethylene (LDPE), linear low density polyethylene (LLDPE) and cross linked polyethylene. Table 2-1 summarizes some characteristics these types of polyethylene (1). The table does not include cross-linked polyethylene because its properties rely mainly on the resin in which it is based.

Table 2-1. Physical and chemical properties of the most important types of polyethylene

Properties	**HDPE**	**LDPE**	**LLDPE**
Density (g/cm^3)	0.94-0.97	0.91-0.94	0.90-0.94
Degree of crystallinity (%)	55-77	30-54	22-55
Tensile modulus (Pa)	22-29	3.6-7.3	5.5-18.9
Tensile yield stress (psi)	2600-4500	1300-2800	1100-2800
Tensile strength at break (psi)	3200-4500	1200-4500	1900-6500
Melting temperature (°C)	125-132	98-115	100-125
Heat of fusion (cal/g)	38-53	21-37	15-43
Degree of branching	+	++	+++

2.1.2 Diesel

Diesel fuel is a middle distillate with a carbon distribution length that ranges from 9-23 carbons (3, 4). The composition of this kind of fuel is dominated by four different kinds of hydrocarbons: n-alkanes (linear saturated hydrocarbons), n-isoalkanes (branched

saturated hydrocarbons), cycloalkanes (saturated cyclic alkanes) and aromatics (5). Table 2-2 shows a typical composition of a diesel fuel. The detailed composition of diesel fuel is very complex and comprise around 4000 different molecules (5).

Table 2-2. Typical composition of diesel fuel

Group of components	Percentage
n-alkanes	24
Isoalkanes/ Cycloalkanes	46
Aromatics	30

2.1.3 Biodiesel

Biodiesel is the fuel that result from the trans-esterification process of triglycerides with an alcohol, typically methanol. Chemically, biodiesel is mainly composed of methyl-esters and its detailed composition will depend mainly on the source of fat used in the process. Some small quantities of triglycerides, diglycerides, mono-glycerides, methanol and glycerol are also found. A typical composition of biodiesel is presented in table 2-3. Traces of elements such as sulfur, nitrogen and phosphorus are usually found in biodiesel at the level of ppm (6).

Table 2-3. Typical composition of biodiesel fuel

Group of components	Approximated Percentage
Methyl esters	96.5
Triglycerides	0.5
Diglycerides	0.5
Monoglycerides	1
Glycerol	0.05
Methanol	0.3

2.2 Microbiology of diesel storage tanks

2.2.1 Characteristics of the ecosystems established in diesel storage tanks

A wide variety of microorganisms are able to use hydrocarbons as source of energy (3, 7–9), catabolism of these molecules can be performed either aerobically or anaerobically (10–12). Fuel storage systems constitute a novel ecological niche, in which complex microbial communities are usually established in the bottom of the tanks (3, 13). For a microbial ecosystem to flourish at least three factors are required: water, macro/micro nutrients and microorganisms under the adequate conditions of pH and temperature (12). In the bottom of fuel storage tanks all these requirements are fulfilled. Figure 2-2 depicts the elements that are commonly found in the ecosystems established in the bottom of fuel storage tanks.

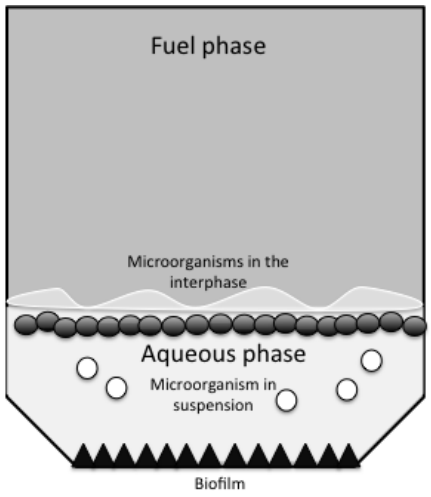

Figure 2-2. Illustration of a microbial ecosystem in a fuel storage tank

The accumulation of water at the bottom of fuel storage tanks is a common phenomenon, usually result of condensation of environmental moisture and also due to de-solubilization of water absorbed in the fuel when the temperature drops (14). Although water is not highly soluble in hydrocarbons, only a small quantity is necessary to generate a small niche for microorganisms to grow. The other important point is that once a community has been established the metabolism of microorganisms can generate more water as a by-product (3).

In terms of nutrients it is clear that the carbon source in a fuel storage tank is in excess, the main nutrient limitations are phosphate, nitrate and iron (3). The only source for these nutrients and also for other microelements such as magnesium, manganese and zinc is the amount of this elements dissolved in the fuel phase. It has to be highlighted that the kind of carbon sources available will necessarily depend on the chemical nature of the fuel. In this work I will focus specifically on diesel and its blends with biodiesel. The hydrocarbons present in diesel fuel been usually in the range from 15 to 22 carbons are more prone to microbial attack than those in other lighter fuels, such as gasoline, that contain shorter molecules that can dissolve cell membranes (3).

Fuel storage tanks are open systems; this implies that microorganisms can come inside from different sources without any constriction. Typical sources of microbial contamination are the fuel itself that can contains up to 10^2 CFU/ml and the air in the surrounding environment (3, 9). Once these microorganisms have found an adequate environment to grow, they start to divide. In general, in this kind of system, three different places for microbial growth are observed (Figure 2-2): some microorganisms establish themselves as biofilms adhering to the surface of the tank, some others remains in suspension and finally some proliferate in the interphase between the water layer and the fuel, where the carbon sources are more readily available (13). It is important to note that the community of the biofilms is not necessarily equal to the community in suspension and that the composition of these two communities will depend on the ability of the microorganisms to adhere to the surfaces available.

Fuel storage tanks are aerobic environments; oxygen is soluble in the fuel and diffuses from it to the water layer in the bottom of the tank, but the existence of biofilms also favors the development of anaerobic spots; limitations in oxygen diffusion from the outer layer of biofilm to the more inner core facilitate creation of some places at the interior of this structure that presents anaerobic metabolism (14).

2.2.2 Microbiology of fuel storage tanks

The microbiology of fuel storage tanks in general and of diesel fuel reservoirs in particular has been studied extensively (3, 7–10, 12, 15). Biodiversity of microorganisms

able to use hydrocarbons in diesel fuel consist of fungi, bacteria and archaea, and includes both aerobic and anaerobic microorganisms (10, 11). Among the anaerobic bacteria methane producers and sulfate reducing bacteria have gained most of the attention due to the negative impact of their metabolic activity on metallic infrastructure (3).

Table 2-4 presents a brief account of some of the bacteria and fungi isolated from diesel storage tanks to give an idea of the biodiversity that could be expected when analyzing these systems.

The great diversity of carbon sources present in petroleum diesel (Table 2-2) leads to a synergistic effect of different microorganisms to degrade this fuel. So it is easy to find that in a complex community of diesel degraders some microorganisms are more prone to degrade paraffins while others might be using less readily available sources of carbon such as aromatic compounds. Metabolic pathways for degradation of n-alkanes, cycloalkanes and aromatics have been described in a variety of microorganisms (5, 10, 12, 16, 17).

It is not surprising that microorganisms are able to use the chemical compounds in diesel as carbon and energy source, these molecules constitutes a reservoir of energy and have been on earth enough time to stimulate the evolution of enzymatic systems that allow microorganisms to use the energy stored in these compounds. Due to its structural and chemical similarity with polyethylene, in the context of the present research project only degradation of linear hydrocarbons is of interest.

The metabolic pathway for degradation of linear hydrocarbons has evolved so the initial molecule is oxidized in such a way that a carboxylic acid is obtained at the end of the process (Figure 2-3) (17, 18). This kind of molecule is a common source of energy and can be used by microorganisms after acetylation by using the β-oxidation pathway. Two different kinds of oxidation processes have evolved: terminal oxidation of the chain and sub-terminal oxidation in the middle of the chain. In this second case an esterase enzyme is required so the metabolic route can proceed.

Table 2-4. Different microorganisms able to use compounds present in diesel as carbon/energy source

Group of microorganism	Species	Reference
Bacteria	*Acinetobacter calcoaceticus*	(3)
	Bacillus sp.	(3, 9)
	Bacillus cereus	(9)
	Brevundimonas	(14)
	Flavobacterium arborescens	(3)
	Micrococcus sp.	(3, 9)
	Pseudomonas sp.	(3)
	Rhodococcus sp.	(9)
	Staphylococcus epidermidis	(9)
Fungi	*Aspergillus flavus*	(3)
	Aspergillus niger	(3, 15)
	Candida famata	(8)
	Candida guilliermondii	(8)
	Candida parapsilosis	(8)
	Fusarium oxysporum	(3)
	Penicillium sp.	(3)
	Rhizopus oryzae	(3)

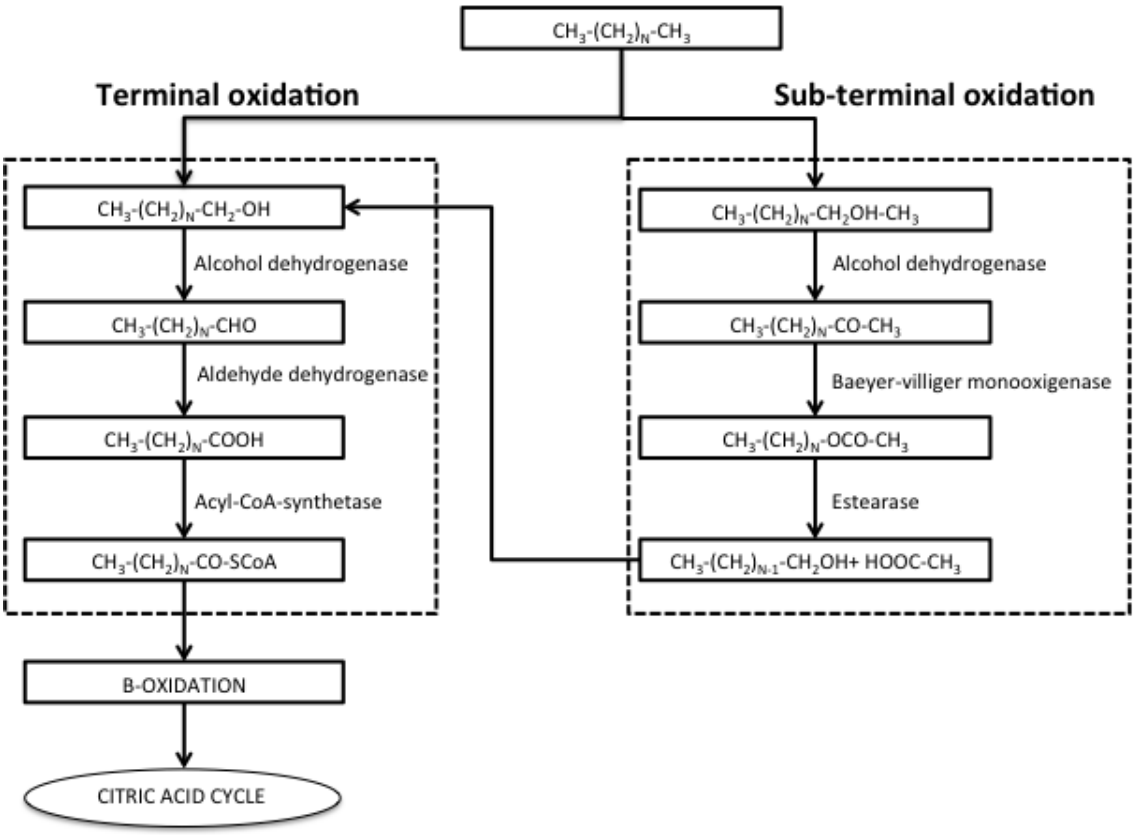

Figure 2-3. Metabolic pathway for the biodegradation of linear hydrocarbons

2.2.3 Impact of biodiesel addition to a diesel storage tank on the ecosystem characteristics

The microbiology of diesel storage tanks, as was already mentioned in this review, is a topic that has been explored in the last 20 years. An area that has not been so well explored is the effect of biodiesel addition on the characteristics of this ecosystem (13, 14, 19–21). Biodiesel is a mixture of methyl esters of fatty acids obtained by trans-esterification of vegetable or animal fat (22). Chemically is a less heterogeneous mixture compared with regular diesel, and it has been claimed that it is more readily available to use by microorganisms because it is already oxidized. Besides, biodiesel has a water holding capacity from 10-25 times higher than regular diesel (23, 24), which implies that pure biodiesel tanks are more prone to develop environments with the necessary conditions to favor microbial growth.

It is expected that introduction of this new carbon source would have an effect on the characteristics of the ecosystem. Changes in at least four variables have been reported as a consequence of biodiesel addition in fuel storage tanks: the rate of growth of microorganisms, the composition of the community, and the metabolism of microorganisms.

The ecology of diesel storage tanks in relation to biodiesel addition is still a new field of research and not many reports are available, reasons for which some of the conclusions have to be borrowed from studies performed in environmental studies devoted to the evaluation of biodiesel biodegradability. Although the methods used in these two kinds of studies are similar the microorganisms present and the physical conditions are different.

2.2.3.1 Effect of biodiesel addition to a diesel storage tank on the growth of microorganisms

The effect of biodiesel on growth characteristics of microorganisms will depend both on the strain and the concentration of biodiesel into the fuel storage tank. The response of a microorganism will depend on the species and metabolic capabilities of the strain; different strains can adapt differently to the presence of biodiesel. If they have preference for methyl-esters instead of hydrocarbons it is likely that an increase in the growth rate is observed when biodiesel is added to the culture media. The concentration of biodiesel is another important factor that has to be considered. A microorganism that is feeding on the aromatic portion of diesel might not be affected by addition of lower concentrations of biodiesel; however, in pure biodiesel will not be able to grow.

When biodiesel effect is evaluated in complex communities rather than in individual microorganisms the outcome will depend on the metabolic capabilities of the community as a whole and on the kind of microorganisms that would benefit from the change in carbon source.

The reports available, both in pure cultures and in complex microbial communities are in agreement with this hypothesis, while in some cases biodiesel addition favors the growth of some microorganisms (25, 26) in some others the effect observed is a decrease of the

growth (13, 21). Other authors have observed that the effect of biodiesel addition on microbial growth can be described using a “U” shape, with maximum growth observed at 0% and 100% biodiesel concentrations (14).

2.2.3.2 Effect of biodiesel addition on the composition of microbial communities

Stability of a microbial community depends on several factors such as temperature, concentration of nutrients, pH among other. In diesel storage tanks the stability of the community can be affected by the presence of a different carbon source. Biodiesel is chemically different from regular diesel and chemically less diverse (it is a blend of methyl-esters of different fatty acids), this can have an impact on the kinds of microorganisms present in the community. The observed result will depend on the ability of microorganisms to switch their metabolisms to the use of hydrocarbons (19).

When biodiesel is added to a diesel storage tank four different outcomes can be expected in the microbial community: in the most unlikely scenario all microorganisms are able to switch their metabolism to biodiesel consumption and this change does not favor the growth of any particular group; in this case no effect on the composition of the community will be observed. In the second case the change in carbon source favors some microorganisms and some others are unable to switch their metabolism; in this case some microorganisms will flourish and some others will perish, this can be observed as a change in the dominant groups and the disappearance of some microorganisms. In the third case all microorganisms are able to switch their metabolism to biodiesel use, but some are actually better at using it as carbon source, in this case a change in the dominant groups of microorganisms will be observed. The final scenario is one in which no member of the community is actually able to switch the metabolism to methyl-ester usage, in this case no growth is observed.

Although no long-term studies on the microorganisms present in a biodiesel storage facility are available it is expected that microorganisms adapted to use methyl-esters rather than hydrocarbons will colonize this microenvironment. Unfortunately, there are

only three studies available in which the effect of biodiesel addition is studied on the composition of complex communities in diesel storage tanks; results presented in literature are contradictory which reveals the necessity of more research in this particular topic (14, 19, 27).

2.2.3.3 Effect of biodiesel addition on the metabolic activity of microorganisms

The metabolic activity of microbial communities depends both on its composition as well as on the metabolic pathways that microorganisms use to metabolize the carbon sources available in the medium. Effects observed are usually due to the presence of by-products result of the metabolism of the carbon sources available. Substitution for methyl esters (Figure 2-3) can lead to the acidification of the medium, due to the production of low molecular weight carboxylic acids (17). These products can promote corrosion reactions on the metallic infrastructure.

It is interesting to note that the addition of biodiesel to the storage tank can alter the metabolic capabilities of the community not only by having an effect on the composition of the microbial community but also by boosting co-metabolism phenomena in different substrates. It has been found in certain strains that the presence of an additional carbon source can boost the use of some substrates (28, 29).

The other important effect that can be observed as a consequence of the addition of new carbon sources is a modification in the ability of the microorganisms to form biofilms (30). It is known that the metabolism of microorganisms depends in an important way in the kind of carbons sources available. For example in *Pseudomonas* sp., it has been demonstrated that the presence of mono and diglycerides (these kinds of compounds are present in low concentrations in biodiesel) can have an impact on the rate of exopolysaccharide production (31), a group of molecules that mediates biofilm formation and surface colonization in many microorganisms.

2.3 Polyethylene biodegradation

Polyethylene is known for being a remarkably resistant polymer to degradation. Its chemical and biological inertness has fostered its application into various products from plastic bags and piping to the construction of fuel storage tanks. From an ecological point of view, the accumulation of plastic debris in the environment is a growing concern, as the rate of plastics product manufacture goes over 25 million tons per year the degradation of its resulting waste is a problem of global proportions (32). However, the study of degradation pathways of polyethylene is not only of interest because of its ecological impact. Polyethylene has become a critical material in the construction of key infrastructure to several industries, making its degradation and deterioration necessary to understand from the viewpoint of stability and integrity.

Degradation of polyethylene can be classified as abiotic or biotic, the former being defined as deterioration caused by environmental factors such as temperature and UV irradiation, while the latter is defined as biodegradation caused by the action of microorganisms that modify and consume the polymer leading to changes in its properties. It is important to highlight that although the damage to polyethylene is classified by only one of these two damage modes, in nature it is typical that both act cooperatively (33). The abiotic mechanisms of deterioration of polyethylene have been described extensively elsewhere (33), and so this review will instead be focusing on the biodegradation of polyethylene and mechanisms associated with this process. Biodegradation of polyethylene has been reported in a number of research studies published over the last 30 years; however, there is general agreement that the process under normal conditions is extremely slow (33–37). The microorganism usage of this polymer is physically limited by its insolubility in aqueous media, lack of functional groups to which microorganisms can attach, and high molecular weight (37). Although there is enough evidence that proves biodegradation of polyethylene there is still a lack of knowledge on the complete metabolic pathways involved in the process and in the structure and identity of all the enzymes involved. Only some advances have been made in this regard and even then the conclusions outlined require verification (38–41).

The present review will cover three different topics, the first being a comprehensive summary of the microorganisms reportedly involved with polyethylene biodegradation; secondly, the effects of these microorganisms on polyethylene properties will be presented; and finally an outline of the degradation process of polyethylene based on published literature will be discussed.

2.3.1 Microorganisms related to polyethylene degradation

Biodegradation of polyethylene is complex and not fully understood. In order to elucidate the potential mechanisms, two different strategies have been followed in the literature. In the first approach, degradation studies have been performed with isolated individual strains specifically able to degrade polyethylene (30, 38–40, 42–51). That approach has the advantage of using pure strains, which is a convenient way to investigate metabolic pathways or to evaluate the effect of different environmental conditions on polyethylene degradation. A disadvantage of this approach is that it ignores the possibility that polyethylene biodegradation can be the result of a cooperative process between different species. These limitations are avoided by the second approach, in which the use of complex environments and mixed communities is applied (32, 52–61). Table 2-5 summarizes some of the different microenvironments that have been employed to study polyethylene biodegradation using mixed and complex microbial communities. Marine water, soil sediments or compost are examples of the environments whereby polyethylene has been investigated under the second approach.

Table 2-5. Different microenvironments used in the study of polyethylene biodegradation

Microenvironment	Reference
Marine exposure conditions	(52–54)
Soil burial conditions	(32, 55–61)
Composting conditions	(59)

The structure of a microbial community isolated on a polyethylene surface during biodegradation experiments can also be influenced by the type of polymer used as substrate. In several studies it has been proven that the physicochemical nature of a surface determines the ability of microorganisms to form biofilm structures (31, 62–64).

The most common polyethylene types are: Low Density Polyethylene (LDPE), High Density Polyethylene (HDPE), Linear Low Density Polyethylene (LLDPE) and Cross Linked Polyethylene (XLPE). They differ in their density, degree of branching and amount of functional groups on the surface. It is important to highlight that polyethylene can be also found mixed with additives such as pro-oxidants or starch (34, 65), both used to improve the degradability of the polymer. The presence of these additives can affect the kinds of microorganisms colonizing the surfaces of these polymers.

Over the past 50 years, a number of strains have been identified for their ability to interact with polyethylene causing some kind of deterioration, this has been done based on the two approaches mentioned before, and using different kinds of polyethylene. Tables 2-6 and 2-7 present an extensive list of the microorganisms that somehow have been related with polyethylene colonization, biodegradation or both. This list has to be approached carefully because in some studies not all the tests required to prove polyethylene biodegradation has been performed. The biodiversity of microorganisms able to degrade polyethylene is so far limited to 17 genera of bacteria and 9 genera of fungi; however, these numbers are likely to increase based on the more sensitive isolation and characterization techniques based on sequencing of rDNA. This technology allows a broader approach to assessing the composition of a community, including the non-culturable fraction of microorganisms that is invisible by traditional microbiology methods yet that constitutes up to the 90% of the real biodiversity in an ecosystem (66).

Table 2-6. Bacterial strains associated with polyethylene biodegradation.

Genus	Species	Reference
Acinetobacter	*baumannii*	(60)
Arthrobacter	spp.	(45, 67)
	paraffineus	(44, 68)
	viscosus	(60)
Bacillus	*amyloliquefaciens*	(60)
	brevies	(69)
	cereus	(60, 67, 70, 71)
	circulans	(69)

	halodenitrificans	(71)
	mycoides	(60, 72)
	pumilus	(60, 67, 71)
	sphericus	(70, 73)
	thuringiensis	(60)
Brevibacillus	*borstelensis*	(51)
Delftia	*acidovorans*	(74)
Flavobacterium	spp.	(74)
Micrococcus	*luteus*	(60)
	lylae	(60)
Microbacterium	*paraoxydans*	(43)
Nocardia	*asteroides*	(48, 75)
Paenibacillus	*macerans*	(60)
Pseudomonas	spp.	(40, 45, 50)
	aeruginosa	(43, 74)
	fluorescens	(60)
Rahnella	*aquatilis*	(60)
Ralstonia	spp.	(74)
Rhodococcus	*ruber*	(39, 42, 47)
	rhodochrous	(48, 49, 75)
	erythropolis	(74)
Staphylococcus	*epidermidis*	(76)
	cohnii	(60)
	xylosus	(60)
Stenotrophomonas	spp.	(74)
Streptomyces	*badius*	(38)
	setonii	(38)
	viridosporus	(38)

Table 2-7. Fungal strains associated with polyethylene biodegradation

Genus	Species	Reference
Acremonium	*kiliense*	(58)
Aspergillus	*niger*	(30, 77, 78)
	versicolor	(58, 79)
	flavus	(48, 80)
Chaetomium	spp.	(81)
Cladosporium	*cladosporioides*	(48, 75)
Fusarium	*redolens*	(55, 56, 58)
Gliocladium	*virens*	(78)
Mortierella	*alpina*	(48)
Mucor	*circinelloides*	(80)
Penicillum	*simplicissimum*	(46)
	pinophilum	(30, 78)
	frequentans	(72)
Phanerochaete	*chrysosporium*	(32, 78, 82)
Verticillium	*lecanii*	(58)

2.3.2 Effect of microbial activity on polyethylene

Microorganisms able to colonize the surfaces of polyethylene substrate have been reported to have diverse effects on its properties; seven different characteristics are

usually monitored for change in order to establish the extent of biodegradation of the polymer: functional groups on the surface, hydrophobicity/hydrophilicity, crystallinity, surface topography, mechanical properties, molecular weight distribution and mass balance. Table 2-8 summarized the main changes observed on polyethylene after microbial attack and the main techniques used to follow these changes. It is important to highlight that modifications to surface chemistry are evidence of interactions by microorganisms with the surface; however, more conclusive evidence of polymer degradation can be obtained when polymer consumption is determined over the course of experiments. So far there have been no studies in the literature that prove incorporation of polyethylene's carbon into a microorganism's macromolecular structure such as its DNA or polysaccharides.

Table 2-8. Changes observed on polyethylene surfaces after treatment with microorganisms

Changes observed	**Techniques used**	**Property measured**	**Reference**
Functional groups on the surface	FTIR	Keto-carbonyl index (I1715/I1565)	(30, 32, 39, 44, 45, 47, 49, 51, 54, 56, 59–61, 68, 70, 75, 77, 78)
		Ester-carbonyl index (I1740/I1465)	(45, 54, 56, 61, 70)
		Vinyl-bound index (I1640/I1465)	(30, 45, 46, 54, 56, 61, 70, 77, 78)
		Double bound index (I908/I1465)	(32, 45–47, 56, 59, 61, 68, 70, 77)
		C-O stretching (I1100)	(67)
Hydrophobicity / Hydrophilicity	Contact angle	Contac angle with water	(70, 71)
		Surface energy	(54)
	Drop deposition	Diameter of a drop	(53)

Crystallinity	FTIR	% Crystallinity	(45, 70)
	DSC	% Crystallinity	(30, 39, 44, 68, 77)
		Melting temperature	(30, 70, 78)
		Relative crystallinity	(78)
		Lamellar thickness	(30)
	XRD	% Crystallinity	(30, 44, 82)
		Lamellar thickness	(44)
Molecular weight distribution	HT-SEC/GPC	Molecular weight distribution	(38, 44, 46, 48, 49, 59, 68, 75)
	Rheology	Molecular weight distribution	(51)
Surface topography	SEM	Topography	(30, 42, 44, 47–50, 57, 59, 60, 75, 78, 80)
	AFM	Topography	(50, 54, 70)
Mechanical properties	Instron	Tensile strength	(38, 50, 52, 60, 70)
		Strain energy	(38)
		% Elongation	(32, 38, 60, 70)
		Ultimate extension	(52)
		Maximum load	(70)
Consumption of the polymer	Gravimetric	Weight loss	(42, 50, 51, 54, 60, 70)
	CO_2 evolution	Weight loss	(55, 56, 58, 72, 80)

2.3.2.1 Functional groups on the surface

The nature and amount of functional groups on the surface of polyethylene substrate is usually studied by FTIR spectroscopy. In the analysis of the polymer's spectral information special emphasis by researchers has been placed on the following functional groups: carbonyls (1715 cm^{-1}), esters (1740 cm^{-1}), vinyls (1650 cm^{-1}) and double bonds (908cm^{-1}). Literature studies concur that changes in these groups are common whenever biological activity on the surface of a substrate has been detected (30, 32, 39, 44, 45, 47, 49, 51, 54, 56, 59–61, 68, 70, 75, 77, 78). In general, it is accepted that in the presence of microorganisms the concentrations of these surface functional groups will decrease,

which is commonly reported as a decrease in the keto, and ester carbonil indexes (47, 51, 61, 77, 78). The other common finding in the literature is that there should be a corresponding increase in the number of double bonds in the presence of microorganisms (45, 47, 60, 77, 78, 83). However, these results have not been universal and some reports have stated that after incubation with microorganisms there will instead be an increase in ketonic groups (30, 45, 60) or a reduction in the number of double bonds (46, 54, 70).

Although the FTIR findings discussed might seem contradictory at first glance, they reveal the degradation of polyethylene to be a complex process that can differ for different microorganisms and different communities. While some microorganisms might have evolved to produce enzymes able to oxidize long polyethylene chains, other microorganisms might only be able to use the already oxidized polyethylene. What is certainly true is that incubation with microorganisms generates changes in the concentrations of functional groups at the surface of a polyethylene substrate either because of their consumption or production. In a complex microbial community in which also abiotic factors are affecting the chemistry of the polymer the net effect observed (accumulation or consumption of functional groups) will depend on the balance of rates of oxidation and degradation, which in turn will depend on the nature of the microorganisms present.

The study of the chemistry of polyethylene surface turns out to be very important, both because oxidized groups are more easily degraded by microorganisms (44), implying this that polyethylene degradation will be boosted if a more oxidized surface is used and also because they modulate microbial attachment by increasing the hydrophilicity of the surface (50).

2.3.2.2 Hydrophobicity/Hydrophilicity

The hydrophobicity/hydrophilicity of a surface depends on the kind, concentration and exposition of the functional groups present in the material. In polyethylene degradation two phenomena can be observed depending on the relation of oxidation and consumption of oxidized groups by microorganisms. If the rate of oxidation processes (due to the action of abiotic factors such as UV light or activity of enzymes) is higher than the rate of

consumption of functional groups then an increase in the hydrophilicity will be observed. Conversely, if the rate of consumption of functional groups is higher than the rate of oxidation then an increase in the hydrophobicity will be observed. Hydrophobicity is an important property of the surface in biodegradation studies, because the relation between surface and microorganisms hydrophobicity will determine the extent of colonization on the polymer substrate. In general, it is accepted that more hydrophobic surfaces are more easily colonized by microorganisms (31, 62–64).

Hydrophobicity is usually determined based on the contact angle of the surface with a probe liquid such as water, the more hydrophilic the surface the smaller the contact angle with water (70, 71). A more advance approach to study hydrophilicity of surfaces is the use of Young-Dupré equation (equation 1), which allows the estimation of the energy of adhesion to the solid as well as its acid (γ_S^+), basic (γ_S^-) and Van der Waals (γ_S^{LW}) components (54).

2.3.2.3 Crystallinity

Polyethylene is a semi-crystalline polymer comprised of crystalline microstructures which are processing history-related, and that are surrounded by amorphous regions. It is generally accepted and it has been corroborated experimentally that amorphous regions are consumed first because it is thought they are more accessible to microorganisms. Experimentally this is observed as an initial increase in the crystallinity percentage due to consumption of amorphous portions (30, 39, 44, 70, 77, 82). Yet there is insufficient research to date to state definitively what happens after the amorphous regions are consumed. Nevertheless, it has been proposed that once the accessible amorphous regions have been depleted microorganisms will progress to consuming the smaller crystals present (82), resulting in an increase in the proportion of larger crystals (30, 44, 70, 82).

2.3.2.4 Molecular weight distribution

One of the main limiters to polyethylene biodegradation is its high molecular weight. One common effect observed after microbial attack is an increase in the average molecular weight as a result of consumption of the lower molecular weight chains (38, 39, 46, 51). This result however is not universal, with some authors only observing a slight if any

change in the molecular weight distribution (49, 75). Some others have concluded that the main factor affecting the molecular weight is the exposition of abiotic factors such as UV irradiation rather than direct microbial attack (49). Some results showing the extent of reduction based the number-average molecular weight (M_n) of polyethylene samples are presented in Table 2-9.

Two different approaches have been used for the determination of molecular weight distribution, the most common one being the use of size exclusion chromatography techniques at high temperature (38, 44, 46, 48, 49, 59, 68, 75). The other possibility is the use of rheological measurements that correlate indirectly with the molecular weight distribution (51).

Table 2-9. Changes in molecular number due to microbial activity in different studies

Substrate	**%Δ Molecular number (M_n)**	**Reference**
LDPE UV irradiated	-34	(51)
LDPE	-15	(39)
LDPE + Starch	-17	(38)

2.3.2.5 Surface topography

Colonization of polyethylene surfaces by microorganisms usually generates changes in the surface topography as have been proven extensively in different research papers. Development of micro-colonies of different microorganisms on the surface of the polymer (42, 47–50, 75, 80) as well as penetration of hyphal structures (30, 77, 82) have been reported as common features after microbial attack. Evidently surface topography will be modified by microbial colonization, but the real question is how the topography is modified if the microorganisms are removed, in other words is it possible to observe cracking and pitting in the polymer surface after biodegradation processes? The answer to this question has not been thoroughly addressed, even though there is enough evidence which proves that some superficial damage will be observed after polyethylene surfaces have been exposed to biodegradation (57, 60, 69).

2.3.2.6 Mechanical properties

Most of the studies on polyethylene biodegradation have focused on thin films, with results showing in this form of substrate that deterioration of the mechanical properties such as breaking load is common. Oxidation induces changes in crystallinity and in the average molecular weight that lead to modification of the mechanical properties. Table 2-10 presents results showing changes in different mechanical properties for polyethylene after biodegradation. The results presented correspond to pure polyethylene not submitted to oxidation treatments before the biodegradation experiments.

Table 2-10. Changes in mechanical properties due to microbial activity in different polyethylene samples

Substrate	Environment	Time	%Δ elongation	%Δ Tensile strength	Reference
LDPE	Waste coal	225	+4%	-16.4	(60)
	Forest soil	225	-4%	-16.4	(60)
	Crater soil	225	-1.5%	-19.5	(60)
	Sea water	365	-12%	-15	(52)
	Sterile sea water + *B. sphericus*	365	+2.7%	-3.8	(70)
	Mineral media + *Pseudomonas* sp	45	NR	-30	(70)
HDPE	Sterile sea water+ *B. sphericus*	365	+8.9	-9.7	(70)

Although rheological analysis can be performed to determine the storage and loss modulus of the polymer, in biodegradation studies authors have been preferred the use of a universal mechanical testing system (UMTS) for determination of mechanical properties of a polymer specimen (52, 60, 70).

The effects of biological activity on polyethylene samples have been studied mainly in thin films; however, thick walls are also a very common application of this polymer in the manufacture of tanks. Therefore the changes in the mechanical properties due to

microbial activity are still an active area or research. Nevertheless, it is likely that microorganisms' effect will only be superficial in that case.

2.3.2.7 Consumption of the polymer

The consumption of a polymer is relevant evidence of that polymer's usage by microorganisms; however, the slowness of that process can make it very difficult to detect. Nevertheless, some studies have reported a reduction in the weight of samples determined either by gravimetric measurements (42, 50, 51, 54, 60, 70) or by CO_2 evolution from the samples (55, 56, 58, 72, 80). Results in weight reduction have to be read with special care when polyethylene mixed with starch is used; in this case initial reduction in weight can be due to starch consumption rather than polyethylene usage. Table 2-11 presents the main results obtained for the extent of biodegradation found in different polyethylene types prepared without any oxidative treatment.

It is important to note that the rate and extent of polymer consumption can be extensively influenced by abiotic factors that promote oxidation. Albertson et al (56) proved that biodegradation rate can increase from 0.2% to 8.4% by irradiating the samples with UV light before biotic treatment.

Table 2-11. Weight loss percentage due to biological action in different polyethylene kinds non-subjected to pre-oxidation treatment

Substrate	**Environment**	**Time**	**% Of weight loss**	**Reference**
LDPE	Waste coal	225	-0.26	(60)
	Forest soil	225	-0.13	(60)
	Crater soil	225	-0.28	(60)
	Sea water	365	-1.9	(54)
	Soil + *Fusarium redolens*	3650	-0.2	(56)
	Soil	800	-0.1	(55)
	Mineral media +	56	-7.5	(42)

	Rhodococcus ruber			
	Mineral media + *Rhodococcus ruber*	30	-2.5	(39)
	Mineral media + *Brevibacillus borstelensis*	30	-2.5	(51)
	Mineral media + *Pseudomonas* sp.	45	-5	(50)
HDPE	Sea water	365	-1.6	(54)
	Soil	800	-0.4	(55)

2.3.3 Mechanisms of polyethylene biodegradation

The mechanisms of biodegradation for polyethylene can be studied from three different perspectives: colonization of the polymer by microorganisms; chemical/biochemical reactive pathways; and the impact of macromolecular structure of the polymer on microbial usage.

Polyethylene is a hydrophobic, high molecular weight molecule, and therefore it is commonly accepted that biofilm colonization is the initial step for degradation of this polymer (47). Biofilms are sessile communities of microorganisms developed on a surface that can be composed of individuals from the same or different species (31). Complex biofilm communities comprised of different microorganisms have been detected on polyethylene surfaces once they were exposed to different biotic environments (32, 42, 44–47, 50, 51, 53, 60, 67, 74, 76, 83–85). Studies on microorganism attachment to polyethylene have identified that the main limitation of the colonization process is the relatively high hydrophobicity of the polymer in contrast to the regularly hydrophilic surfaces of most microorganisms (47, 50). It has been proposed that strains with more hydrophobic surfaces can play an important role in the initial colonization of the polymer. The other metabolic adaptation that can be important in polymer colonization is the production of surfactants, molecules that can mediate the attachment process of microorganisms to the hydrophobic surface (50, 58).

Theoretically, polyethylene can be used as a carbon source for microorganisms similar to many other hydrocarbons; however, its high molecular weight is a limitation for enzymatic reactions to take place. In terms of the chemical/biochemical processes involved in polyethylene biodegradation it can be stated that there are two key reactions, the first one being the reduction of its molecular weight and the second being the oxidation of the molecules. Reduction of molecular weight is required for two reasons, firstly to enable transport of molecules through the cell membrane, and secondly because enzymatic systems present in the microorganisms are only able to attack certain molecular weights, usually in the range of 10 to 50 carbons, though there has been a report of enzymatic activity up to 2000 carbons (40). Once the size of the molecule is reduced, oxidation is required in order to transform the hydrocarbon into a carboxylic acid that can be metabolized by means of β-oxidation and the Krebs cycle (61). Figure 2-4 presents the proposed mechanisms of biodegradation for polyethylene.

Both oxidation and molecular weight reduction during the biodegradation process are a result of synergistic effects between biotic and abiotic factors (photoxidation or heat treatment). There are several papers reporting both the formation of carbonyl groups (oxidation) and reduction of molecular weight after treatment with UV light (48, 49, 56, 58, 61). The biotic factor is determined by groups of enzymes able to degrade oxidized or reduced polyethylene molecules. However, there are very few works devoted to studying the enzymes involved in this processes. Breaking down large polyethylene molecules can be accomplished by enzymatic action, as proven by Santo et al (39), who found that by incubation with the enzyme laccase the molecular weight of polyethylene was reduced and its keto-carbonyl index increased. These two factors indicated that both scission and oxidation reactions were taking place by the same enzyme. In regards to the oxidation process there was another important work, this one by Yoon et al (40), which isolated an alkane hydroxylase from the AlkB family that was active to polyethylene samples with molecular weights up to 27000 Da. It is interesting to note that enzymes of this family have been described as microorganisms that are able to degrade hydrocarbons. In general, it is accepted that alkane hydroxylase performs the first oxidation that leads to the subsequent degradation of a hydrocarbon (18).

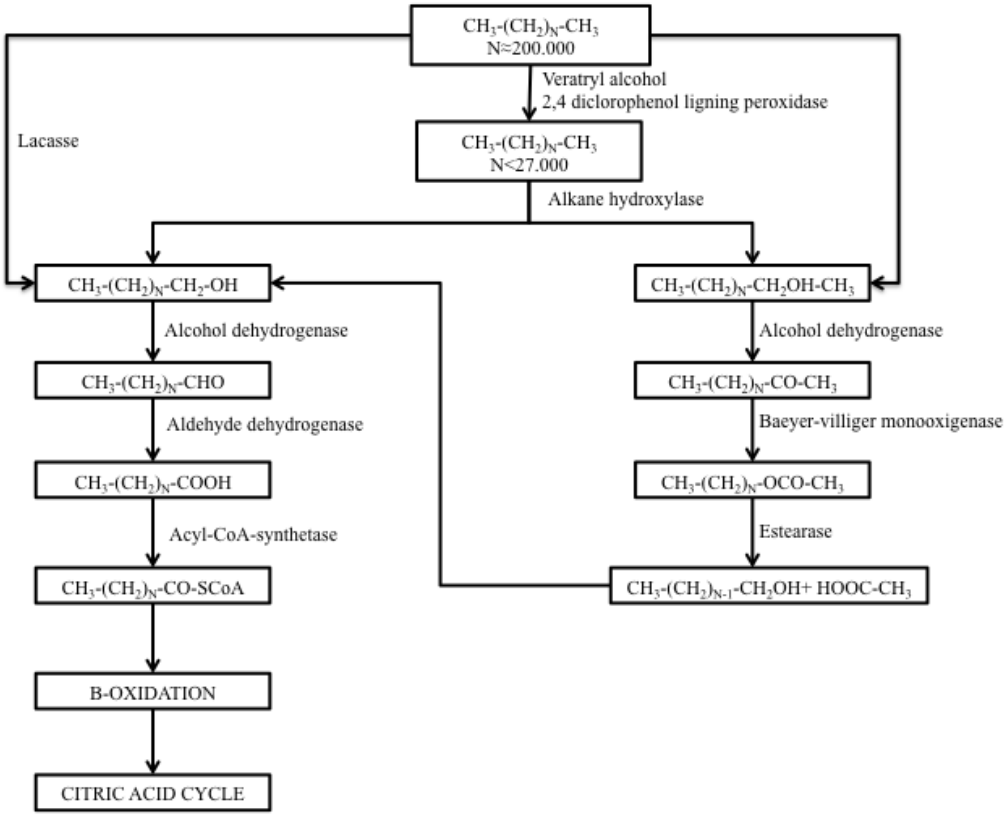

Figure 2-4. Hypothetical mechanisms of polyethylene degradation

2.3.4 Conclusions and perspectives

Research performed in polyethylene biodegradation, both using pure strains as well as complex microbial communities has proved that biodegradation of this material, although slow, is actually happening in nature. The rate of this process is modulated by the intensity and presence of abiotic factors such as temperature and UV light as well as by the physical and chemical properties of the polymer. Factors such as crystallinity, degree of oxidation and molecular weight distribution can have an important impact on the degree and rate of usage of the polymer by microorganisms.

Research performed so far is mainly of a descriptive nature, with a few works devoted to the study of polyethylene degradation mechanisms or the isolation of enzymes related to this process. However, further evidence is required to conclude on the complete mechanisms of polyethylene degradation. It is likely that future works will use a more mechanistic approach to the problem of polyethylene biodegradation. Isolation and

identification of the enzymes able to oxidize and break polyethylene chains as well as the size of polyethylene chains that they are able to use as substrate is a primary goal to elucidate the mechanisms of degradation of polyethylene.

Another important area of research is the identification of the fate of polyethylene inside microorganisms, so far it has been suggested that it is metabolized by means of the tricarboxylic acid cycle (TCA), however isotopic marking has not been used to prove that this is actually happening.

The effect of microbial degradation on the morphology of the polyethylene it is only partially studied, it is known that amorphous regions are more easily degraded and that small crystals are likely used by microorganisms, however it is still unknown if highly organized crystalline regions would be also susceptible to microbial attack and at what rate.

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Chapter 3

3 Effect of Biodiesel Addition on Microbial Community Structure in a Simulated Fuel Storage System

Biodiesel is an alternative fuel that can replace diesel partially or completely. It is produced by trans-esterification of fatty acids with an alcohol (usually methanol) in the presence of a catalyst. A recent report indicated that its production increased 169% going from 326 to 878 million gallons between 2009 and 2011 in the United States alone (1). This increase in production has been driven by growing concerns on the stability of petroleum supply as well as by volatility of the price of crude oil. This trend has led to a transition for the diesel industry from 100% diesel to blends at different ratios with biodiesel. In Europe, the objective is to reach a 10% replacement by 2020 (2) and in Canada there is already a requirement for a 2% blend.

Research on the effect of biodiesel on microbial community structure has been mainly focused in biodegradation of biodiesel, with an emphasis on bioremediation (3–5). However, there is little reported research on the effects that biodiesel may have on the microbial communities developed in storage and transport infrastructure. The few studies available do not present conclusive evidence on the effect of biodiesel over microbial community structure (2, 6–8). Some authors have found either by denaturing gradient gel electrophoresis (DGGE) and plating techniques that the dominant groups of microorganism in a fuel system were influenced by biodiesel addition (2, 7), while some others using quantitative PCR of selected groups found that a consortium isolated from soil remains stable independently of the biodiesel concentration used as carbon source (6). This lack of agreement in results can be explained if it is considered that the source of the communities used in these studies was different, whereas the first one used a community obtained from diesel storage tank, the second one used a more diverse soil community. Although the results can be community dependent the question for the effect of biodiesel on microbial community structure remains open for discussion and more research is necessary in order to gain a deep insight of the problem.

Microbial communities are usually developed at the bottom of fuel storage tanks due to the presence of moisture, which accumulates as a product of condensation when temperature drops (7). This microenvironment is well suited for growth of microorganisms able to use either olefins or aromatics as carbon sources. Presence of biodiesel in storage tanks may enhance microbial activity and lead to a change in ecosystem composition. An increase in microbial activity once biodiesel is added is expected because its higher bioavailability and hygroscopicity (2). An impact on the kind of microbial communities is also expected because blending of diesel and biodiesel changes the chemical nature of the fuel.

Typically, microbial communities can be studied by using three different strategies: culturing of microorganisms in selective media, analysis of community level physiological profiles (CLPP) or thirdly, the analysis of data from the 16/18 sRNA gene generated by PCR using universal primers. Culture in selective media is a classic approach; however, it has the limitation that only 1-5% of the microorganisms present in the environment can grow in synthetic culture media (9), and so the results obtained are skewed to microorganisms able to grow in the culture media used. CLPP is a technique that uses a microplate containing a number of different carbon sources, microbial communities are inoculated directly in the plate and differences between them are determined based in the pattern of carbon utilization. Although CLPP is also skewed for microorganisms able to growth in culture it has proved to be very effective for studying shifts in microbial communities as a result of ecological perturbations (10); however, the technique does not give an indication of the kind of microorganisms that are present in the community. Finally, amplification of 16 sRNA sequence has been used as a strategy to identify the kind of microorganisms present in a community. PCR products can be sequenced or run in a denaturing gel to generate a fingerprinting. This approach is very labour intensive nevertheless it is useful when the identities of the microorganisms are required. Tracking changes in fuel storage tanks will require the simultaneous use of these techniques in such a way that the maximum amount of metabolic and genetic information is obtained and relevant conclusions can be made both in for the composition and metabolic capabilities of the community under study.

In this work a simulated fuel storage tank (mesocosm) is used to study the effects of biodiesel addition on microbial structure and function in a community obtained from a diesel storage facility. Changes and evolution of the microbial community were tracked with a combined strategy using the three approaches described above: community level physiological profiling, construction of libraries for the 16s RNA gene and culturing in selective media. Growth and pH were also followed during the course of the experiment.

3.1 Materials and methods

3.1.1 Experimental set up

A mesocosm was designed to simulate the bottom of a fuel storage tank in which an excess of water was accumulated as product of condensation. An Erlenmeyer flask (500 mL) was prepared containing 50 mL of Richard and Vogel's culture medium (All chemicals were obtained from Sigma-Aldrich, St. Louis, USA) pH 7 (11), 50 ml of a water sample collected from the bottom of a tank used for diesel storage (2L of water were collected during the spring of 2012 by Imperial Oil Ltd in their operation facilities in Sarnia, ON) and 100 mL of a diesel/biodiesel blend. Two immiscible phases were developed: one containing water and hydrophilic compounds, and the top one containing the less dense fuel. Mixtures with 0%, 25%, 50%, 75%, 100% (v/v) of biodiesel were evaluated. Experiments were carried out for 200 days continuously, microbial growth and composition were analyzed. Samples were prepared in triplicate.

The Richard and Vogel's medium is a mixture of mineral salts that fulfill minimum requirements of nitrogen, phosphate and microelements; the only carbon source available was the fuel blend that diffuses to the water layer. The water sample that was added contained an inoculum with a real microbial community metabolically adapted for the use of diesel as a carbon source. The system was kept in darkness at 25°C without aeration; however, oxygen diffusion was allowed. Fuels were sterilized before the experiment by means of a 0.2 μm filter (Millipore, Billerica, USA,), and the culture medium was sterilized at 15 psi and 121°C during 15 minutes.

3.1.2 Properties of the water layer

Microbial growth was measured as an increase in the optical density at 590 nm of the water layer. Tests were performed with a plate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland) at 0, 50, 75, 100 and 200 days of storage. Acidity of the water layer was determined with a pH meter (Phi 40 pH meter, Beckman Coulter Inc., Indianapolis, USA) at 50, 75, 100 and 200 days. Statistical analysis was performed using a mixed model (included analysis of fixed and random effects) using the statistical package SAS Version 9.3 (SAS Institute INC., Cary, USA). The structure of variance was chosen among a compound symmetric, unstructured or autoregressive by using the bayesian information criteria (BIC). An ANOVA for the treatments was performed to evaluate the significance of both simple effects and interactions. A t-test ($p<0.01$) was used for comparison between treatments.

3.1.3 Microbial counts

Colony forming units were determined for bacteria and anaerobes in selective culture media. Decimal dilutions from 10^{-1} to 10^{-8} of the communities were prepared in peptone water (0.1g/L) pH 7. Bacterial counts were determined by plating in agar “Plate Count” pH 7 (Sigma-Aldrich, St. Louis, USA) after incubation at 30°C for 48 h. Anaerobes were determined by plating in agar “Wilkin-Chalgren” pH 7.1 (Hmedia, Mumbai, India) after incubation at 25°C for 120h (12); anaerobic conditions were obtained by means of oxygen capture bags in a sealed jar. Culture media were sterilized by autoclaving at 121°C, 15 psig for 15 minutes. Statistical analysis was performed as described in the preceding section.

3.1.4 Community level physiological profiling (CLPP)

CLPP was performed every 25 days during the first 100 days of the experiment for communities developed in the water layer. Three replicates of each biodiesel concentration were analyzed. For the experiment 96 well plates (ECOPLATE, Biolog Inc., Hayward, USA) were used. These plates contain 31 different carbon sources and a blank. Each well was inoculated with 150 µL of the undiluted community (after

incubating for 0, 25 and 50 days) or a 1/5 dilution of the community (after incubating for 75, and 100 days). The dilution at higher storage times was necessary to avoid color development in the blank. Plates were incubated at 25°C and absorbance readings at 590 nm were performed approximately every 8 hours over a 96 hour period in a microplate reader (Tecan Group Ltd., Seestrasse, Switzerland).

Data were transformed for principal component analysis using Taylor power law in order to improve the normality and homogeneity of the variance (13). The "b" value in the transformation was calculated in such a way that the ratio between the highest and the lowest variance was as close to unity as possible. Normality, homoscedasticity and the number of significant linear correlations were calculated as in Weber et al (13). Principal component analysis was performed using the statistical package R version 2.15.2 (R Foundation for Statistical Computing,Vienna, Austria). A single point with the same average well color development (AWCD) (AWCD=0.45 for storage times of 25 and 50 days and AWCD=0.26 for storage times of 75 and 100 days) was chosen for analysis and comparison between communities instead of a single point in time in order to avoid bias due to inoculum density (14).

Substrate richness, defined as the number of wells with a corrected absorbance greater than 0.25 (10), was calculated every 25 days for all of the communities studied. In order to study the divergence of the microbial communities compared to the initial community a one-dimensional metric was used as suggested by Webber and Legge (15). In their approach the Euclidean distance between two multidimensional data sets is used as a measurement of the degree of divergence between two communities. Distances were calculated between data sets at which the AWCD was 0.25.

3.1.5 16s rDNA sequencing

Three libraries for 16s DNA gene were generated for the microbial communities developed within the water layer of the mesocosm studied: one for the undisturbed community at the beginning of the experiment (106 clones accession numbers KF135678 - KF135783) and two for communities obtained from the water layer in the experimental system at 0% (90 clones Accession numbers KF135784 - KF135873) and 100% biodiesel

(82 clones Accession numbers KF135874 - KF135949) after 100 days of exposure to the fuel.

Power Water Kit (MoBIO, Carlsbad, USA) was used for DNA extraction following the manufacturer instructions. Briefly, microorganisms were collected by filtration of 50 ml from the water layer (0.2 μm) (Millipore, Billerica, USA,). Then a lysis step by bead beating was followed by purification and elution using a silica column. DNA was quantified by spectrophotometry at 280/260 nm using the Nanoquant system (Tecan Group Ltd., Seestrasse, Switzerland) and a plate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland). Universal primers specific for amplification of a 1029 pb region of the 16 sRNA gene of bacteria were designed using ARB software Release 5.5 (The ARB project, Munich, Germany). Bacteria coverage of the primers based on Silva database sequences was calculated and maximized.

PCR was performed using a touchgene gradient thermocycler system (Techne, Staffordshire, England). The thermal cycle consisted of an initial denaturation at 94°C followed by 35 cycles of denaturation at 94°C for 60 seconds, annealing at 60°C for 45 seconds and extension at 72°C for 70 seconds. A last extension step at 72°C for 8 minutes followed. The reaction mixture consisted of MgCl2 3 mM, 0.2 mM of each deoxynucleoside triphosphate (Life technologies, Carlsbad, USA), 0.3 μM of each primer (Forward 5'-ACTCCTACGGGAGGCAGC-3' and Reverse 5'-CCCGGGAACGTATTCACCG-3 with matching efficiencies for bacteria of 75% and 46% respectively) and 1 unit of AmpliTaq 360 DNA polymerase (Life technologies, Carlsbad, USA). In order to avoid false positives caused by residual DNA (16) reaction mixtures were treated before template and primers addition with 0.1 units of DNAse (Sigma-Aldrich, St. Louis, USA) for 30 minutes at 37°C followed by an inactivation step at 90°C for 50 minutes. Negative controls were analyzed for all reactions.

PCR products were visualized and purified using the flash gel recovery system (Lonza, Basel, Switzerland). Ligation and cloning of PCR products were performed using pGEN easy cloning system following the instruction of the manufacturer (Promega, Fitchburg, USA). Plasmid extraction was done using a PureLink® Quick Plasmid Miniprep kit (Life

technologies, Carlsbad, USA). Products were sequenced in the Robarts Research Institute (London, Canada). Processing of the sequences for removal of vector residues and quality control was performed using a trial version of Sequencher Software Version 5.0.1 (Gene Codes Corporation, Ann Arbor, USA) and eBiox Version 1.5.1 (Pleasanton, USA). Sequences with less than 700 pb were removed from the libraries as well as sequences with a quality index lower than 80%. Libraries were compared for genus biodiversity using the Libcompare function of RDP release 10 (Michigan State University, East Lansing, USA) (17). For all the libraries Shannon diversity index was calculated. The results in the libraries were analyzed by principal component analysis, each genus was identified with one variable, and each library was represented in a plane that has the two most important principal components as axes.

3.2 Results and discussion

3.2.1 Properties of the broth

Growth was measured as an increase in absorbance in the water for each of the treatments (Figure 3-1). Statistical analysis revealed that there were no significant differences among treatments during the first 100 days of the experiment. At 200 days significantly higher growth ($p<0.01$) was detected in samples using pure biodiesel as a carbon source. Growth was observed both in the water layer and in the interphase of the fuel as a dense mat. This mat was dispersed in the culture media before absorbance measurements in order to account for all microbial activity in the system. Analysis of the growth kinetics (Figure 3-1) showed that all treatments but the biodiesel started their stationary phase of growth around 100 days after the beginning of the experiment.

Although growth measured by absorbance of the broth is an indirect measurement of microbial activity on the water layer, diesel and biodiesel are highly insoluble in water, and so the only source of new material in the water was the microbial activity of the community inoculated at the beginning of the experiment. Then any increase in the absorbance of the broth was due to metabolism rather than mass transfer from the fuel phase to the water phase. Higher growth in biodiesel samples coincides with results

presented by Bücker et al., 2011 who report higher biomass production for molds and fungi when pure biodiesel was used as a carbon source, as well as with the results obtained by Sørensen et al., 2011 which reported a higher growth in biodiesel samples measured by DAPI staining.

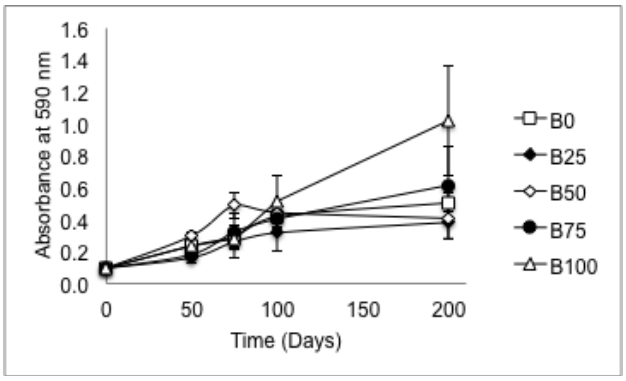

Figure 3-1. Microbial growth at different biodiesel concentrations during the 200 days of the experiment. Significant higher accumulation in pure biodiesel samples was observed at the end of the experiment.

Statistical analysis of pH trend (Figure 6) shows that there were significant differences among the treatments and that these differences were dependent on time. Acidification of the culture media was detected 75 days from the beginning of the experiment for samples growing in pure biodiesel and after 200 days for samples growing in 75% biodiesel. On the other hand, a slight alkalization of the culture media was observed after 100 days of culture for samples growing in pure diesel.

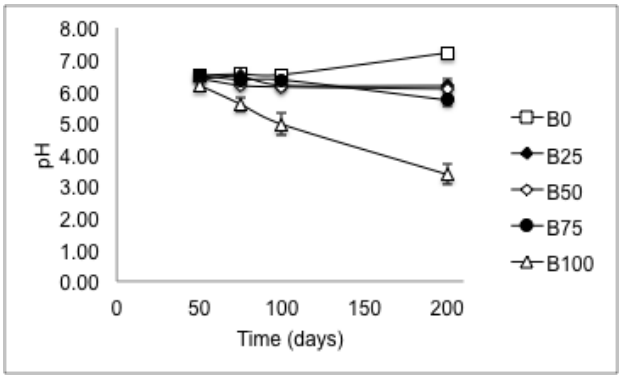

Figure 3-2. Change in the pH of the water layer during the 200 days of the experiment. Acidification of the water layer in pure biodiesel samples and alkalization of pure diesel ones was observed.

Acidification at higher biodiesel concentrations (Figure 6) was probably caused by the metabolism of the microorganisms. It is known that production of low molecular weight acids by some bacteria can lead to a pH drop in fuel systems (18). In this study this trend was especially clear for the group of samples containing pure biodiesel as a carbon source. A similar acidification trend was observed in pure diesel by Bento and Gaylarde, 2001. This kind of behavior is particularly important in terms of corrosion of metals and deterioration of the fuel. However, this behavior can be community dependent, which implies that future research work with communities obtained from different ecosystems is required to conclude if biodiesel addition in general leads to microbial communities with the ability to decrease the pH of water environments in fuel systems. A synergistic effect between bacteria and fungi could have happened in the pure biodiesel system; acid-producing bacteria could have caused a pH drop in the media that favored fungi and yeast development in later stages of the experiment (after 50 days). This hypothesis is supported by the microbial mats observed in the interphase of the system which is very characteristic of fungi growth, and it fits with the results of Schleicher et al., 2009 that found that in pure biodiesel the dominant community was composed mainly by yeast and fungi.

3.2.2 Microbial counts

Statistical analysis of both bacteria and anaerobes showed that there was a significant interaction ($p<0.01$) between the treatments and storage time, which implied that analysis for significant differences between treatments should be performed independently each time. The logarithm of colony forming units for mesophilic heterotrophic bacteria and anaerobes is presented in Tables 3-1 and 3-2; treatments with no statistical difference are marked with the same letter.

Since all experimental units were inoculated with the same community, initial microbial counts were identical. The logarithm of colony forming units (CFU) was 3.6 for bacteria and 4.1 for anaerobes at the beginning of the experiment in all experimental units. Microbial counts present a sharp increase during the first 25 days probably due to an excess of carbon source. For most of the storage times there were no significant

differences between microbial counts (both for aerobes and anaerobes) among samples growing in B0, B25 and B50. At higher biodiesel concentration a statistically significant decrease in microbial counts was observed. For samples growing in B75, a statistically reduction in microbial counts was found after 200 days, and in pure biodiesel samples (B100) this trend was evident after 50 days of storage.

Table 3-1. Logarithm of colony forming units of mesophilic heterotrophic bacteria growing in the water layer of the system at different biodiesel concentrations measured at different times during the course of the experiment

	Mesophilic heterotrophic bacteria (log10(CFU))				
	Time 25	Time 50	Time 75	Time 100	Time 200
B0	9.6±0.1 B	7.7±0.2 A	7.8±0.4 A	7.8±0.1 A	7.5±0.1 A
B25	10.1±0.2 A	7.6±0.3 A	7.3±0.3 A	7.7±0.2 A	7.8±0.3 A
B50	10.1±0.1 A	7.7±0.3 A	7.9±0.2 A	7.8±0.2 A	7.5±0.1 A
B75	10.2±0.1 A	7.5±0.3 A	7.9±0.1 A	7.4±0.7 A	2.2±3.8 E
B100	10.1±0.1 A	2.1±3.6 E	0.0±0.0 E	0.4±0.8 E	0.0±0.0 E

Table 3-2. Logarithm of colony forming units of anaerobes growing in the water layer of the system at different biodiesel concentrations measured at different times during the course of the experiment

	Anaerobes (log10(CFU))				
Blend	Time 25	Time 50	Time 75	Time 100	Time 200
B0	8.0±0.3 A	7.1±0.1 A	6.6±0.2 A	5.7±0.1 A	6.5±0.2 A
B25	8.1±0.1 A	6.7±0.3 B	6.5±0.1 A	5.7±0.2 A	6.1±0.4 A
B50	8.0±0.1 A	6.9±0.1 A	6.1±0.2 B	5.8±0.03 A	6.5±0.4 A
B75	8.0±0.1 A	7.0±0.2 A	6.6±0.1 A	5.7±0.4 A	5.0±0.3 B
B100	8.2±0.02 A	0.0±0.00 E	0.0±0.00 E	0.0±0.00 B	4.3±0.2 C

The main objective of the culturing experiments was to observe divergence in the microbial communities caused by the addition of biodiesel. Using this culture based approach with selective media for bacteria and anaerobes it was possible to determine some divergence in microbial communities due to biodiesel addition (Tables 3-1 and 3-2), however this could only be observed for samples at high biodiesel concentrations after at least 50 days of the experiment.

An important phenomenon arose in the present experiment, on the one hand a maximum increase in absorbance at 200 days was observed for samples obtained from systems with pure biodiesel as a carbon source (Figure 3-1) yet on the other hand, microbial counts both for anaerobes and bacteria went to zero or close to zero after 50 days (Tables 3-1 and 3-2). This result could imply that the kind of species that were predominant in the samples using pure biodiesel were not able to growth in the culture media used in the present study, it is common that microbial biodiversity is poorly represented in culturing techniques (9). A similar decrease in the number of culturable bacteria was obtained after acidification of the water layer in a fuel system by other authors (19). It has also been reported that aerobes and anaerobes colony forming units were very close to zero in systems using pure biodiesel as a carbon source, and significantly lower than in other biodiesel concentrations (20).

3.2.3 Community level physiological profiling

Principal component analysis of community level physiological profiles for samples of the water layer using different biodiesel/diesel blends and different storage times are presented in Figure 3-3. Samples clustering together based on their carbon consumption patterns are presented within rectangles or ellipses.

CLPP is claimed to be a very sensitive technique for clustering microbial communities based on carbon consumption patterns. The technique has received some criticism that questions the ability of the test to obtain relevant information to make conclusions on the metabolic capabilities of a community (21–23); however, in this work the tool has been used only to compare communities rather than to make inferences on the kind of metabolisms within the communities under study.

In the present study it was found after performing a principal component analysis of the CLPP that the original community diverged in different ways for samples growing at different biodiesel concentrations. This divergence was observed for samples after 25 days after the experiment has started (Figure 3-3). Different levels of clustering could be observed. For samples at 25, 50 and 75 days it was possible to group together samples obtained at the same biodiesel concentration (Figure 3-3-A, 3-3-B and 3-3-C). A second level of clustering was observed in all the experimental time points (Figure 3-3) where it was always possible to identify at least 3 clusters, one corresponding to samples obtained from pure biodiesel, one to pure diesel and one to samples obtained from mixtures of diesel and biodiesel.

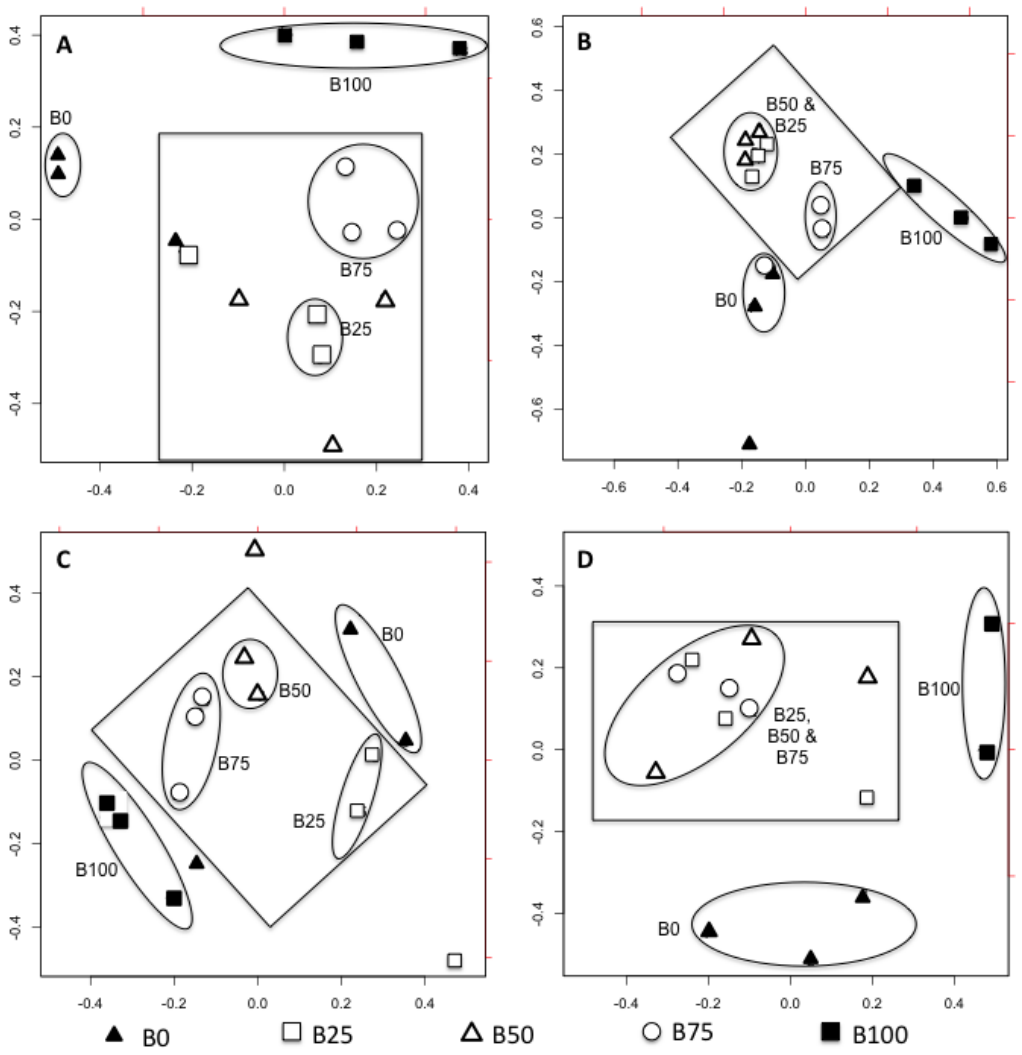

Figure 3-3. PCA for samples at 25 days (A), 50 days (B), 75 days (C) and 100 days (D)

Clustering of results suggest that the community diverged in different ways depending on the amount of biodiesel that was added in the fuel. Communities with different metabolic capabilities based on carbon consumption profiles were developed depending on the carbon sources available for microbial growth. This implies that the structure of the communities explored in this study was influenced by the addition of biodiesel in the fuel system.

Euclidean distance analysis, as suggested by Webber and Legge (15), showed that all the communities presented an initial period of fast divergence during the first 25 days, followed by a period of 50 days of recovery in which divergence (referring to the initial community) was slightly reduced, and finally 100 days after the beginning of the experiment a new increase in the distance was observed (Figure 3-4). At the end of the analysis period, samples in pure biodiesel presented the greatest extent of divergence compared to the initial community.

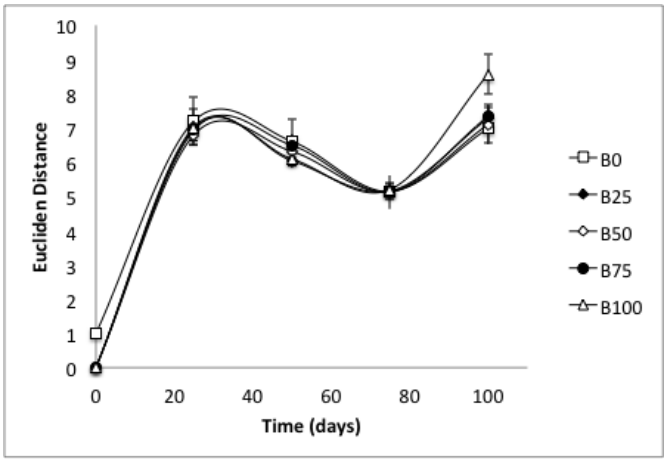

Figure 3-4. Euclidean distance between community level physiological profiles (CLPP) of the initial community and microbial communities at different times during the experiment

It is interesting to note that although communities were revealed to be different as shown by plating experiments and PCA analysis, the divergence measured as the Euclidean distance to the initial community followed very similar trends (Figure 3-4) for all the communities under evaluation. This implies that although the communities were diverging in a different way from the initial community the extent of this divergence was

similar for all biodiesel concentrations. Results presented in Figure 3-4 confirmed that communities in pure biodiesel presented the highest degree of divergence by the end of the experiment.

Data from CLPP were analyzed for substrate richness. Results are shown in Figure 3-5. All communities presented an initial period in which the richness decreases, followed by a recovery of their metabolic capabilities at different rates except for samples growing in pure biodiesel in which a very pronounced decrease in the ability to use the carbon sources present in the microplate was observed.

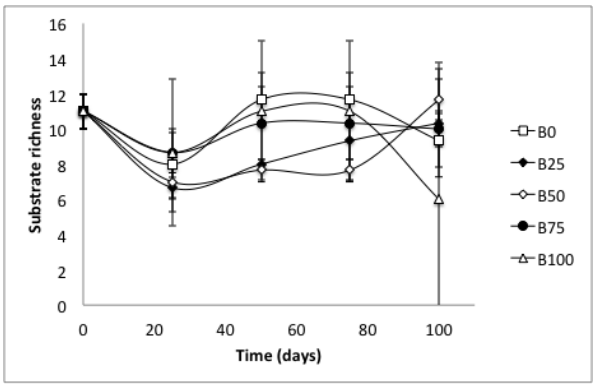

Figure 3-5. Number of carbon sources used by the communities based on CLPP patterns

Substrate richness for communities growing at different biodiesel concentrations presented a different trend in the recovery phase, which is evidence of the different community structure that was developed as a result of perturbation with biodiesel. It must be noted that results of substrate richness for pure biodiesel samples are in agreement with those for microbial counts in selective culture media previously presented. It can be stated that although some microbial activity was present in samples with pure biodiesel as a carbon source, as can be inferred from the drop in pH and the increase in absorbance, some members of the community that was developed were unable to grow in synthetic culture media and were unable to use a wide variety of carbon sources, as observed by the CLPP carbon usage patterns. A different explanation for the low substrate richness of samples growing in pure biodiesel could be that the community is mainly composed of filamentous fungi and yeast, it is known that these microorganisms are unable to oxidize tetrazolium dyes so their growth can not be accounted by the assay (24).

3.2.4 16s rDNA sequencing

Library comparisons among the initial community and samples obtained 100 days afterwards for B0 and B100 are presented at the level of phylum and genus in Table 3-3. The results showed that the three libraries were different, which confirmed the earlier stated results obtained by principal component analysis of the community level physiological profiles.

Table 3-3. Relative frequency of isolation in libraries constructed at the beginning of the experiment (L1) and 100 days after in pure diesel (L2) or pure biodiesel (L3)

	L1	L2	L3
Phylum comparison			
Spirochaetes	0	3.3	0
Actinobacteria	0	63.0	0
Bacteroidetes	0	7.6	0
Proteobacteria	96.4	23.9	92.8
Unclassified	2.7	2.2	7.2
Genus comparison			
Brevundimonas	0.0	7.6	34.9
Breoghania	0.0	1.1	0
Hyphomonas	0.9	0.0	0
Marinobacterium	0.0	1.1	0
Pseudomonas	0.0	1.1	41
Pseudoxanthomonas	0.0	7.6	0
Pusillimonas	0.0	0.0	8.4
Rhizobium	1.8	1.1	1.2
Rhodococcus	0.0	62.0	0
Rhodovulum	16.2	0.0	0
Spirochaeta	0.0	3.3	0
Thalassolituus	50.5	0.0	0
Thalassospira	9.0	0.0	7.2
Unclassified	21.6	15.2	7.3
Shannon index	1.30	1.28	1.37

Data on composition obtained for the three rDNA libraries generated proved that the communities studied in this paper present different structure, confirming what was already observed by CLPP analysis. The presence of biodiesel led to a change in the microbial composition within the simulated fuel storage system. This was probably due to the different nature of the carbon sources available for microbial growth. While diesel is a complex mixture of olefins, aromatic and polycyclic compounds, biodiesel is chemically more homogeneous consisting only of methyl esters of different fatty acids.

Both at the level of phylum and genus it was possible to observe higher richness of phylum and genera in samples growing in pure diesel. Eight different genera and four different phyla were identified in pure diesel samples in contrast with five genera and 1 phylum in pure biodiesel ones. However, the Shannon biodiversity index in the samples was similar and slightly higher for samples growing in pure biodiesel. The difference in the richness of genera isolated in diesel and biodiesel can be explained considering that the community used as inoculum was obtained from a diesel storage tank adapted to use diesel compounds as carbon source, so a higher number of dormant species were able to became metabolically active in pure diesel than in pure biodiesel once the required nutrients were supplied. The broad differences between pure diesel and pure biodiesel communities after 100 days of exposure to fuel proved that only a portion of the initial community was able to adapt to use methyl esters as carbon source.

Roughly it can be said that the initial community was dominated by Proteobacteria of the genus *Thalassolituus*, an obligate oil degrading bacteria (25) undetectable at the end of the experiment both in pure diesel and pure biodiesel communities. Pure diesel communities at the end of the experiment were dominated by Actinobacteria belonging to the genus *Rhodococcus* while pure biodiesel ones by Proteobacteria of the genera *Pseudomonas* and *Brevundimonas*. Three metabolic features were very common among the genera isolated (Table 3-4): ability to use hydrocarbons as carbon source, ability to degrade aromatic or polycyclic compounds, and production of surfactants. Some of the genera isolated have previously being reported in diesel-enriched environments, such as *Marinobacterium* and *Pseudomonas* (Schleicher et al., 2009; Yakimov et al., 2007). Differences between the initial community (isolated from a diesel storage tank) and the

community growing in pure diesel 100 days after the experiment can be due to the excess of nutrients supplied at the beginning of the experiment that could favor the development of fast growing species adapted to divide at high nutrient concentrations.

Table 3-4. Metabolic features of genus isolated in this study

Genus	Features	Reference
Production of surfactants	*Brevundimonas, Pseudomonas Pseudoxanthomonas, Rhodococcus, Thalassospira*	(26–28)
Degradation of oil or hydrocarbons	*Brevundimonas, Breoghania*, *Pseudomonas, Rhodococcus, Spirochaeta, Thalassolituus*	(25, 29–33)
Degradation of aromatic compounds	*Marinobacterium, Pseudomonas, Pseudoxanthomonas, Pusillimonas, Rhizobium, Rhodococcus, Rhodovulum, Thalassospira*	(33–37)

Results obtained by principal component analysis of the three libraries are presented in Figure 3-6, the distribution of the three libraries in the plane with the two most important principal components corroborates the statement that the three libraries are different.

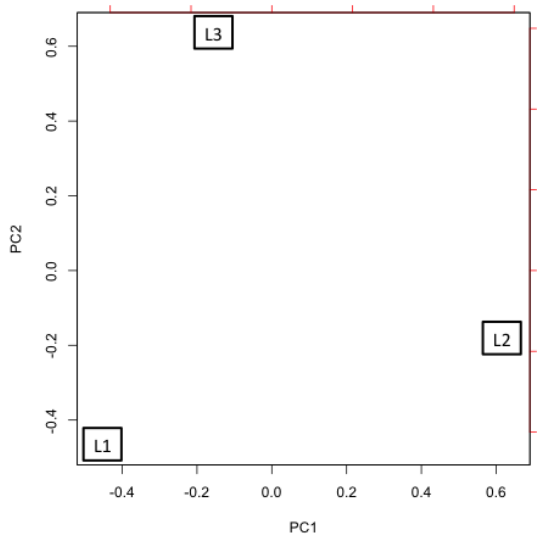

Figure 3-6. Results of principal component analysis for the three libraries constructed: (L1) at the beginning of the experiment, (L2) 100 days after in pure diesel and (L3) 100 days after in pure biodiesel

Comparison of the library obtained at the beginning of the experiment (library 1) with communities obtained 100 days after the beginning of the experiment for pure diesel (library 2) and pure biodiesel (library 3) (Table 3-3) are in agreement with the observations presented for distance analysis of CLPP (Figure 3-5). In both analyses it is observed that communities diverged from the initial community and that they did it in a different way for pure diesel and pure biodiesel.

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Chapter 4

4 Investigation of Biofilm Formation on Polyethylene in a Diesel/Biodiesel Fuel Storage Environment

Depletion of fossil fuels has led to the development of alternatives such as biodiesel. Full implementation of these technologies requires complete understanding of their performance in engines as well as of their corrosive properties and compatibility with different kind of materials. There is considerable research on biodiesel corrosive effects on metals such as carbon steel, stainless steel, copper and aluminum among others (1–5). Nevertheless, there is still a lack of information on the effects that biodiesel addition may have on polymeric materials, which are now commonly used in the fuel industry.

Among commercial polymers, polyethylene is the most commonly used material in fuel storage; it is generally considered to be inert both chemically and biologically. However, some recent evidence has suggested that this polymer may be prone to deterioration of its physical properties in fuel storage systems. Two mechanisms have been proposed to explain this deterioration. On the one hand, the fuel can be absorbed into the material, possibly leading to loss of its mechanical properties (6–8). Conversely, biodegradation studies have shown that that the metabolic activity of some microorganisms can result in deterioration or degradation of polyethylene (9–17).

Polyethylene is completely insoluble in water; consequently its biodegradation process is possibly mediated by biofilms (17), which are structures of sessile microorganisms associated as a community (18, 19). So far there are a number of research papers that have been published dealing with polyethylene biodegradation by biofilms, however all of them have been performed in microenvironments far different from those observed in fuel storage facilities. Available research contains data of polyethylene degradation either under soil burial conditions or in partial immersion under conditions similar to those found in the open sea, were plastic accumulation is an ecological problem (9, 13–15, 17, 20). Though these efforts are of great interest for the scientific community they are not

useful to obtain conclusions applicable to fuel storage facilities, because the environmental conditions and biodiversity are very different in those ecosystems. Therefore, there is a potential need for research focused on biofilm development on polyethylene surfaces and on the factors that affect this process in fuel storage systems.

Fuel storage systems are complex microenvironments with a wide variety of microorganisms present at the bottom of storage tanks where there is accumulation of water produced through condensation of environmental moisture. These conditions are suitable for microbial development, both in suspension or as a biofilm on the surfaces available (21, 22). In these systems factors such as the chemical nature of the fuel and the kind of the materials used for the manufacture of the fuel storage tank can have an influence on the composition and metabolism of the biofilms developed. Fuel composition will determine the kind of carbon sources available for microbial growth thus influencing the dominant microbial groups encountered in the system. On the other hand polymer type will determine characteristics such as hydrophobicity, degree of branching, molecular weight, cross-linking and crystallinity. These characteristics will influence the strength of microorganism-material interactions, as well as the accessibility and biodisponibility of the polymer, conditioning in this way the species that are able to growth or that will predominate in the biofilm.

Blending biodiesel with regular diesel and changing the material used in the manufacture of a diesel storage tank may affect the composition and activity of the microbial community in the biofilm developed, potentially resulting in different microbe/polymer interactions. Understanding the extent and nature of these changes in the microbial communities may help to manage and design efficient strategies to completely or partially substitute diesel for biodiesel without comprising the integrity of the infrastructure.

In this work a simulated diesel/biodiesel storage tank was designed to study the effect of biodiesel concentration over biofilm development, community structure and composition on linear low-density polyethylene (LLDPE), cross-linked polyethylene (XLPE) and bilayer construction of polyamide-11 and linear low-density polyethylene (LLDPE/PA).

The results reported here represent the first attempt to understand the effect of biodiesel addition on biofilm community structure and colonization capabilities on polymeric surfaces under conditions similar to those found in an industrial fuel storage system. Viability and microbial growth were determined and the structure of the resulting communities was evaluated by community level physiological profiling (CLPP) and sequencing of 16s rDNA gene. Images of the biofilm developed on the different polymers were obtained by Scanning Electron Microscopy (SEM).

4.1 Materials and methods

4.1.1 Experimental set up

A simulated fuel storage tank was used in which water of condensation was present at the bottom. The system consisted of a 500 ml Erlenmeyer flask containing 50 mL of mineral medium Richard and Vogel's pH 7 (23) (all chemicals were purchased from Sigma-Aldrich, St. Louis, USA), 50 mL of water collected in the bottom of an industrial diesel storage tank (sampled during the spring of 2012 by Imperial Oil Ltd at their facilities in Sarnia, ON), and 100 mL of a diesel/biodiesel blend. The water sample was used as inoculum of a microbial community adapted to the use of diesel as a carbon source. The diesel fuel was a low sulfur, summer grade purchased from an ESSO station (London, ON) while the biodiesel was kindly supplied by the University of Guelph (Ridgetown site, ON); characterization of the biodiesel fuel was included in an earlier paper (6); the system developed two phases, the upper one containing the fuel and the lower aqueous phase containing the culture medium and the inoculated microorganisms. Biodiesel concentrations of 0%, 25%, 50%, 75% and 100% (v/v) were used. Three different kinds of polymer samples were evaluated: linear-low-density polyethylene (LLDPE; LL™ 8460, ExxonMobil), cross-linked polyethylene (XLPE; Paxon™ 7004, ExxonMobil) and a bilayer construction of the same LLDPE with polyamide-11 (LLDPE/PA). All polymers were first molded at McMaster University (Hamilton, ON) by means of a rotational molding system. Small slabs (approximate dimensions 1cm x 1cm x 0.5cm) of the polymers were prepared and were immersed in the water layer of the simulated fuel storage tank by means of a stainless steel wire; the wire was required because the density of the polymer was lower than water. Experimental units (defined as a polymer slab in an

independent fuel-medium system) were prepared in triplicates and were kept in darkness at 25 °C during 200 days to allow biofilm development on the hydrophobic polymer surface. Fuels, culture media and polymers were sterilized before inoculation. Fuel sterilization was performed by filtration at 0.2 μm (Merk-Millipore, Billerica, USA). Culture media was autoclaved before inoculation at 121°C for 15 minutes. Polymer slabs were sterilized by immersion in 95% ethanol for a period of 4 hours, after which the residual alcohol was allowed to completely evaporate in a vented laminar flow hood. Once sterile the polymer slabs were immersed in the water phase of the system. Controls of all experimental units containing only Richard and Vogel's media with no fuel and no microorganisms were prepared and kept under the same conditions.

4.1.2 Biofilm development

4.1.2.1 Biofilm growth quantification

Quantification of biofilm development on the plastic surfaces was performed after 200 days of incubation following a modified procedure of a procedure described elsewhere (24). Slabs of the polymers were taken out from the simulated fuel storage system and washed once with water to remove unattached cells. Then they were immersed in a 1% crystal violet solution for 5 minutes (Merck-Millipore, Billerica, USA), during this time the dye was absorbed by cells present in the community. Excess dye was washed with distilled water and then samples were incubated for 15 minutes in 3mL of isopropanol-acetone 80:20 (Merck-Millipore, Billerica, USA). Finally, the absorbance at 570 nm was determined in a microplate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland) as an indirect measurement of the amount of microorganisms present in the biofilm. Absorbance measurements were corrected by subtracting the control value and then normalized with respect to the surface area of a slab. Surface area of the rectangular cuboid slab was determined using a caliper. Statistical differences between treatments were evaluated by means of a two way Analysis of Variance (ANOVA) using a factorial design with two factors: concentration of biodiesel with 5 levels (of 0%, 25%, 50%, 75% and 100%) and type of polyethylene with 3 levels (LLDPE, XLPE and LLDPE/PA). Differences among treatments were evaluated using a Turkey test ($p<0.01$). All data

processing was performed in the statistical package R version 2.15.2 (R Foundation for Statistical Computing; Vienna, Austria).

4.1.2.2 Viability

Viability is a measurement of the metabolic activity of cells. When comparing microbial communities a relative value of this variable can be determined as the hydrolysis of fluorescein diacetate to fluorescein, a colored product. This reaction is catalyzed by several kinds of enzymes such as hydrolases and proteases which are commonly expressed in most microorganisms, in such a way that it is able to account for the global microbial activity of a community (25). In this study, viability was determined following the method used by Orr et al (26) to evaluate biofilm activity on polyethylene surfaces. Briefly, the slabs of polymer were immersed in 30 mL of a fluorescein diacetate solution (10 μg/mL) (Merck-Millipore, Billerica, USA) in 60 mM phosphate buffer (pH 7.6) (Sigma-Aldrich, St. Louis, USA). Fluorescein diacetate was dissolved in acetone (3mg/ml) (Caledon, Georgetown, Canada) before addition to the phosphate buffer. Samples were incubated for 23 hours at 30°C with agitation (100 rpm) in a rotary shaker, and after this time their absorbance were read at 494 nm in a microplate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland). Data correction and statistical analysis were performed using the same procedure described above.

4.1.2.3 Biofilm imaging by scanning electron microscopy (SEM)

Images of biofilms growing on the surface of all polymers studied were taken for those samples growing in pure diesel, pure biodiesel and 50% biodiesel, following the procedure describe by Karcz et al (27). Briefly, samples were fixed in 3% glutaraldehyde (Sigma-Aldrich, St. Louis, USA) buffered in 0.1M phosphate buffer (pH 7.2) and then washed 3 times. This procedure was followed by a post fixation step with 1% osmium tetroxide (Sigma-Aldrich, St. Louis, USA) in 0.1M phosphate buffer (pH 7.2) during 2 hours. Samples were then dehydrated by serial incubations of 10 minutes in solutions of increasing ethanol concentration (Sigma-Aldrich, St. Louis, USA) starting at 30% and finishing at 100% by increasing 10% each step. Critical point drying with CO2 was performed (Electron microscopy technologies, Hatfield, USA). Samples were coated with

10 nm of osmium tetroxide in an osmium plasma coater (OPC80T, Filgen, Nagoya, Japan) before observation by SEM (Leo 1530 Gemini, Zeiss, Oberkochen, Germany).

4.1.3 Biofilm community structure

4.1.3.1 Community level physiological profiling (CLPP)

CLPP analysis was performed for samples obtained from the biofilm established on the surfaces of the polymers. Three different samples per polymer were evaluated, one incubated in pure diesel, one incubated in 50% (v/v) biodiesel and one incubated in 100% biodiesel, which gave a total of 9 different treatments. Three replications per treatment were used.

For the detachment of cells, polymer slabs were immersed in 20 ml of phosphate buffer (pH 7, 10mM supplemented with NaCl 8.5g/L) and incubated at 30°C for 1 hour at 100rpm in a rotary shaker, as recommended by Weber et al (28). This was followed by 1 minute of vortexing. The suspension obtained in this way contained a sample of the biofilm community and was used to evaluate community level physiological profiles in ECOPLATES (Biolog Inc., Hayward, USA). This system contains 31 different carbon sources and a blank. Each well of the ECOPLATE was inoculated with 150 µL of the suspension. Incubation was carried out for 160 hours at room temperature. The absorbance at 590 nm was determined every 12 hours in a microplate reader (Tecan Group Ltd., Seestrasse, Switzerland).

Two different strategies were used for data analysis. In the first approach, a single point in time at 101 hours was used as a metric. In the approach, all of the samples corresponding to pure biodiesel were excluded and a single point with an average well color development (AWCD) close to 0.36 was used. Samples corresponding to pure biodiesel had to be excluded because microbial growth in most of the carbon sources was very close to zero in such a way that the AWCD never reached a value close to 0.36. In both approaches data were normalized and transformed using Taylor power law (29). Data were analyzed by Principal Component Analysis (PCA) using the statistical software R version 2.15.2 (R Foundation for Statistical Computing, Vienna, Austria). Substrate richness defined as the number of wells with a corrected absorbance greater

than 0.25 (30) was calculated for all treatments. Statistical differences for substrate richness between treatments were evaluated by using a factorial ANOVA as described above.

4.1.3.2 16s rDNA characterization of communities

Six libraries for 16s DNA gene with approximately 20 clones each (Accession numbers KF361885 - KF362015) were generated for the microbial communities developed on the surface of the polymers. Three libraries were from the polymer samples incubated in pure diesel as a carbon source and three from the polymer samples incubated in pure biodiesel. DNA extraction was performed using Power Biofilm Kit (MoBIO, Carlsbad, USA) following the manufacturer's instructions; this kit is designed so biofilm is used as substrate for DNA extraction without a detachment procedure. DNA was quantified by spectrophotometry at 280/260 nm using the Nanoquant system (Tecan Group Ltd., Seestrasse, Switzerland) and a plate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland). PCR conditions are described elsewhere (Restrepo-Florez, Bassi, Rehmann and Thompson, 2013). PCR products were visualized using a flash gel system (Lonza, Basel, Switzerland) and purified using UltraClean PCR Clean-Up Kit (MoBIO, Carlsbad, USA). pGEN easy cloning system was used for ligation and cloning (Promega, Fitchburg, USA) and PureLink® Quick Plasmid Miniprep kit (Life technologies, Carlsbad, USA) for plasmid extraction. Products were sequenced at the Robarts Research Institute (London, Canada).

Quality control, vector removal and editing of sequences were performed using a trial version of Sequencher Software Version 5.0.1 (Gene Codes Corporation, Ann Arbor, USA) and eBiox Version 1.5.1 (Pleasanton, USA). Sequences with less than 700 pb or with a quality index lower than 80% were removed. Multiple unknown bases at the beginning and the end of the sequences were removed to improve quality. Libraries were analyzed to taxonomic identification by using the classifier service of RDP release 10 (Michigan State University, East Lansing, USA) (31). Rarefaction curves using the RDP software were constructed to verify the completeness of the libraries. For all the libraries Shannon diversity index was calculated. The results in the libraries were analyzed by principal component analysis, each genus was identified with one variable, and each

library was represented in a plane that has the two most important principal components as axes.

4.2 Results

4.2.1 Biofilm development

4.2.1.1 Biofilm growth quantification

Crystal violet method staining is a technique that allows the relative determination of biomass accumulation on biofilms. Comparative results for biofilm growth on the different polymers after 200 days of incubation in different biodiesel concentrations are shown in Figure 4-1. Statistical analysis ($p<0.01$ proved that for all polymers evaluated there was greater accumulation of biofilm on samples growing in pure biodiesel (B100) compared to other biodiesel concentrations. Samples of LLDPE growing in pure biodiesel presented lower accumulation of biofilm compared with the other two polymeric materials under that biodiesel concentration.

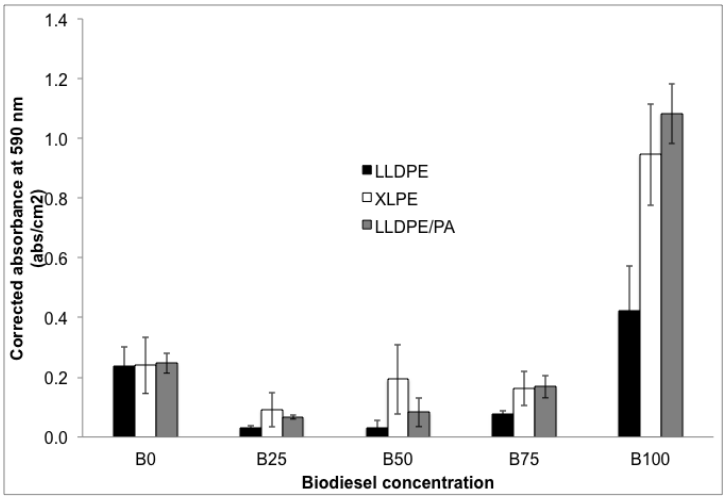

Figure 4-1. Biofilm growth quantification on the surfaces of the three polymers studied after 200 days of incubation: low-density polyethylene (LLDPE), Cross-linked polyethylene (XLPE) and polyethylene half coated with nylon (LLDPE/PA)

4.2.1.2 Viability

Viability is a measurement of the metabolic activity of the biofilm. Results on this variable for biofilm developed on the surface of the polymers used in this study and incubated at different biodiesel concentrations are presented in Figure 4-2. Statistical analysis showed that ($p<0.01$) the highest viability was found for samples growing in pure diesel. The other samples did not present statistically significant differences among them.

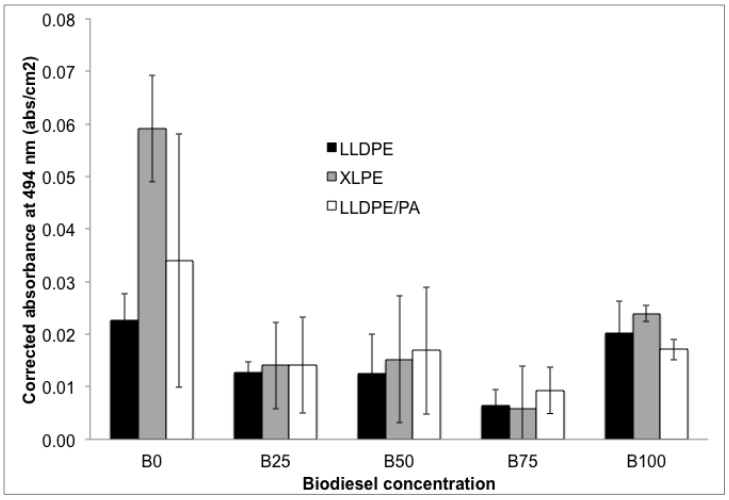

Figure 4-2. Relative Biofilm viability quantified by hydrolysis of fluorescein diacetate for the biofilm developed on the three polymers studied after 200 days of incubation: linear-low-density polyethylene (LLDPE), cross-linked polyethylene (XLPE) and polyethylene half coated with nylon (LLDPE/PA).

4.2.1.3 Biofilm imaging by scanning electron microscopy (SEM)

Pictures by SEM of the biofilms developed on the surface of the different polymers used in this study incubated under different biodiesel concentrations are presented in Figure 4-3. Complete colonization of the polymers is observed as well as the presence of micro-colonies, typical structures in biofilm development, biodiversity of the biofilm is observed as different morphologies are present in the pictures that were taken.

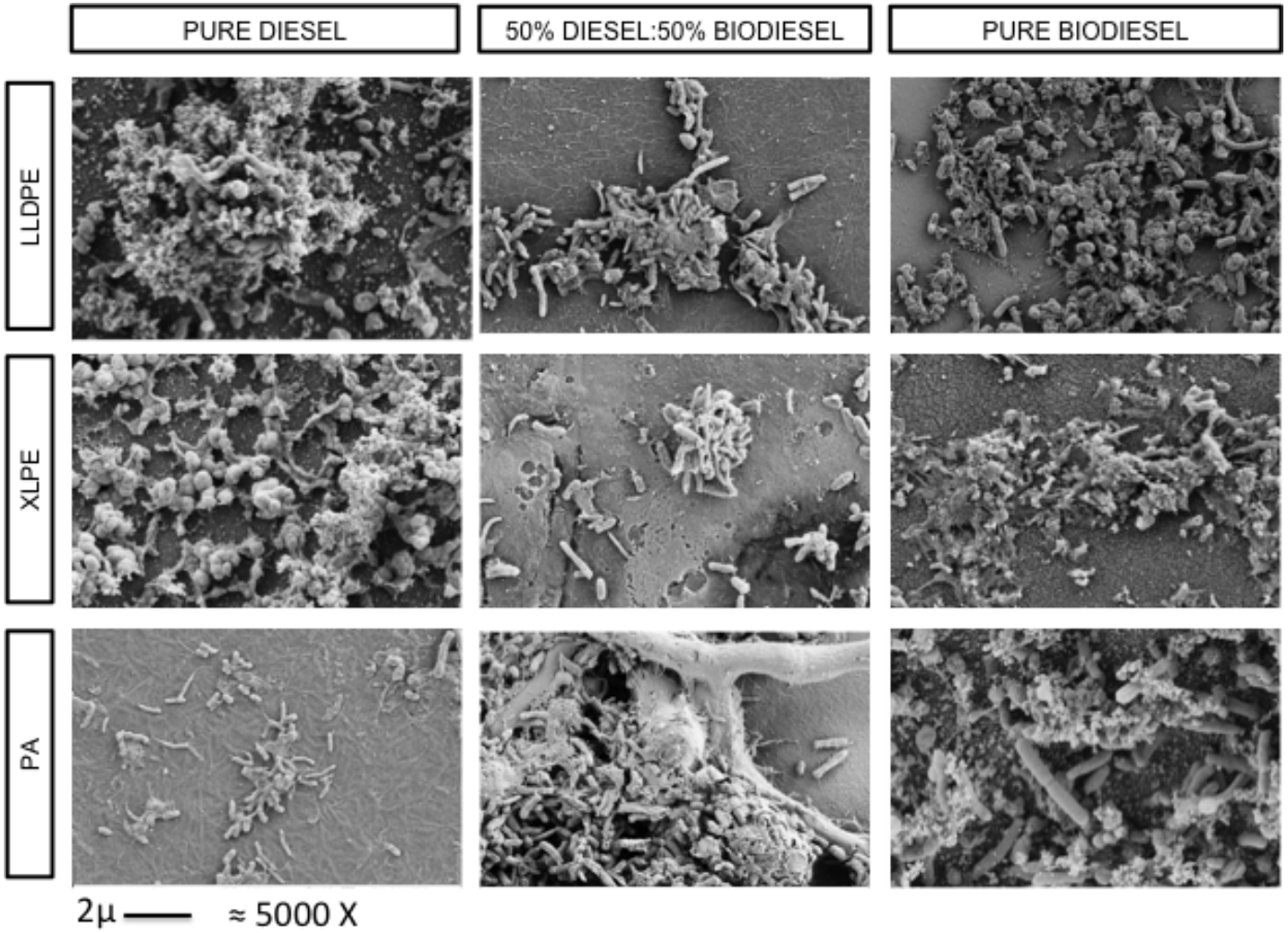

Figure 4-3. Images for biofilms after 200 days of incubation for different polymers incubated at different biodiesel concentrations

4.2.2 Biofilm community structure

4.2.2.1 Community level physiological profiling (CLPP)

Results of PCA when the first approach of analysis described in the materials and methods session is used are presented in Figure 4-4. Two groups were observed, one clustering samples growing in pure biodiesel (B100) and one clustering samples growing either in B0 or B50 blends, no other pattern of clustering could be observed in the analysis. Pure biodiesel samples are very different from the others, presenting an AWCD significantly lower than those growing either in B0 or B50. For this reason, another approach for data analysis in which pure biodiesel samples are excluded is needed in order to observe further clustering effects, either due to polymer type or fuel concentration (second approach described in the materials and methods session).

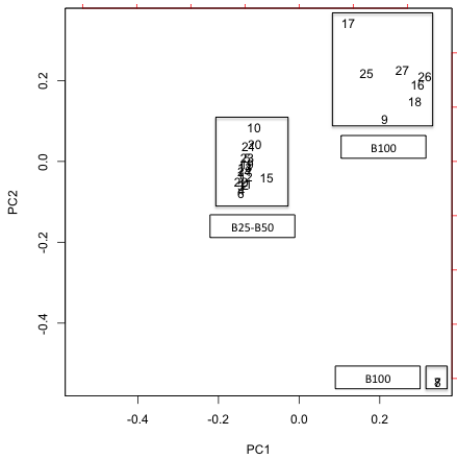

Figure 4-4. Principal component analysis of CLPP when pure biodiesel samples are included. Two clusters are observed (shown as squares in the figure) one corresponding to pure biodiesel samples and one containing both pure diesel or blends 50% biodiesel.

Figure 4-5 presents the results of PCA when the pure biodiesel samples are excluded from the analysis. Two different patterns of clustering are identified in this case, one based on the nature of the polymer and other on the fuel used as a carbon source. In Figure 4-5-A three different groups can be observed, corresponding to the polymers used in the study. Figure 4-5-B shows another way of grouping the samples based on the concentration of biodiesel used as the carbon source in the experiment.

Figure 4-6 present the results of substrate richness for communities growing at different biodiesel concentrations. Statistical analysis showed no interactions between the two factors evaluated. Significant differences ($p<0.01$) were found among the different biodiesel concentrations but not among polymer types.

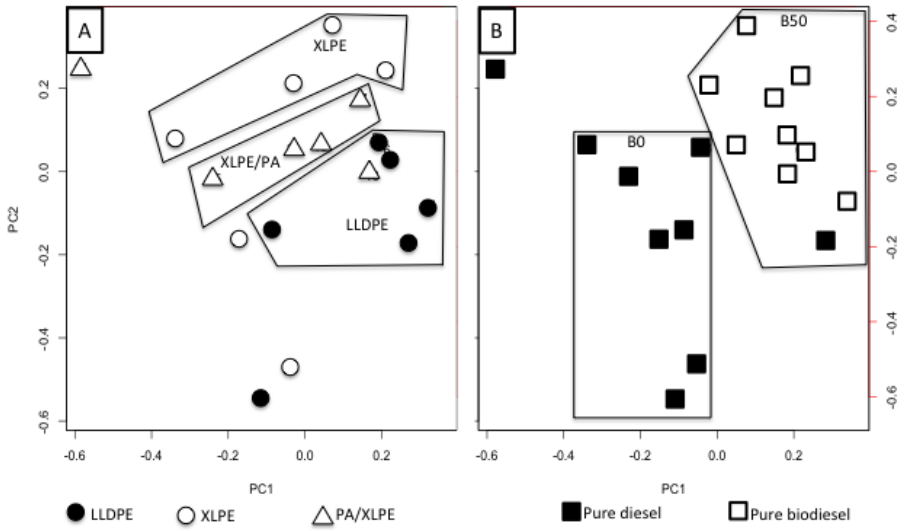

Figure 4-5. Principal component analysis of CLPP patterns for biofilm communities once pure biodiesel samples were excluded. (A) Grouping pattern based on the nature of the surface. (B) Grouping pattern based on biodiesel concentration in the fuel phase.

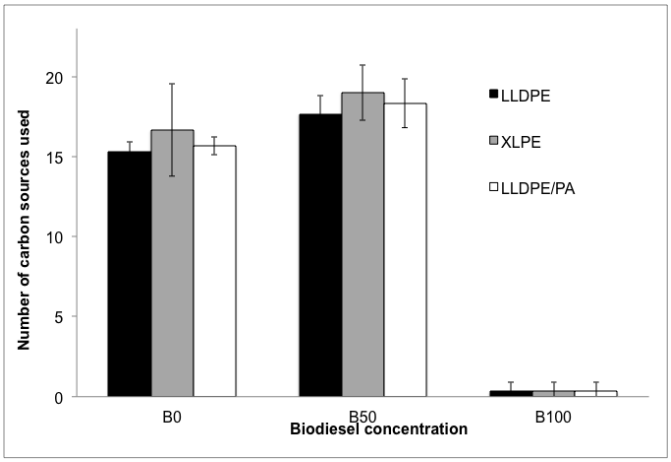

Figure 4-6. Number of substrates used in the CLPP profiles for samples obtained from different polymers growing under different biodiesel concentrations. Error bars indicated for standard deviation

4.2.2.2 16s rDNA characterization of communities

Analysis of the 16s rDNA libraries constructed for the biofilm communities is presented in Table 4-1. The completeness of the libraries was verified by rarefaction constructed at 5% distance (data not shown). Results show that the libraries were different, which suggested that both the polymer type as well as the biodiesel concentration had an influence on the structure of the microbial community established, confirming the results obtained by PCA of the CLPP.

Table 4-1. Relative frequency of isolation of microbial genera in the libraries constructed for different polymers under different concentrations of biodiesel

	LLDPE		**PA**		**XLPE**	
	B0	B100	B0	B100	B0	B100
Agromyces	0.0	0.0	0.0	0.0	5.0	0.0
Bacillus	0.0	4.3	0.0	12.5	0.0	0.0
Brevundimonas	0.0	4.3	0.0	0.0	5.0	0.0
Corynebacterium	0.0	0.0	0.0	0.0	0.0	4.3
Micrococcus	0.0	47.8	0.0	0.0	0.0	0.0
Ochrobactrum	5.0	21.7	0.0	12.5	0.0	17.4
Propionibacterium	0.0	13.0	0.0	0.0	0.0	8.7
Pseudomonas	0.0	0.0	0.0	0.0	10.0	0.0
Pseudoxanthomonas	90.0	0.0	50.0	8.3	60.0	4.3
Pusillimonas	0.0	4.3	0.0	0.0	5.0	8.7
Rhizobium	5.0	4.3	0.0	12.5	5.0	8.7
Rhodococcus	0.0	0.0	45.0	0.0	10.0	43.5
Spirochaeta	0.0	0.0	5.0	0.0	0.0	0.0
Staphylococcus	0.0	0.0	0.0	8.3	0.0	4.3
Streptophytaa	0.0	0.0	0.0	8.3	0.0	0.0
Tumebacillus	0.0	0.0	0.0	37.5	0.0	0.0
Shannon index	0.39	1.49	0.86	1.77	1.37	1.71

Results for principal component analysis of the rDNA libraries generated in this study are presented in figure 4-7. It is interesting to note that results confirm what was stated regarding the differences between the six libraries. The other important point is that samples appear to be separated in the first component based on the biodiesel concentration, in the left region of the plane are grouped samples growing in pure diesel wile in the right region are samples growing in pure diesel.

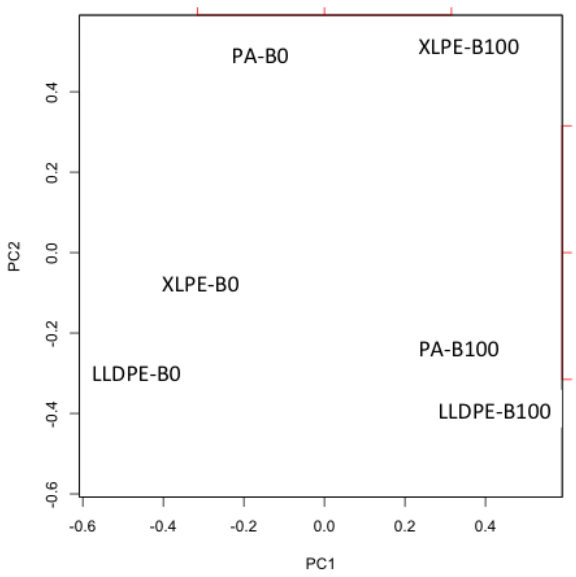

Figure 4-7. Principal component analysis of the six 16S rDNA libraries generated in this study

4.3 Discussion

4.3.1 Biofilm development

4.3.1.1 Biofilm growth and viability

Results obtained by crystal violet indicate that pure biodiesel can favor the accumulation of biofilm on plastic surfaces (Figure 4-1). At lower concentrations of biodiesel (samples from B0 to B75) the biofilm accumulation process is not affected by the presence of biodiesel. This behavior can be explained if we assume the presence of certain biofilm forming groups of microorganisms that can grow well in biodiesel but are inhibited by petrochemical diesel.

In addition to the measurements with crystal violet, determination of relative viability by hydrolysis of fluorescein diacetate was also performed (Figure 4-2). Maximum viability was found for samples growing in pure diesel. It has to be considered that while the crystal violet method (Figure 4-1) is designed to account for all of the biomass present in the system regardless of its metabolic state, including dead, dormant or inactive biomass; viability methods only accounts for metabolically active cells. This indicated that after 200 days most of the biomass accumulated in samples with pure biodiesel was in a low activity metabolic state.

This low metabolic activity in pure biodiesel samples is explained either by a high concentration of dead or dormant cells or by considering a higher yield of exopolysaccharides; these kinds of compounds are not metabolically active but are counted as microbial biomass by the crystal violet essay used in this study. Emulsifiers such as mono and di-glycerides, present in low concentrations in biodiesel can affect the rate of exopolysaccharide production, as proven in recent studies with biofilms of *Pseudomonas* spp. on polyethylene (32).

Based on what was presented it can be seen that a higher biofilm accumulation is observed in pure biodiesel samples but a higher number of active cells are found in pure diesel ones. For the conditions evaluated and the community under study it is clear that addition of biodiesel will affect the amount of biofilm developed on a surface as well as its metabolic activity. This last statement is of particular interest in polyethylene degradation, because changes in the metabolic capabilities of a community might result in changes in the way microorganisms interact with such materials.

4.3.1.2 Structure of the biofilm communities by SEM

Biofilms developed on the surfaces of the different polymers were analyzed by SEM. Three different qualitative criteria were used to characterize and compare the samples: the 3D structure of the biofilm, the extent of the colonization of the surface, and the composition based on the morphology of the microorganisms observed. The structure of the biofilms developed presents a typical pattern of micro-colonies and complex 3D structures similar to those found when pure cultures of other microorganisms such as

Rhodococcus rodococcus and *Rhodococcus ruber* are growth on polyethylene surfaces (17, 32). The degree of colonization of all surfaces was high after 200 days for all of the polymers evaluated. The complexity of the community and the kind of interaction between the different microorganisms that form the biofilm is evident in the pictures, microorganisms with different morphologies (rods, spheres) are usually found as part of the same structure, as exemplified in the biofilm growing on polyamide at B50 concentration. It is possible to observe hyphal growth as well as a dense mat of rod shaped microorganisms in the image. In most of the samples polysaccharide matrices, as the ones depicted in Figure 4-8, are found as the mediation strategy between the different members of the community.

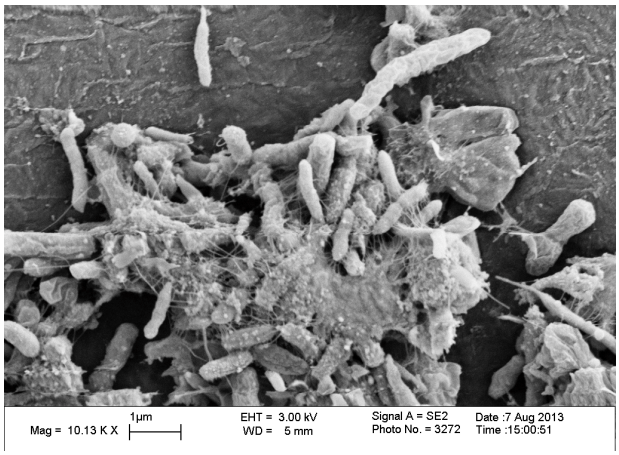

Figure 4-8. Typical structure of a micro-colony, polysaccharide mediated interaction can be observed in the picture.

4.3.2 Community structure

4.3.2.1 Community level physiological profiling (CLPP)

Community Level Physiological Profiling (CLPP) is a technique that allows the differentiation of communities based on their carbon consumption profiles. CLPP when analyzed by Principal Component Analysis (PCA) led to the identification of clusters of samples with similar metabolic behavior in the test. The underlying hypothesis is that if two communities cluster together they have a similar structure and composition. As mentioned before, two different strategies were used for data analysis in this study. In the

first one, all samples collected were included (Figure 4-4) and in the second one, the pure biodiesel samples were excluded.

The main limitation to the first approach was that the response of the communities growing in pure biodiesel was significantly lower than the other communities (Figure 4-5). This behavior had a skewing effect on the PCA in such a way that the only conclusion that could be made when pure biodiesel samples were included was that they were in fact different from the other communities. In Figure 4-4 this is observed as the existence of only two clusters of samples, one of which contains most of the B100 samples.

The lack of response of samples growing in pure biodiesel can be explained by three different causes. First, it is possible that the active microbial community developed on surfaces of the polymers was unable to reduce tetrazolium dyes; if this happened the test would be unable to account for the growth of the community. Second, the conditions used for detachment and incubation (phosphate buffer pH 7) were not optimal for the microorganisms used. Third, the microbial metabolism in these communities was anaerobic; given that the profiling assay was conducted at aerobic conditions the test could inhibit the microorganisms. In any case, the results confirm what was stated in the preceding section, the addition of biodiesel had an impact on the kind of communities and metabolic activity of the communities developed upon a polyethylene surface. Independent of these reasons for the behavior observed in the pure biodiesel samples it is clear that the second approach for data analysis, the one in which biodiesel samples are excluded, was necessary to study further clustering patterns among the samples, either due to polymer type or to fuel concentration.

When this second approach is used PCA analysis revealed two different patterns of clustering completely hidden in the preceding analysis. One pattern based on the polymer nature and the other one based on the kind of fuel used as a carbon source (Figure 4-5). The main implication of the existence of these two ways to cluster the samples is that the structure of the community obtained at a diesel storage tank will be determined both by the material selected for tank manufacture and by the chemical nature of the fuel.

The nature of the material selected for tank manufacture will determine the characteristics of the finished surface, both in regards to roughness and hydrophobicity, factors that can have an impact on the kind of community able to colonize on it as well as on the strength of the attachment by such microorganisms (18,33–34). Hydrophobic characteristics of the polymers evaluated in this study are different being LLDPE the more hydrophobic and PA the less. Effect of polymer type on the structure of the community is observed in Figure 4-5-A as the existence of three different groups based on the polymer nature.

On the other hand, it is expected that the nature of the carbon source will have an impact on the kind of communities present in a system, and also in the ability of microorganisms to attach to a surface, as it has being previously found for different bacterial species such as *Enterobacter sakazakii*, *Salmonella* spp and *Listeria monocytogenes* (35, 36). As it was already mentioned, biodiesel concentration impact on the microbial communities developed could be due to the presence of surface-active compounds (mono and di-glycerides) commonly present in low concentration after trans-esterification process (26, 32) and that can mediate interaction of microorganisms with hydrophobic surfaces.

4.3.2.2 16s rDNA characterization of communities

It was determined by analysis of CLPP that the metabolic behavior of microbial communities in diesel storage tanks will depend both on the material of the tank and on the chemical nature of the fuel. These results are confirmed by 16S rDNA analysis of the different communities obtained (Table 4-1). It can be seen that although some similarities are present among the samples all communities are different from each other. Both polymer type as well as the concentration of biodiesel had an influence in the composition of the biofilm. This proved that there was a complex community composed of microorganisms from different genera presumably cooperating in the colonization process.

Diversity indexes were different for all the treatments evaluated, and it was found that for all samples growing in pure diesel were lower than for samples growing in pure biodiesel. This behavior was due to the existence of a dominant genus

(*Pseudoxanthomonas*) in pure diesel samples that accounts for more than 50% of the isolates in all the polymers.

Analysis by principal component analysis of the 16S rDNA genetic libraries revealed that samples are different in structure, the kind of fuel used as a carbon source proved to be very important in the distribution of samples in the principal components plane, as can be observed in figure 4-7.

Independent of the polymer type the predominant genus in the samples growing within pure diesel was *Pseudoxanthomonas*. Samples growing in pure biodiesel did not present a common dominant genus; however, genera *Ochrobactrum*, *Pusillimonas* and *Rhodococcus*, were found in them. It is interesting to note that the genera isolated in this study have been known for having one or two of the following metabolic capabilities: degradation of hydrocarbons or degradation of polymers (polyethylene or polyamides). These support the hypothesis outlined in this study that two main ecological factors are determining the nature of the biofilm fuel storage systems, those being the polymer type and the composition of the fuel.

Most of the genera isolated in this study have been known for their ability to use hydrocarbons as a carbon source, and some of them such as *Pseudoxanthomonas*, *Micrococcus* and *Ochrobactrum* are known for their ability to produce surfactants (37–39), a group of molecules that can aid in the solubilization of hydrocarbons in the aqueous phase. Surfactants can also be important in the biofilm colonization process onto the polymeric materials. Degradation of either polyethylene or polyamides is the other feature common in the genera isolated. Genera such as *Pseudomonas*, *Bacillus* and *Rhodococcus* are known for their ability to degrade polyethylene and use it as a carbon source (26, 32, 40), while *Brevundimonas*, *Bacillus* and *Agromyces* have been reported as polyamide degraders (41).

Although the extent and effect of the metabolic activity of these communities on the polymeric materials is not yet established it seems that fuel storage environments are suitable for the growth of microorganisms that at least theoretically are able to degrade polymers. The first step in this biodegradation process is surface colonization and biofilm

formation. Results presented in Table 4-1 prove that the kind of microorganisms involved in this first step as well as its metabolic capabilities will be influenced both by the nature of the surface and the fuel.

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Chapter 5

5 Effect of biodiesel on biofilm activity on linear low density polyethylene (LLDPE) in a simulated fuel storage tank

Biodiesel has emerged over the last 20 years as an attractive substitute and replacement for conventional diesel due to its renewable nature. In the European Union as an example it is expected that by 2020 a 10% replacement of regular diesel for biodiesel (2) will occur.

Most of the research performed in the field of biodiesel has been focused on the development and improvement of production strategies that allow a cost reduction (3–5). However, the compatibility of biodiesel with different materials has not been significantly studied to date. Among the materials used in fuel infrastructure, polyethylene is of particular importance because it is used in the manufacture of storage tanks (6). Polyethylene is widely used, primarily due to it being an inert material both chemically and biologically.

Polyethylene is a polyolefin resulting from the condensation of ethylene units, with a molecular weight distribution that ranges from few hundred up to ten million Da (1). The molecule presents a certain degree of branching, which influence the mechanical and physical properties of the polymer. Structurally it is a semi-crystalline material, with a complex morphological structure in which crystalline regions are embedded in amorphous ones (1).

The presence of biodiesel in fuel storage tanks can lead to loss in the mechanical properties of polyethylene(7). Two mechanisms have been proposed to explain this behavior. Firstly, it is possible that fuel absorption in polyethylene walls cause deterioration of the mechanical properties(7–9). Secondly, it is possible that biodiesel is

favoring the development of microorganisms able to use polyethylene as carbon source, and therefore boosting deterioration of the material.

Evidence of the ability of microorganisms to use polyethylene as carbon source has been collected over the last 30 years (10–26). It has been found that presence of a biotic phase in contact with polyethylene can lead to loss of mechanical stability, changes in crystallinity, molecular weight distribution, chemistry and topography of the polymer (10–25). The biochemical pathways involved in the process are still not completely known; however, it is clear that oxidation of polyethylene chains is a fundamental step required to accelerate the use of this substrate by microorganisms (26, 27). The resemblance of polyethylene structure with paraffins can imply that once the molecular weight of the polymer has been reduced to the range in which enzymes involved in alkane degradation are active (typically 5 to 50 carbons (28)), then the biochemical machinery used in hydrocarbon metabolism may be used for polyethylene degradation.

As polyethylene is a highly hydrophobic polymer which is insoluble in water it has been suggested that its mechanism of degradation needs the formation of biofilms, which is likely the first step for biodegradation (19). Alternatively it is possible that extracellular hydrolytic enzymes are produced, in any case an efficient degradation process would require the attachment of microorganisms to the substrate. Therefore biodegradation studies in diesel storage tanks require a special focus on the biofilm forming capacities of the microbial communities developed.

Diesel storage tanks typically possess conditions that are conducive for microbial growth, particularly at the bottom where water due to condensation accumulates (2, 29). Both diesel and biodiesel possesses a variety of micronutrients as well as potential carbon sources. Microorganisms have been found to flourish both in the interphase of fuel and water layer and as biofilms attached to the walls of the tank (29, 30). It has been previously proven that addition of biodiesel to a regular diesel storage tank can lead to changes in the microbial community of these regions which would potentially have an impact on the biofilm forming capacity of the community as well as on the biodegradation rate of polyethylene (31).

Although a large degree of literature exists on polyethylene biodegradation, the studies available cannot be extrapolated to fuel storage infrastructure analysis for three primary reasons. The first limitation is that almost all work to date has been conducted using thin films (10–25), however fuel infrastructure is characterized by the use of thick walls during tank manufacture; this is important as the effect of microorganisms is dependent on their ability to penetrate the material, thick walls may only be vulnerable to superficial microbial attack. The second limitation is the common use of pro-oxidant additives or accelerated photoxidation processes in an attempt to improve the degradability of the polymer and reduce the environmental impact of its disposal (11, 21, 25, 26, 32); this practice is common as it accelerates the biodegradation process, however in fuel storage conditions photoxidation is not a relevant phenomena and the only oxidation would be due to either microbial activity or by chemical species present in the system. Finally, the third limitation is that the most common practice for selecting the biotic phase in polyethylene degradation studies is the use of a single strain; this approach ignores the possibility that polyethylene biodegradation may be the result of a cooperative microbial community process rather than the action of an individual microorganism. In the few studies in which complex microbial communities has been used polyethylene has been exposed to soil burial conditions, marine environment or composting systems but to date a microbial community resembling that in fuel storage tanks has not been studied (11, 16, 18, 20, 26, 27, 32–36). This illustrates the importance of studying a realistic microbial community on representative storage tank under realistic bioprocess time scales.

In this investigation, polyethylene samples were exposed over a period of 100 days to an aging process in a biotic environment that simulates the one observed in the bottom of diesel storage systems. Diesel/biodiesel blends, with biodiesel concentrations ranging from 0% to 100% were used in the fuel phase in order to observe the effect of this disturbance on polyethylene metabolism capabilities of a microbial community. Biofilm formation on linear low-density polyethylene slabs was measured by using the crystal violet method. Deterioration of polyethylene due to the presence of this biofilms was studied by monitoring changes in surface functional groups by FTIR, surface free energy by contact angle, crystallinity by DSC and topography by SEM.

5.1 Materials and methods

5.1.1 Experimental set up

A simulated fuel storage tank with realistic microbiological characteristics was designed. The system consisted of three main components (Figure 5-1): a fuel layer containing a diesel/biodiesel blend with biodiesel concentrations ranging from 0% to 100% (v/v); a water layer composed of mineral media Richards and Vogel (all chemicals were purchased from Sigma-Aldrich, St. Louis, USA)(37) inoculated with a microbial community obtained in a real diesel storage facility and finally a piece of polyethylene with approximate dimensions of 1x1x0.5 that resembled the tank walls (LLDPE; LL™ 8460, ExxonMobil). Polyethylene was molded (McMaster University, Hamilton, ON) by means of a rotational molding system and then cut to the required dimensions. A stainless steel wire was placed around the slabs of polymer in order to obtain full immersion in the water layer. The inoculum was obtained during the spring of 2012 from local refinery facilities in Sarnia, ON. The diesel fuel was a low sulfur, summer grade purchased from ESSO and the biodiesel was supplied by the biodiesel pilot plant from the University of Guelph (Ridgetown site, ON); characterization of the biodiesel fuel was included in an earlier paper(7). Fuel, culture media and polymer slabs were sterilized before inoculation. Fuel sterilization was performed by filtration throught 0.2 μm pore size filters (Merk-Millipore, Billerica, USA). The culture media was autoclaved at 121°C for 15 minutes. Polymer slabs were sterilized by immersion in 95% ethanol for a period of 4 hours. Experimental units were prepared in triplicates and kept in darkness at environmental temperature during 75 days to allow biofilm development on the polymer surface and eliminate photo-oxidation. Samples for analysis were taken every 25 days. Controls containing only Richard and Vogel's media with no fuel and no microorganisms were prepared and kept under the same conditions.

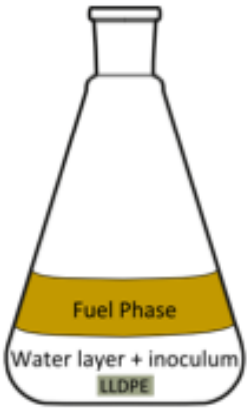

Figure 5-1. Experimental set up used in this study

5.1.2 Biofilm growth quantification

Quantification of biofilm growth was performed every 25 days following a modified procedure of the crystal violet protocol described by Stepanovic et al 2000 (38). Slabs of the polymer were removed from system and washed with distilled water to remove unattached cells and were immediately immersed in a 1% crystal violet solution for 5 minutes (Merck-Millipore, Billerica, USA) and then washed with distilled water to remove excess of dye; the samples were then incubated for 15 minutes in 3ml of isopropanol-acetone 80:20 (Merck-Millipore, Billerica, USA). Absorbance at 570 nm was determined in a microplate reader (Infinite M200 pro, Tecan Group Ltd., Seestrasse, Switzerland) as an indirect measurement of the amount of biofilm. Absorbance measurements were corrected by subtracting the control value (polyethylene without any microorganisms) and then normalized with respect to the surface area of the slab. Surface area of the rectangular cuboid slab was determined using a caliper.

5.1.3 Polyethylene sample preparation

Prior to measurement of the effect of microorganisms on polyethylene, biofilms were removed from the surfaces. Polymer slabs were immersed in an SDS solution (20% w/v) during 12 hours followed by incubation with hydrochloric acid (6.6% v/v) during one hour. Finally, samples were sonicated in acetone for 1 hour to remove any residual organic material attached to the surface. Although some chemical modification of the

surface could result from this treatment all controls were submitted to the same procedure, besides the conditions used were mild to minimize this effects.

5.1.4 Study of microbial effect on the chemistry of the surface

5.1.4.1 Fourier Transformed Infrared Spectroscopy (FTIR)

Formation and/or consumption of oxidized groups on the surface was determined using FTIR with attenuated total reflectance (ATR) in a Nicolete 6700 equipment (Thermo Scientific, Waltham, USA). Formation of ketones, aldehydes and esters was followed by determination the Keto-Carbonyl index measured as the ration between the peaks at 1718 cm^{-1} and 1471 cm^{-1}.

5.1.4.2 Contact angle measurements

Hydrophobicity was studied by measuring contact angle with Millipore grade distilled water using a goniometer (Ramé-Hart, Succasunna, USA). Contact angle of each sample was determined as the average of three measurements taken in different parts of the surface. Surfaces that are more hydrophilic are more easily wetted by polar fluids like water and tend to form smaller contact angles when drops of water are formed on their surface.

In order to characterize the surface free energy the XDLVO approach was used. Van der Waals (LW) and acid-base interaction components (AB) of the surface free energy were determined by use of Young-Dupré equation (equation 1) and data of contact angle with three different fluids of known surface tension(39). The three probe liquids used were water Millipore grade (γ^{LW}=21.8 mN/m, γ^{+}=25.5 mN/m, γ^{-}=25.5 mN/m), formamide (γ^{LW}=39 mN/m, γ^{+}=2.28 mN/m, γ^{-}=39.6 mN/m) and diiodomethane (γ^{LW}=50.8 mN/m, γ^{+}=0 mN/m, γ^{-}=0 mN/m). Contact angle of each liquid was determined as the average of three measurements taken in different parts of sample surface. Non-linear fitting of the data was performed to regress the unknown tension components of the polyethylene film.

$$(1 + \cos(\theta)) = 2(\sqrt{\gamma_S^{LW}\gamma_L^{LW}} + \sqrt{\gamma_S^{+}\gamma_L^{-}} + \sqrt{\gamma_S^{-}\gamma_L^{+}}) \quad \text{(Equation 1)}$$

5.1.5 Changes in the crystallinity of the polymer

Percentage of crystallinity was determined by differential scanning calorimetry (DSC) in a Q200 Equipment (TA instruments, New Castle, USA). Following the ASTM standard D3418-12. A crystallization heat of 64.6 J/g was used as reference for 100% crystalline polyethyelene(40).

5.1.6 Gravimetric analysis

To study the rate of polymer consumption by microorganisms the weight of samples was measured during the course of the experiment using a gravimetric method. Samples were weighted upon inoculation and then consumption was calculated at the end of the experiment once biofilm was removed. A 5 digits scale (Mettler Toledo, Columbus, USA) was used. After biofilm removal samples were dried for 8 hours at 70°C to remove any residual moisture.

5.1.7 Polymer topography

5.1.7.1 Surface free of microorganisms

Topography of polyethylene samples once biofilm was removed was studied by scanning electron microscopy (SEM) (Leo 1530 Gemini, Zeiss, Oberkochen, Germany) at a magnification 5000 X and 3 KV. Samples were coated before observation with 10 nm of osmium tetroxide in an osmium plasma coater (OPC80T, Filgen, Nagoya, Japon) before observation.

5.1.7.2 Microorganisms interaction with the polymer

In order to find out if the observed changes in the topography of the polymer were somehow related with the presence of microorganism, samples were submitted to the same treatment described in section 4.2.1 without biofilm removal, observations were made by SEM using the procedure describe by Karcz et al 2012(41). Samples were fixed in 3% glutaraldehyde (Sigma-Aldrich, St. Louis, USA) buffered in 0.1M phosphate buffer (pH 7.2) and then washed with milliQ water 3 times. A post fixation step with 1%

osmium tetroxide (Sigma-Aldrich, St. Louis, USA) in 0.1M phosphate buffer (pH 7.2) during 2 hours was used. Then samples were dehydrated by serial incubations of 10 minutes in solutions of increasing ethanol concentration (Sigma-Aldrich, St. Louis, USA) from 30% to 100% by increasing 10% each step. Critical point drying with CO2 was used before imaging (Electron microscopy technologies, Hatfield, USA). Samples were osmium coated as described previously.

5.1.8 Statistical analysis

Statistical comparison of variables studied in this work at different times under different biodiesel concentrations was performed by longitudinal data analysis using a mixed model (included analysis of fixed and random effects) in the statistical package SAS Version 9.3 (SAS Institute INC., Cary, USA). The structure of variance was chosen among a compound symmetric, unstructured or autoregressive by using the bayesian information criteria (BIC). A t-test with a significance level of 1 % ($p<0.01$) was chosen as the criteria to determine differences among treatments.

5.2 Results

5.2.1 Biofilm growth

Results of biofilm growth curves during 100 days at different biodiesel concentrations are shown in figure 5-2. Statistical analysis shows no significant differences among the treatments during the first 75 days of culture. At 100 days higher accumulation of biofilm was found in samples growing at higher biodiesel concentrations B75 and B100, when compared with samples at medium B50 and lower biodiesel concentrations B0 and B25.

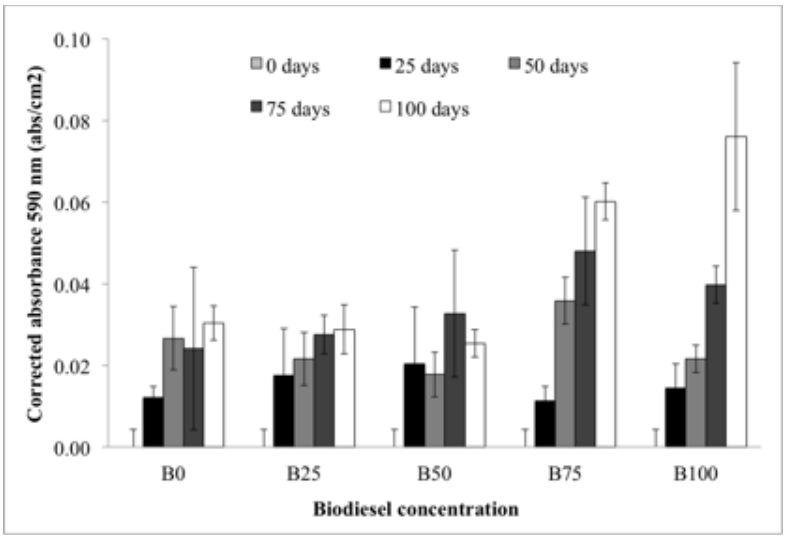

Figure 5-2. Biofilm growth on polyethylene surfaces at various biodiesel-diesel rations over 100 days of incubation

5.2.2 Study of microbial effect on the chemistry of the surface

5.2.2.1 Fourier Transformed Infrared Spectroscopy (FTIR)

Formation and or consumption of carbonyl groups was followed by FTIR, results corresponding to keto-carbonil index of different treatments in the course of the experiment are shown in figure 5-3. In general, it was observed consumption of oxidized groups as a reduction in the keto-carbonil index. However, this trend was statistically significant only for samples growing in B0, B50 and B100. The fact that the statistical differences are not found for samples in B25 and B75 is likely due to experimental variability rather than to a mechanistic process related with the concentration of biodiesel. It is interesting to note that in B50 and B0 samples it is observed an initial period of consumption followed by an increase in the keto-carbonyl index, this can be due to oxidation processes that are taking place after depletion of the initial amount of oxidized groups available for microbial growth. Controls do not present significant changes in the keto-carbonil index during the period of the experiment, indicating microbial activity as the sole driver of this change.

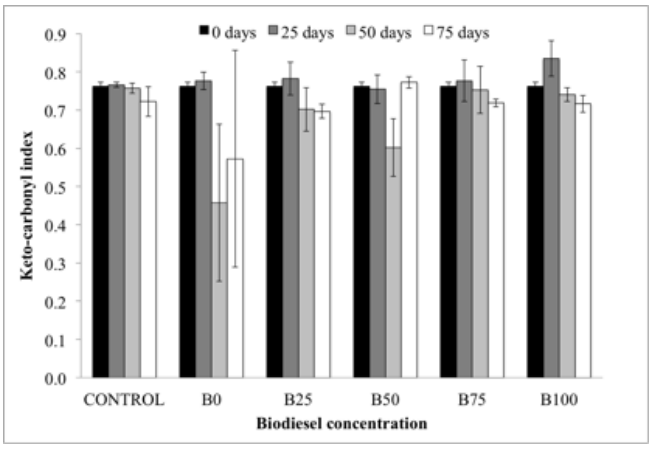

Figure 5-3. Keto carbonyl index of polyethylene samples during the first 75 days of the experiment

5.2.2.2 Contact angle measurement and surface free energy determination

Water contact angle results obtained for the different treatments in the course of the experiment are presented in Figure 5-4, statistical analysis of the results shows that both treatments and time are factors that influence the value of contact angle, the two factors do not present interaction. It is observed that contact angle increases with time for all the treatments, indicating an increase in hydrophobicity. Significant statistical differences ($p<0.05$) between samples incubated at low biodiesel concentrations B0, B25 and samples at high biodiesel concentrations B75 and B100 were found.

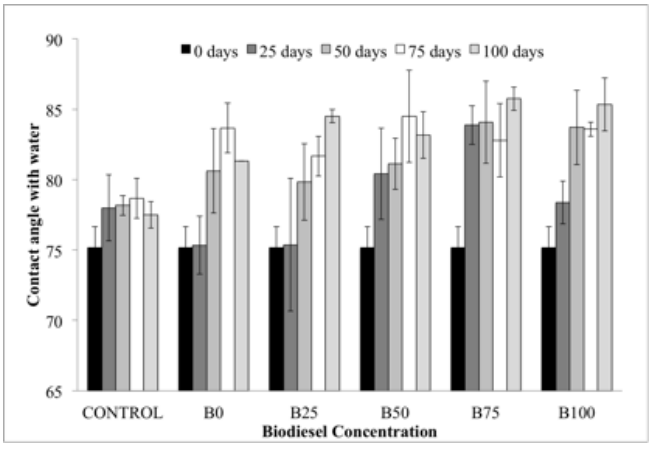

Figure 5-4. Contact angle with water of polyethylene surfaces

No significant changes were found for the van der Waals component and the acid component of the surface free energy regressed via the young-Dupré equation, the last one remained close to zero in all the measurements. Results obtained for the basic component are presented in figure 5-5. Statistical analysis showed that both time and biodiesel concentrations had significant affect in the variable ($p<0.01$). No interaction between factors was found from ANOVA analysis. All samples but the controls showed a decrease in basic groups (electron donors). Statistically it was found that a higher decrease in the basic component occurs when samples are incubated at higher biodiesel concentrations (B75 and B100).

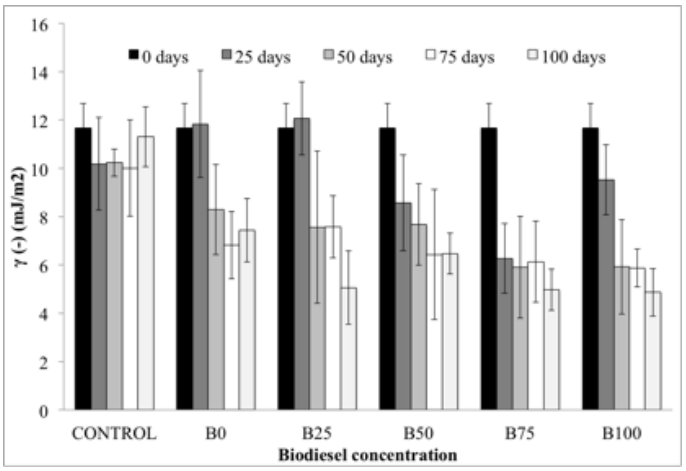

Figure 5-5. Basic component of the surface free energy calculated by using Young-Dupré equation

5.2.3 Changes in crystallinity

Results for changes in crystallinity are presented in table 5-1, statistical analysis of the results remonstrate that there is no effect due to the treatments but that time does have an impact on the crystallinity of the polymer. Results show an initial increase in crystallinity up to 50 days and then a decrease. There is no evidence in this study that the changes observed are due to microbial activity.

Table 5-1. Changes in crystallinity percentage over the course of the experiment

	0 days	**25 days**	**50 days**	**75 days**	**100 days**
Control	48.0±0.8	50.9±0.4	52.1±0.2	49.3±1.4	46.7±2.7
B0	48.0±0.8	49.3±3.3	51.8±1.4	49.8±3.4	48.9±1.8
B25	48.0±0.8	50.7±1.1	51.1±1.2	45.7±0.6	48.7±3.8
B50	48.0±0.8	51.6±0.3	50.5±2.0	47.1±1.9	48.7±0.4
B75	48.0±0.8	49.1±2.0	50.4±2.5	45.3±2.0	48.9±2.2
B100	48.0±0.8	50.5±2.1	51.4±2.1	49.3±0.8	49.7±0.9

5.2.4 Gravimetric analysis

No significant changes, compared with the controls, in the weight of the samples was observed during the course of the experiment. This behavior is an indication that the microbial attack is only superficial without any deep penetration by microorganisms and that polymer consumption is happening at a very slow rate. In general a small weight increase with a maximum of 0.3% was observed in all samples (controls inclusive). This increase is explained mainly by experimental error in the gravimetric analysis rather than by an absorption process in the polymer.

5.2.5 Changes in the topography of the polymer

5.2.5.1 Topography of the surface free of microorganisms

No significant changes on the surface of the polymer were observed once microorganisms were removed. Results by SEM at different biodiesel concentrations once microorganisms are removed are presented in figure 5-6.

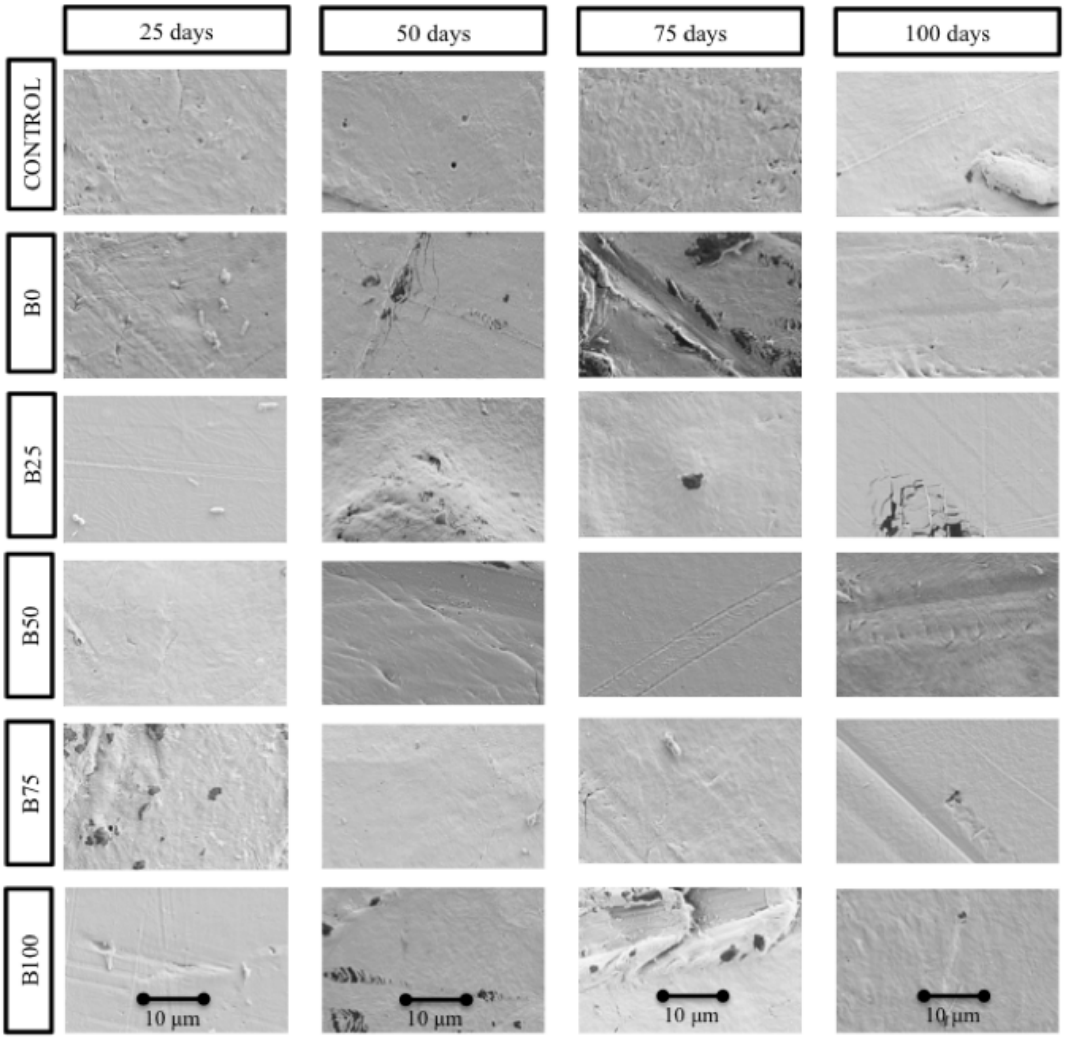

Figure 5-6. SEM images of polyethylene samples incubated at different biodiesel concentrations

5.2.5.2 Topography of the surface in the presence of microorganisms

The colonization of the polymer by microorganisms is depicted in Figure 6, slight penetration on the surface of the is observed, these pictures show how the action of microorganisms is only superficial, which is in agreement with the negligible change in mass measured by gravimetric analysis. No visual signs of deterioration were observed for control samples.

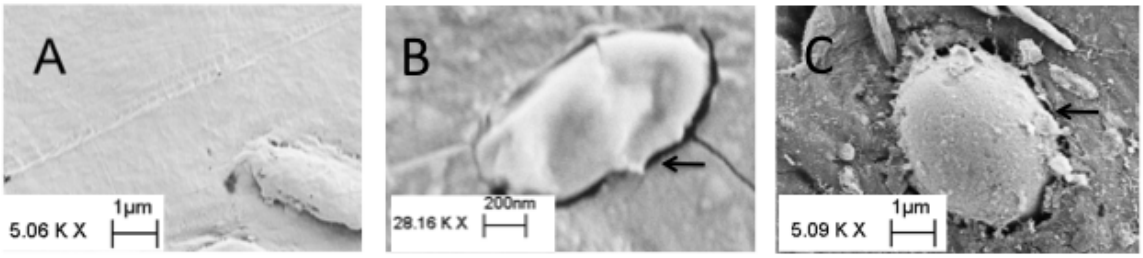

Figure 5-7. SEM images of biofilms growing on polyethylene, Bacteria penetrating the surface of the polymer are indicated by arrows

5.3 Discussion

5.3.1 Biofilm growth

Results proved that biofilm development was favored in the last part of the study at high biodiesel concentrations (B75 and B100). Biodiesel can have an impact on the amount of biofilm accumulated by three different mechanisms: it can lead to a shift in the microbial community; it can stimulate metabolic routes related with polymer colonization or can be source of surfactants in the form of mono and diglycerides that facilitate colonization of hydrophobic surfaces. In a previous study it was shown that biodiesel presence generates a shift in the composition of a community in a diesel storage tank(31), it is likely that biodiesel presence is favoring the development of microorganisms with high biofilm forming capabilities. There are two metabolic pathways that can be stimulated by the presence of biodiesel related to polymer degradation. First, the exopolysaccharide production route can be activated by mono and diglycerides (present in low concentrations in biodiesel), as has been previously proved in *Pseudomonas putida*(42). Polysaccharides are known to mediate the interaction of microorganisms with surfaces and among microorganisms in biofilms(24, 43). Second, the production of surfactants can be boosted in microorganisms, these compounds with its amphiphilic nature can mediate the interaction of the hydrophilic microbial surface with the hydrophobic polymer and facilitate the initial colonization of polyethylene by microorganisms(32). Mono and diglycerides present in low concentration in biodiesel can also act as surfactants, favoring as described before the colonization process of polyethylene by microorganisms.

5.3.2 Surface chemistry

Changes in the functional groups on the surface of the polymer indicate that microorganisms are metabolizing polyethylene. Consumption of carbonyl groups was observed as a general trend in the FTIR profiles. This kind of behavior has been observed in other works in which microbial degradation of polyethylene is studied(14, 19, 26, 44, 45). The presence of carbonyl groups indicates that oxidation is taking place in the polymer either during the molding process, in which the polymer is melted at high temperatures, or due to the presence of enzymes. Enzymatic oxidation of polyethylene has been demonstrated in a strain of *Rhodococcus ruber*(12). In any case the ability of microorganisms to utilize the polymer will depend on their ability to re-oxidize the polymer once the initial pool of carbonyl groups is depleted.

It was observed an increase with time in the contact angle with water, this indicates that the surface of the polymer is turning more hydrophobic due to the presence of microorganisms, this results are in agreement with the findings obtained by FTIR, as consumption of carbonyl groups (hydrophilic) will have an impact increasing the hydrophobicity of the surface. These results are also corroborated by the analysis of the basic component of the surface free energy, which shows a significant reduction in the course of the experiment. It is important to note that the basic component of the surface free energy is associated with electron donor groups (39), reduction in this kind of compounds is an indication of microbial respiration, and therefore evidence that the polymer is been used by microorganisms as a carbon and energy source.

Analysis of van der Waals and acid component of surface free energy reveals that these components remains virtually unchanged during the course of the experiment. Van der Waals forces (Keesom, Debye and London forces) are mainly due to dipole interactions(39). It is not likely that this kind of forces get modified due to the chemical modifications caused by microbial attack. On the other hand, the acid component of the surface free energy (electron acceptor) (39), is associated in with the presence oxygen, nitrates or sulfates that get reduced when respiration occurs, this kind of chemical species

are not likely present in the polymer which explains why their value remains constant and close to zero during this study.

Both water contact angle and basic component of surface free energy reveals that higher microbial activity is present in samples under higher biodiesel concentration (B75 and B100), it was already mentioned that addition of this fuel to the system might be driving changes in the composition of the community and probably generating activation of metabolic routes related to polyethylene degradation. Results on polymer degradation are in agreement with those obtained for biofilm formation and indicate that the higher accumulation of biodiesel can have an impact on the rate of polymer consumption.

5.3.3 Changes in the crystallinity of the polymer

Other studies have found that the crystallinity of polymer films changes with time due to the action of microorganisms(15, 17, 46), a mechanisms in which an initial increase in crystallinity due to the consumption of amorphous regions followed by a decrease in crystallinity once microorganisms start to consume small size crystals have been described (17), however in this study the effect of biotic treatment was not observed and changes in the crystallinity cannot be attributed to microbial action.

5.3.4 Changes in the topography of the polymer

The damage found in the polymer was only of a superficial nature, with no deep penetration found by the analysis, as can be observed in Figures 5 and 6. This explains why surface chemistry is modified but weight loss was not detected in the experiments. Longer experiments are required in order to get more conclusive evidence but it seems that although microbial activity on the polymer was present, it occurs very slow. In the short terms this mode of damage to plastic storage tank do not seem to be a risk; however, conclusions in the long term can not be outlined from the data collected in this study.

Many studies has been published in which important modifications of the topography of the polymer due to the action of microbial activity have been reported [40,45,46];

however, most have not described protocols to remove organic matter from the surface, it is likely that most of the effects of microorganisms described in this studies are caused by the presence of organic matter.

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Chapter 6

6 Conclusions and Recommendations

6.1 Conclusions

The community structure in the bottom of a diesel storage tank was influenced by biodiesel concentration with three different communities identified by community level physiological profiling. These results were confirmed by 16s rDNA analysis, that revealed different microbial composition after 100 days of storage for samples in pure diesel and pure biodiesel. Microbial activity in high biodiesel concentration was also different, which was confirmed by acidification of the culture media. Biodiesel community was unable to grow either in selective culture media or in most Ecoplate substrates. This outcome could be attributed to fungal and yeast development or to loss of viability due to pH drop.

The structure and composition of the community of biofilms developed on the surface of different polymer surfaces in diesel/biodiesel storage tanks is affected by the nature of the polymer and by the concentration of biodiesel used as a carbon source. This could be confirmed by analysis of the 16s rDNA libraries constructed in this study and by principal component analysis of CLPP for sessile communities, which revealed two levels of clustering, one based on the concentration of biodiesel present in the fuel phase and one based on the nature of the polymer.

Biodiesel concentration can affect both the amount of biofilm accumulated as well as the metabolic activity of the microorganisms growing on the surface of a fuel storage tank made of a polymeric material such as LLDPE, XLPE or LLDPE/PA, as can be confirmed from results of crystal violet assay and viability measurements by hydrolysis of fluorescein diacetate.

It is likely that communities developed at the bottom of storage tanks will be composed by microorganisms characterized by having one or more of these three different

metabolic features: ability to degrade hydrocarbons, production of surfactants and/or degradation of polymeric materials such as polyethylene and polyamides.

High biodiesel concentration favors the development of microbial biofilms in the bottom of fuel storage systems. In this study it was observed that this behavior was correlated with higher polymer biodegradation, and was verified as consumption of oxidized groups on the surface measured as a reduction in the keto-carbonil index, and by reduction in the electron donor groups as calculated from Yong-Dupré equation. However, the observed damage was only superficial.

6.2 Future work

The results obtained in this study can be community specific. It would be interesting to repeat this experimental procedure with communities from different sources, in order to observe if the results obtained are consistent independent on the community.

Other factors such as temperature and illumination can affect the rate of microbial degradation of polyethylene, these factors were kept constant in the present experiment results varying them can reveal further details on the susceptibility of the material to microbial attack.

In the introduction of this text it was stated that two mechanisms has been proposed to understand the effect of biodiesel on polyethylene degradation. So far all studies deal with one or the other mechanisms however a synergistic approach has not been explored and could reveal new insights in this research area.

In the area of polyethylene biodegradation in fuel systems a more mechanistic approach would be beneficial for the understanding of the mechanisms underlying the deterioration process. Studies focused on identification of enzymes related with this process or with the fate of polyethylene in the metabolism of microorganisms are still needed.

7 APPENDICES

7.1 APPENDIX I: Design of universal primers for bacteria

Universal primers for the 16s rDNA were designed in the present study, coverage and specificity were checked by comparing the probes against SILVA database. Specificity and coverage of the primers designed for this study are presented in table 7-1.

Table 7-1. Specificity and coverage of primers designed in this study

Primer	Coverage Bacteria	Coverage Archaea	Coverage Eucharia
Forward	75%	0.06%	2.5%
Reverse	46%	0.22%	0.43%

In table 7-2, the characteristics of the primers designed are summarized. Note that the annealing temperature selected for the PCR was chosen around 5 degrees below the melting temperature of the primers.

Table 7-2. Properties of the primers used in this study

Primer	Sequence	Primer length	Fragment length	GC%	Dimers	T_m
Forward	ACTCCTACGGGAGGCAGC	19	1029 PB	66.67	No	67
Reverse	CCCGGGAACGTATTCACCG	18		61.11	No	66

Amplification of bacterial RNA with this primers at different annealing temperatures is presented in figure 7-1.

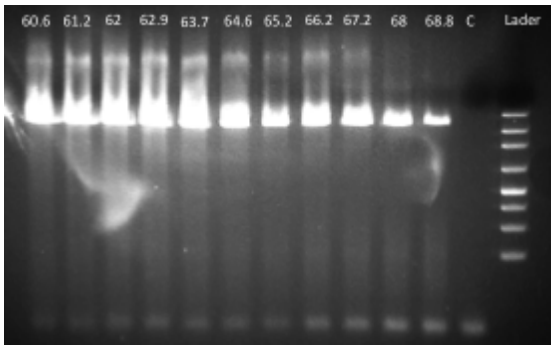

Figure 7-1. Amplification of bacterial DNA at different annealing temperatures with the primers designed in this study

7.2 APPENDIX II: DNA-ase treatment in PCR to avoid false positives

The sensitivity and universality of the primers that amplify 16s rDNA makes PCR reactions that use this kind of primers very prone to problems of contamination, either from the polymerase mixture, that can contain traces of bacterial DNA, or from the environment. This is why a pre-treatment with DNAse as detailed described in chapter 3 was developed in order to minimize this inconvenient.

Figure 7-2 shows how the treatment with DNAse helps to avoid false positives in the PCR reaction. Lines 2 and 3 correspond to a PCR reaction pre-treated with DNAse while lines 4 and 5 correspond to a PCR reaction without the pretreatment. Lines 3 and 5 are the negative controls and lines 2 and 4 are the positive controls. The results clearly show that non-treated samples present false positives.

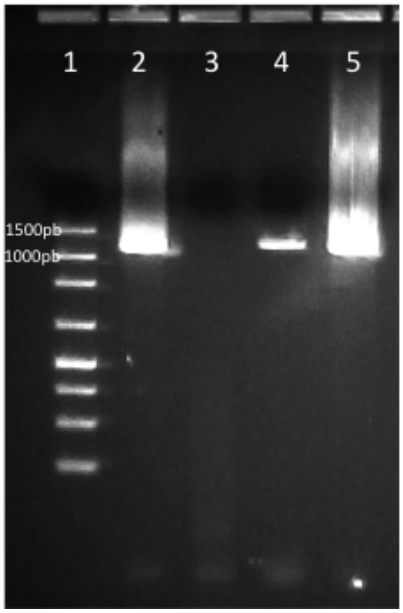

Figure 7-2. Agarose gel for treated and untreated samples with DNAse

It is interesting to note that only 0.1 units of DNAse were necessary to perform this experiments, however the dose required in a different set of conditions will depend on the concentration of initial contaminants in the PCR reaction mixture.

7.3 APPENDIX III: Calibration curve for crystal violet method

The method used for biofilm quantification is based on the unspecific absorption of crystal violet by different kinds of microorganisms; the basic idea is that the absorbance is proportional to the amount of biomass present in the biofilm. In figure 7-3 is presented a linear relation between the relative amount of biofilm and the values for absorbance obtained.

In the assay polyethylene samples with constant area were incubated with microorganism during one month to allow biofilm development, after this period of time the biofilm quantification assay described in the methodology in chapter 3 was applied to the samples. It is assumed that each samples developed the same amount of biofilm. The assay was applied first increasing amount of samples to corroborate the linearity of the test. The result obtained proves that the absorbance is proportional to the amount of biomass on the surface of the polymer.

It has to be noted that performing the test with known amounts of a strain of a microorganism (i.e *Escherichia coli*) would be irrelevant in this case, because in this research the biofilms are composed of complex microbial populations. This is why the relative amount of biofilm developed on the surface of a know area was chosen as a variable to evaluate the linearity of the test.

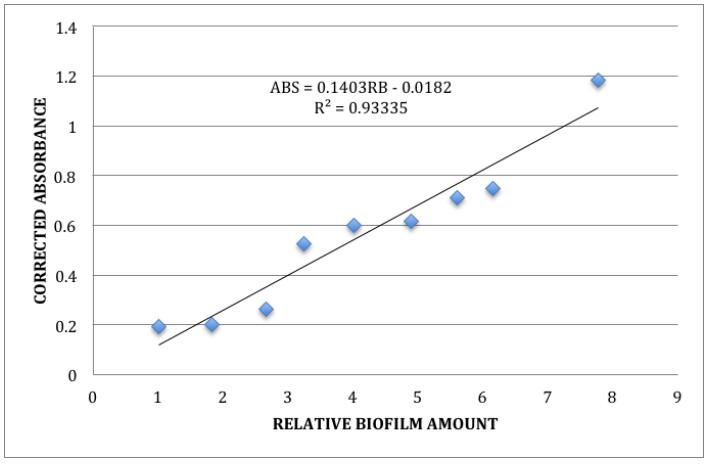

Figure 7-3. Linear relation between absorbance and relative amount of biofilm

7.4 APPENDIX IV: Changes in acidity in the fuel phase

Oxidation in the fuel phase during the experiment was followed by titration with KOH, results for different biodiesel concentrations in the fuel phase are presented in figure 7-4.

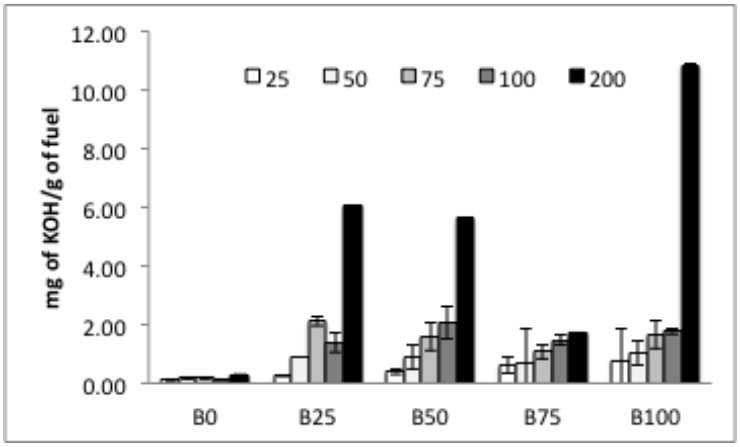

Figure 7-4. Acidity of the fuel phase determined by titration with KOH

7.5 DETAILS OF STATISTICAL ANALYSIS FOR DIFFERENT VATIABLES

7.5.1 STATISTICAL ANALYSIS FOR VARIABLES RELATED TO SUSPENDED COMMUNITY

7.5.1.1 Growth by absorbance

Table 7-3ANOVA table for the variable growth.

Effect	P-value
[Biodiesel concentration]	<0.0291
[Time]	<0.0001
[Biodiesel concentration][Time]	<0.0001

Table 7-4. Results for comparison of growth among treatments at different times during the experiment. Significant differences among treatments are shown with different letters

	0 days	50 days	75 days	100 days	200 days
B0	A	A	A	AB	A
B25	A	A	A	B	AB
B50	A	A	A	AB	A
B75	A	A	A	A	B
B100	A	A	A	AB	C

7.5.1.2 pH

Table 7-5. ANOVA table for pH analysis of the water layer during 200 days of the experiment

Effect	P-value
[Biodiesel concentration]	<0.0001
[Time]	<0.0001
[Biodiesel concentration][Time]	<0.0001

Table 7-6. Results for comparison of the pH among treatments at different times during the experiment. Significant differences among treatments are shown with different letters.

	Time 50 days	**Time 75 days**	**Time 100 days**	**Time 200 days**
B0	A	A	A	B
B25	A	A	A	A
B50	A	A	A	A
B75	A	A	A	C
B100	A	B	B	D

7.5.1.3 Heterotrophic bacteria

Table 7-7. ANOVA table for the logarithm of heterotrophic bacteria plate counts

Effect	**P-value**
[Biodiesel concentration]	<0.0001
[Time]	<0.0001
[Biodiesel concentration][Time]	<0.0001

7.5.1.4 Anaerobic bacteria

Table 7-8. ANOVA table for the the logarithm of anaerobic bacteria plate counts

Effect	**P-value**
[Biodiesel concentration]	<0.0001
[Time]	<0.0001
[Biodiesel concentration][Time]	<0.0001

7.5.2 STATISTICAL ANALYSIS FOR VARIABLES RELATED TO BIOFILM COMMUNITY

7.5.2.1 Biofilm growth by crystal violet

Table 7-9. ANOVA table for biofilm growth on different polymers at different biodiesel concentrations

Effect	P-value
[Biodiesel concentration]	<0.0001
[Polymer type]	<0.0001
[Biodiesel] [Polymer type]	<0.0001

Table 7-10. Results for comparison of biofilm growth for samples incubated at different biodiesel concentrations in the polymers used in this experiment. Significant differences among treatments are shown with different letters

	XLPE	LLDPE	PA/XLPE
B0	A	A	A
B25	A	A	A
B50	A	A	A
B75	A	A	A
B100	B	B	B

7.5.2.2 Relative viability

Table 7-11. ANOVA table for relative viability of biofilm growing on different polymers at different biodiesel concentrations

Effect	P-value
[Biodiesel concentration]	<0.0001
[Polymer type]	<0.0164
[Biodiesel concentration] [Polymer type]	0.0504

Table 7-12. Results for comparison of biofilm growth for samples incubated at different biodiesel concentrations. Significant differences among treatments are shown with different letters

B0	A
B25	BC
B50	BC
B75	B
B100	C

7.5.3 STATISTICAL ANALYSIS FOR VARIABLES RELATED TO POLYETHYLENE DEGRADATION

7.5.3.1 Biofilm growth by crystal violet

Table 7-13. ANOVA table for biofilm growing on LLDPE at different biodiesel concentrations

Effect	**P-value**
[Biodiesel concentration]	0.2059
[Time]	<0.0001
[Biodiesel concentration] [Time]	0.0046

Table 7-14. Results for comparison of biofilm growth for samples incubated in the presence of microorganisms at different biodiesel. Significant differences among treatments are shown with different letters

	0 days	**25 days**	**50 days**	**75 days**	**100 days**
B0	A	A	A	A	A
B25	A	A	A	A	A
B50	A	A	A	A	A
B75	A	A	A	A	B
B100	A	A	A	A	B

7.5.3.2 Keto-Carbonyl index

Table 7-15. ANOVA table for keto-carbonyl index of LLDPE surface for samples incubated in the presence of microorganisms at different biodiesel concentrations

Effect	P-value
[Biodiesel concentration]	0.1183
[Time]	<0.0001
[Biodiesel concentration] [Time]	<0.0001

Table 7-16. Results for comparison of keto-carbonyl index among samples incubated with microorganisms at different biodiesel concentrations at different times during the experiment. Significant differences among treatments are shown with different letters

	B0	B25	B50	B75	B100	Control
T0	A	B	A	A	A	A
T25	A	B	A	A	B	A
T50	B	AB	B	A	C	A
T75	A	A	A	A	D	A
T100	A	A	A	A	D	A

7.5.3.3 Contact angle

Table 7-17. ANOVA table for contact angle of LLDPE surface for samples incubated in the presence of microorganisms at different biodiesel concentrations

Effect	P-value
[Biodiesel concentration]	<0.0001
[Time]	<0.0001
[Biodiesel concentration] [Time]	0.0501

Table 7-18. Results for comparison of contact angle among samples incubated with microorganisms at different biodiesel concentrations. Significant differences among treatments are shown with different letters

C	A
B0	B
B25	B
B50	C
B75	C
B100	C

7.5.3.4 Basic component of the surface free energy

Table 7-19. ANOVA table for the basic component of the surface free energy of LLDPE surface for samples incubated in the presence of microorganisms at different biodiesel concentrations

Effect	**P-value**
[Biodiesel concentration]	0.0007
[Time]	<0.0001
[Biodiesel concentration] [Time]	0.0978

Table 7-20. Results for comparison of basic component of the surface free energy among samples incubated with microorganisms at different biodiesel concentrations. Significant differences among treatments are shown with different letters

C	A
B0	B
B25	B
B50	BC
B75	C
B100	C

7.5.3.5 Crystallinity

Table 7-21. ANOVA table for crystallinity of LLDPE for samples incubated in the presence of microorganisms at different biodiesel concentrations

Effect	P-value
[Biodiesel concentration]	0.6721
[Time]	<0.0001
[Biodiesel concentration] [Time]	0.1312

7.5.3.6 Weight loss

Effect	P-value
[Biodiesel concentration]	0.8283
[Time]	<0.0001
[Biodiesel concentration] [Time]	0.1626

Curriculum Vitae

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Universidad Nacional de Colombia
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2003-2008
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Publications

Conference papers

Restrepo-Florez JM. Bassi A. Thompson M. 2013. Effect of biodiesel addition on microbial community in diesel storage tanks. Preceedings of NACE international corrosion society. (USA).

Journal papers

Restrepo-Flórez JM. Bassi A. Thompson M. 2013. Degradation and deterioration of polyethyelene by the action of microorganisms-a review. International Biodegradation and Biodeterioration (Submitted)

Restrepo-Flórez JM. Bassi A. Rehmann L. Thompson M. 2013. Effect of biodiesel addtition on microbial community structure in a simulated fuel storage system. Bioresource Technology 147:456-463.

Restrepo-Flórez JM. Bassi A. Rehmann L. Thompson M. 2013. Investigation of biofilm formation on polyethylene in a diesel/biodiesel fuel storage environment. Fuel (Submitted).

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