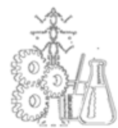
Degradation of Refractory Organic Compounds in Aqueous Wastes employing a combination of biological and chemical treatments

ANUTA CHINDRIS



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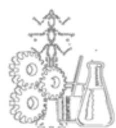

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"….I have never dedicated
anything to you because you left too early
in life but today I dedicate to my dear
father with all my heart my PhD-thesis.
I always remember what you
told me a long time ago : 'it is better to
add life to your days than to add days to
your life….",
your daughter, Anuta.

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Abstract

In this study the removal of refractory organic compounds (ROCs) in Aqueous Wastes (AW) employing a combination of biological and chemical treatment were investigated at Department of Chemical Engineering and Materials Science, University of Cagliari, Italy and Department of Engineering, Oxford University, UK.

The main objectives were to stimulate and optimise the degradation of ROCs with efficient removal of them in AW. This project is divided in two sections, a theoretical section which included a brief literature overview and subsequently an experimental section which is divided in two parts.

The first part deals with the solubilisation and biodegradation of polycyclic aromatics hydrocarbons. *Phenanthrene* was selected as model pollutant in this study which was solubilised in synthetic aqueous wastes using the mobility agents like surfactants (Tween80, Triton X100), or organic solvents (Methanol, Acetone, n-Hexane) and aerobic bacteria mixed cultures.

The second part deals with the degradation of Metal Working Fluids MWFs in real aqueous wastes employing biological and chemical treatments. Chemical and/or biological treatments are usually used to destroy the refractory compounds. The MWFs aqueous waste were provided by Microbial Solution, Oxford (UK).

The systems used for investigation consist of batch reactors using an aerobic mixed pure cultures for biological treatment and oxidants, *potassium permanganate* $KMnO_4$ and *hydrogen peroxide* H_2O_2 for a chemical oxidation treatment.

Results show the influence of both the refractory compounds and recalcitrant intermediaries on systems.

Section I

Theoretical section

Chapter 1

Introduction

The impact of industrial activities on the environment and the most relevant European Union legislation and policies for refractory organic compound are presented in this Chapter.

Introduction

Since a long time, natural processes have not been able anymore, to rectify the environmental load caused by the ever-increasing world population.

The Industrial Revolution, the creation of increasingly large manufacturing facilities has meant that the extent of potential environmental impact is escalating. Large-scale of incidents involving industrial wastes including the ROCs have focussed public attention on industrial environmental pollution.

The fate of refractory organic compounds ROCs in the environment is now well understood and government legislations are becoming increasingly the treatment and disposal of aqueous waste from commercial and industrial activities like chemicals (polycyclic aromatic hydrocarbons PAHs, MWFs), pharmaceuticals [22], [48] etc.

PAHs have received much attention since they are known to be potentially mutagenic or carcinogenic to humans and other living organisms [48, 90].

The biological and chemical processes are generally still the treatment option of choice and can be very successful in degrading ROCs [7], [36]. However, with legislative focus increasing on the fate of compounds with low biodegradability, there is an increasing demand for a combination of different treatment processes or more advanced treatment processes to "*break* " such molecules, and improve reducing environmental impact.

Several studies have been identified degradation of ROCs as a vital elimination process in wastewater treatment WWTs so improved WWTs to utilize a combination of biological and chemical treatments may be an efficient way to reduce pollution.

1.1 European Union legislations and policies

For the last 35 years the water purification research has been extensively growing. Rigorous pollution control and legislation in many countries [EEC directives] have resulted in an intensive search for new and more efficient water treatment technologies. Since the first European directive in 1976 [24], much progress has been made in tackling point source contamination of Europe's waters, but severe pressure remains regarding Priority Substances (PS).

Water, pre-requisite for life and key resource of humanity, is abundant on earth (about 1400 million km3) but 97.5% is salt water. Of the remaining 2.5% that is fresh water, only less than 1% of the world's fresh water resources are

readily available for human use and this resource is unevenly distributed [114].

The European Union (EU) pressure on water supply is especially high in arid regions with scarce fresh water resources such as the Mediterranean Basin [33].

Contamination by refractory organic substances significantly affects the viability of sustainable water reuse strategies applying treated municipal or industrial water effluents as potential alternative water sources. Since long, plant protection products, substituted phenols, non-biodegradable chlorinated solvents (NBCS) and surfactants are recognised as examples for relevant substances [24].

Adverse health and other potential effects of many contaminants present in water are still uncertain and lacking investigation. EU legislation takes into account increasing knowledge and continuously adapts the legal framework to protect and improve the quality of Europe's fresh water resources. The most recent update was the European Water Framework Directive (WFD) [29]. The most important instruments applied is the identification of Priority Substances (PS) and Priority Hazardous Substances (PHS)or "black list of the EU" table 1.1 [27], [32] that are considered to be of crucial impact concerning the future situation of the pollution of Europe's aquatic environment. Depending on the classification the identified substances are subject to strict regulation (PS) or to a planned complete phase-out of their application until 2020 (PHS).

EU water legislation is set out in the following main directives: the Dangerous Substances Directive [24],[32] and the Integrated Pollution Prevention and Control Directive (IPPC) [26] controlling pollution of surface water with dangerous substances from large industrial installations; the Drinking Water Directive [25] safe guarding human health by establishing strict standards for the quality of water intended for human consumption.

In the Lisbon strategy the EU set itself the goal of becoming "*the most competitive and dynamic knowledge-based economy in the world, capable of sustained economic growth with more and better jobs and greater social cohesion*" [28, 35]. Later, at the Gothenburg European Council in 2001 [30] and enlarged to a global scale in the Johannesburg Summit on Sustainable Development in 2002 [115] the EU committed itself to the EU sustainable development strategy based on the principle that economic growth and environmental protection should go growth too.

Group	Included substances
Policyclic aromatic hydrocarbons PAHs	Benzo-pyrene, Anthracene, Naphthalene, etc
Chlorophenols	Monochlorophenol, 2,4-dichlorophenol, 2-amino-4-chlorophenol, pentachlorophenol, 4-chloro-3-methylphenol, trichlorophenol, etc.
Chloroanilines and nitrobenzenes	Monochloroaniline, 1-chloro-2,4-dinitrobenzene, dichloroaniline, chloronitrobenezene; chloronitrotoluene, etc.
Chloride hydrocarbons	Chlorobenezene, chloronaphthalene, chloroprene, chloropropene, chlorotoluene, etc.
Solvents	Benezene, carbon tetrachloride, chloroform, toluene, ethylbenzene, dichlorometane, ethylbenzene, tetracholoroethylene, trichloroethylene, etc.
Pesticides	Cyanide chloride, 2,4-dicholorophenoxyacetic acid and derivates, DDT, pirazone, phoxime, parathion, dimethoate, dichlorvos, trifularin and derivates etc.
Other	Benzidine, chloroacetic acid, chloroethanol, vinyl chloride, xilene, diethylamine, dimethylamine, epichlorohydrine, isopropylbenzene, trichlorotrifluoroethane
Inorganic substances	Arsenic and its compounds, cadmium and its compounds, mercury and its compounds.

Table 1.1 *Black list of chemical substances selected by EU.*

There are also a number of additional wastewater problems regarding non biodegradable wastewater. The IPPC Directive [26] has requested the development of technologies and management practices for specific industrial sector for the minimisation of pollution and for the development of water recycling.

The environmental technologies play an important role in de-coupling environmental impacts from economic growth [31].

As a consequence, simple, low cost and at-hand technologies are strongly necessary to treat non-biodegradable wastewater.

A series of innovative processes and process combinations with great potential benefit compared to state-of-the-art water technologies have been identified, e.g. the combination of chemical treatment and biological treatment.

1.2 Objectives

The main purpose of this Ph.D thesis were to enhance the available knowledge based on WWTs contained the ROCs with different objectives to be achieved on:

1° Solubilisation of *phenathrene PHE*, a refractory organic compound in synthetic aqueous waste employing different mobility agents, surfactants *Tween 80*, *Triton X100*, and organic solvents *Methanol, Hexane* and *Acetone* in order to increase its bioavailability;

2° Biocompatibility and bioavailability of mobility agents used on the solubilisation of PHE against the microorganisms employed;

3° Degradation of *PHE*, in synthetic aqueous waste employing a biological treatment aerobic mixed bacteria cultures *Bulab 5733* and *Bulab 5738* in order to reduce the concentration of PHE;

4° Degradation of *Metalworking Fluids Wastewater MWFs*, a mixed refractory compounds in real aqueous waste employing a combination of biological and chemical methods in order to reduce the highest COD level. The biological method employed a pure mixed bacteria culture or consortium consisting of five different bacteria, *Agrobacterium radiobacter*, *Comamonas testosterone, Methylobacterium mesophilicum, , Microbacterium esteraromaticum, , Microbacterium saperdae*,. The chemical method employed chemical oxidants *potassium permanganate, KMnO4* and *hydrogen peroxide* H_2O_2.

1.3 Outline of the thesis

This thesis is organized into 6 chapters. The first chapter consists of a brief introduction on the impact of industrial activities on the environment and the most relevant European Union legislation and policies for refractory organic compound ROCs. The second chapter focuses on literature overview of the biological and chemical processes in fundamentals of wastewater treatments and a selection of pollutants. The third chapter focuses on the mobility agents used to increase the bioavailability of pollutants.

The fourth chapter illustrates the materials and methods used in the experimental setup of this thesis.

The fifth chapter is divided into two parts. The first part is focused on the degradation of pollutants in synthetic wastewater and it is organized around three major topics: (i) the solubilisation of selected pollutant *PHE*, using the mobility agents, (ii) the biocompatibility and bioavailablity of the mobility agents against the bacterial mixed cultures, (iii) the biodegradability of PHE and

mobility agents.

The second part of this chapter focuses on real wastewater, *Metalworking Fluids Wastewater MWFs,* and it is organised around three topics : (i) the biodegradability of *MWFs* using a aerobic mixed pure culture, (ii) the chemical degradability of *MWFs* using oxidant agents, (iii) the development of combined biological and chemical systems for biorecalcitrant *MWFs*.

Finally, the sixth chapter focuses on the conclusions and future purposes.

Chapter

2

Literature overview

In this Chapter is a brief summary of the various fundamentals processes that have been developed to treat aqueous waste and literature summary of pollutants.

2.1 Introduction

The processes and technologies available at present for wastewater treatment are very diverse. In general, the conventional processes, are often classified as preliminary, primary, secondary and tertiary treatment [53].

-Preliminary treatment is designed to remove the debris and sandy materials from the wastewater.

-Primary treatment is the second step in treatment and separates solids and greases from wastewater. Wastewater is held in a sedentary tank for several hours allowing the particles to settle to the bottom and the greases to float on the top. The solids drawn off the bottom and skimmed off the top receive further treatment as sludge. The clarified wastewater flows on to the next stage of wastewater treatment. Clarifiers and septic tanks are usually used to provide primary treatment.

-Secondary treatment is a biological treatment. In the biological treatment of wastewater the objectives are to coagulate and remove the nonsettleable colloidal solids and to stabilize the organic matter. For domestic wastewater the major objective is to reduce the organic content. For industrial wastewater, the objective is to remove or reduce the concentration of organic and inorganic compounds [2]. The microorganisms absorb organic matter from sewage as their food supply. Two approaches are used to accomplish secondary treatment by aerobic means: fixed biomass and suspended biomass systems.

- Fixed biomass systems grow microorganisms on solid supports such as rocks, sand or plastic. The wastewater is spread over the supports, allowing the wastewater to flow past the film of microorganisms fixed to the solid supports. As organic matter and nutrients are absorbed from wastewater, the film of microorganisms grows and thickens. Trickling filters, rotating biological contactors, and sand filters are examples of suspended biomass systems.

- Suspended biomass systems stir and suspend microorganisms in wastewater. As the microorganisms absorb organic matter and nutrients from the wastewater they grow in size and number. After the

microorganisms have been suspended in the wastewater for several hours, they are settled out as sludge. Some of the sludge is pumped back into the incoming wastewater to provide "seed" microorganisms. The remainder is wasted and sent on to the sludge process. Activated sludge, extended aeration, oxidation ditch, and sequential batch reactor system are all examples of suspended biomass systems.

-Tertiary treatment focuses on removal of disease-causing organisms from wastewater. Treated wastewater can be disinfected by adding chlorine or by using ultraviolet light. High levels of chlorine may be harmful to aquatic life in receiving streams. Treatment systems often add a chlorine-neutralizing chemical to treated wastewater before stream discharge.

The conventional process treating domestic (DWW) and industrial degradable wastewater (IWW) are presented in figure 2.1.

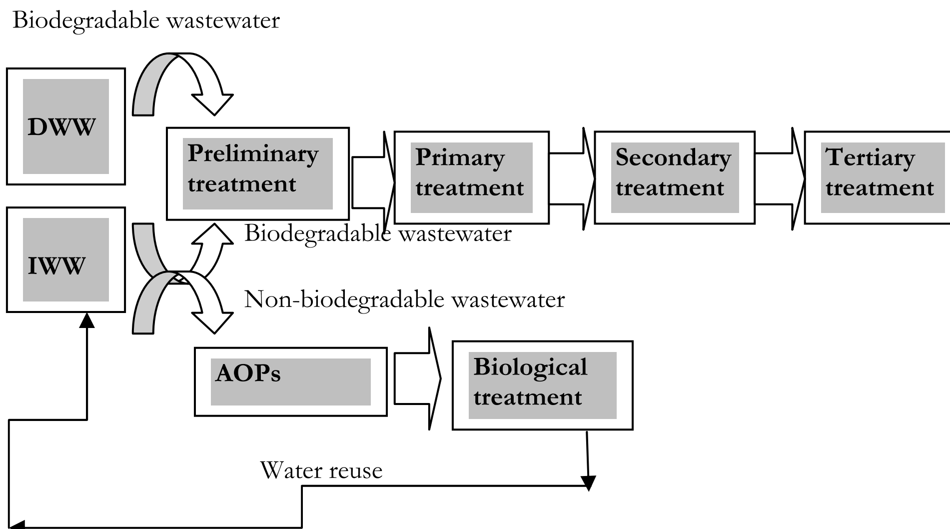

Figure 2.1 *Schematic representation of conventional wastewater treatment.*

2.1.1 Secondary treatment or biological treatment

2.1.1.1 Introduction

The biological treatment [51] is recognised as the best available technology by the EU for many applications.

Nevertheless, there exists a great number of anthropogenic activities that

generate wastewater containing non-biodegradable or even toxic substances, which cannot be treated with biological treatment. Example for such activities are agriculture [14], [39], industry [26] or the household [3].

The biological wastewater treatment systems are designed to remove the dissolved organic load from the waters using microorganisms. The microorganisms used are responsible for the degradation of the organic matter. Biological treatment encompasses basically aerobic and anaerobic treatment.

2.1.1.2 Aerobic treatment

Aerobic wastewater treatment converts the organic pollutants (COD, BOD) in wastewater into a fair amount of excess sludge, and oxidizes the rest with oxygen (air) to carbon dioxide. In aerobic operation the oxygen is essential for successful operation of the systems.

The most common aerobic processes are activated sludge and lagoon, active or trickling filters, and rotating contactors.
However, the operating costs for aeration, sludge treatment and disposal are high so that, whenever feasible, anaerobic treatment systems are preferred.

2.1.1.3 Anaerobic treatment

Anaerobic treatment proceeds with breakdown of the organic load to gaseous products that constitute most of the reaction products and biomass. Anaerobic treatment is the result of several reactions; the organic load present in the waste water is first converted to soluble organic material which in turn is consumed by acid producing bacteria to give volatile fatty acids and carbon dioxide and hydrogen. Further more anaerobic processes are also sensitive to temperature [100].

In general, when comparing a conventional aerobic treatment with anaerobic treatment systems the advantages of anaerobic systems are [100]:

- Lower treatment costs and production energy;
- High flexibility, since it can be applied to very different types of effluents;
- Smaller volume of excess sludge;
- High organic loading rate operation;
- Anaerobic organisms can be preserved for a long time, which make it

possible to treat wastewater that is generated with longer pauses in between.

2.1.1.4 Some fundamentals of microbiology

2.1.1.4.1 Introduction

Basic to the design of a biological treatment process, or to the selection of the type of process to be used, is an understanding of the form, structure, and biochemical activities of the important microorganisms, [75].

The microorganisms are commonly grouped into three kingdoms : protista, plants and animals as shown in table 2.1

While the most significant differences among protists, plants and animals, the three kingdoms are similar. The cell is the basic unit of life for each, regardless of the complexity of the organism and the most living cells are quite similar as shown in figure 2.2.

They have a cell wall, which may be either a rigid or a flexible membrane and if they are motile, usually possess flagella. The interior of the cell contains a colloidal suspension of carbohydrates, proteins and other complex organic compounds, called cytoplasm.

Kingdom	**Representative members**	**Characterization**
Protista	Prokaryotic cells (bacteria, blue-green algae, etc) Eukaryotic cells (algae, fungi, protozoa	Unicellular or multicellular, without tissue differentiation
Plant	Mosses Ferns Seed plants	Multicellular, with tissue differentiation
Animal	Crustaceans Rotifers	

Table 2.1 *The three kingdoms of microorganisms.*

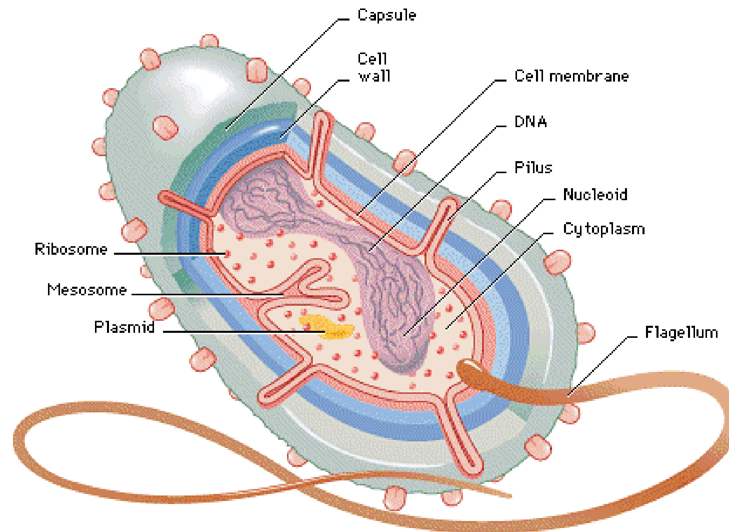

Figure 2.2 *Internal structure of bacterial cell.*

The cytoplasmic area contains ribonucleic acid (RNA), whose major role is in the synthesis of proteins. Also within the cell wall is the area of the nucleus, which is rich in deoxyribonucleic acid (DNA). DNA contains all the information necessary for the reproduction of all the cell components. In the eukaryotic cells the DNA is enclosed by a membrane and the nucleus is clearly defined. In the prokaryotic cells the nucleus is poorly defined.

In order to reproduce and function properly the organisms must have [75]:

- A source of energy;
- A source of carbon for synthesis of new cellular material;
- Inorganic elements or nutrients such as nitrogen and phosphorus, and other trace elements such sulphur, potassium, calcium, and magnesium are also vital to cell synthesis.

Since all these elements must be derived from environment, a shortage of any of these substances would limit and, in some cases, alter growth. If an organism derives its cell carbon from carbon dioxide it is called autotrophic, if it uses organic carbon it is called heterotrophic. For autotrophic organisms, the energy needed in the synthesis of new cellular material, it can be supplied by the sun as in photosynthesis or by an inorganic oxidation-reduction reaction, in this

case the organisms are called autotrophic chemosynthetic. For heterotrophic organisms, the energy needed for cell synthesis is supplied by the oxidation or fermentation of organic matter [78].
The organisms can also be classified according to their ability to utilize oxygen in:

- Aerobic organisms –they can exist only when there is a supply of molecular oxygen;
- Anaerobic organisms – they can exist only in an environment that is void of oxygen;
- Facultative organisms – they have the ability to survive with or without free oxygen.

The organisms used in this research belong to protista kingdom, prokaryotic cells and they are called bacteria microorganisms.

2.1.1.4.2 Bacteria

Bacteria are single cell protists and they utilize soluble food. Their usual mode of reproduction is by binary fission. Their general form falls into one of three categories: spherical, cylindrical and helical and they vary widely in size 0.5 to 1.0 μ in diameter for the spherical, 0.5 to 1.0 μ in width by 1.5 to 3.0 μ in length for the cylindrical (rods) and 0.5 to 5 μ in width by 6.0 to 15.0 μ in length for the helical (spiral) [47], [75].

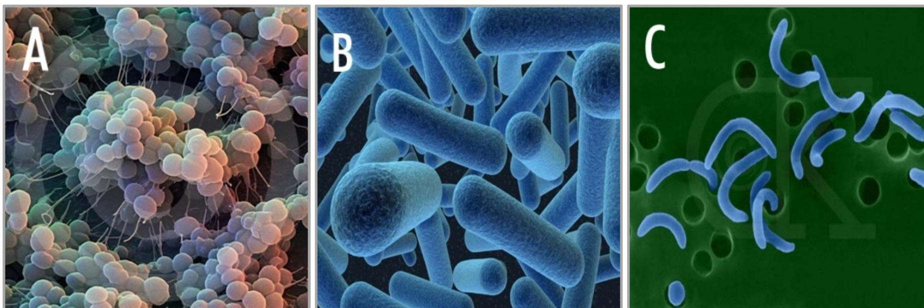

Figure 2.3 *General forms of bacteria (A-spherical form; B-cylindrical form and C-spiral form).*

Tests on a number of different bacteria indicate that they are about 80% water and 20% dry material, of which 90% is organic and 10% is inorganic. An

approximate formula for the organic fraction is $C_5H_7O_2N$, and as indicated by the formula about 53% by weight of organic fraction is carbon. Temperature and pH play a vital role in the life and death of bacteria. According to the temperature range, bacteria may be classified as psychrophilic (-2-30 ºC), mesophilic (20-45ºC) and thermophlic (45-75ºC).

The pH of a solution is also a key factor in the growth of organisms. Generally, the optimum pH for growth lies between 6.5 and 7.5. In a batch culture, pH can change during growth as a result of metabolic reactions that consume or produce acidic or basic substances as wastes [75].

2.1.1.4.3 Bacterial growth

The effective environmental control in biological waste treatment is based on an understanding of the basic principle governing the growth of microorganisms.

Generally, bacteria reproduce by binary fission where the original cell becomes two new organisms. The time required for each fission, which is termed the generation time, can vary from days to less then 20 minutes. The more cells present, the more turbid the suspension [47], [75]

The general growth pattern of bacteria is shown in Fig.2.4.

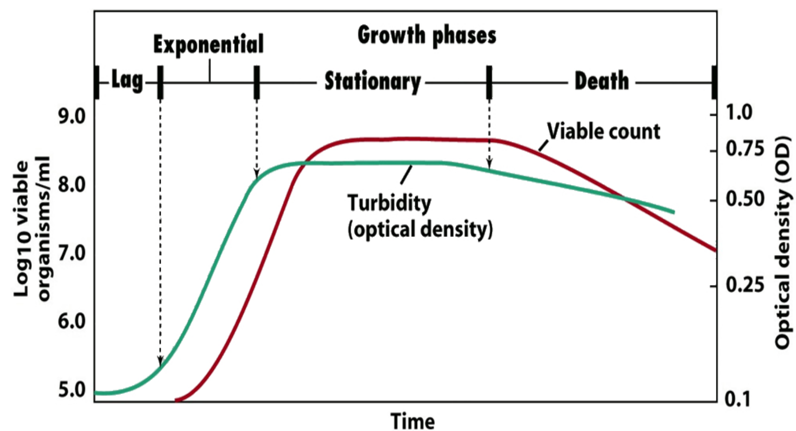

Figure 2.4 *Typical bacterial growth curve.*

Initially, a small number of organisms are inoculated into a culture medium, and the number of viable organisms is recorded as a function of time. The growth cycle of the batch culture can be divided into several distinct phases

called [47],[75], [78]:

- *The lag phase* represents the time required for the organisms to acclimate to their new environment upon addition of inoculums to a culture medium.
- *The log growth phase or exponential log phase* is marked by rapid exponential growth. During this period the cells divide at a rate determined by their generation time and their ability to process food. The nutrients are metabolised at maximum speed until one of the nutrients is depleted and starts limiting growth.
- *The stationary phase*. During this phase the population remains stationary and this phenomenon is explained by i) that the cells have exhausted the substrate or nutrients necessary for growth, ii)the growth of new cells is offset by the death of the old cells.
- *The log death phase* is usually a function of the viable population and environmental characteristics. During this phase the bacterial death rate exceeds the production of new cells.

The growth patterns especially the rate of the exponential growth could be influenced by environmental conditions such as temperature and components of the culture medium as well as the genetic characteristics of the bacteria [47].

Different growth rate can be used for bacterial growth, like growth in terms of bacterial mass, growth in terms of bacterial numbers and growth in terms of bacterial mixed cultures.

The survival and growth of a microbial species in a mixed culture depends on the nature and extent of interactions with the other species present. Amongst the types of interactions which can occur between different species in a mixed culture are neutralism, competition, commensalisms, mutualism, symbiosis and predation [109].

To ensure that the microorganisms will grow, they must be allowed to remain in the system enough to reproduce. This period depends on their growth rate, which is related directly to the rate at which they metabolise or utilize the waste. Controlling the growth rate of the microorganisms can ensure effective waste stabilisation.

In batch and continuous culture systems the rate of growth of bacterial cells can be define by the following relationship:

$$r_g = \mu X \qquad (2.1)$$

where r_g is the rate of bacterial growth g L^{-1} s^{-1};
μ is specific growth rate, s^{-1}
X is concentration of microorganisms g

Because $\frac{dX}{dt} = r_g$ for both culture the following relationship is also valid for batch reactor:

$$\frac{dX}{dt} = \mu X \qquad (2.2)$$

In batch culture, if one of the essential requirements (substrate and nutrients) for growth were present is only limited amounts, it would be depleted first and growth would cease, in continuous culture, growth is limited. Experimentally it has been found that the effect of limiting substrate or nutrient can often be defined adequately using the following expression proposed by Monod (1949):

$$\mu = \mu_m \frac{s}{k_s + s} \qquad (2.3)$$

where μ is specific growth rate, s^{-1};
μ_m maximum specific growth rate, s^{-1};
s-concentration of growth limiting substrate in solution, g L^{-1};
k_s half velocity constant, substrate concentration at one-half the maximum growth rate, g L^{-1}.

If the value of μ is substituted in Eq (2.1), the resulting expression for the rate growth rate is:

$$r_g = \frac{\mu_m X s}{k_s + s} \qquad (2.4)$$

In both batch and continuous-growth culture systems, a portion of substrate is converted to new cell and a portion is oxidized to inorganic and organic end products [78]. Because the quantity of the new cell produced has been observed to be reproducible for given substrate, the following relationship has been developed for the rate of substrate utilization and the rate of growth.

$$r_g = -Y r_{su} \qquad (2.5)$$

Y-maximum yield coefficient, g/g (defined as the ratio of the mass of cell formed to the mass of substrate consumed);

r_{su}-substrate utilisation rate, g L^{-1} s^{-1}.

On the bases of laboratory study, it has been concluded that yield depends on (i) the oxidation state of the carbon source and nutrient elements, (ii) the degree of polymerization of the substrate, (iii) pathways of metabolisms, (iv) the growth rate, and (v) various physical parameters of cultivation.

If the value of rg from Eq.2.3 substituted in Eq.2.4, the rate of substrate utilization can be defined as follows:

$$r_{su} = -\frac{\mu_m XS}{Y(k_s+S)} \qquad (2.6)$$

In Eq. (2.6) the term μ_m/Y is often replaced by the term k, defined as the maximum rate of substrate utilization per unit mass of microorganisms:

$$k = \frac{\mu_m}{Y} \qquad (2.7)$$

If the term k is substituted for the term μ_m/Y in Eq (2.7), the resulting expression is:

$$r_{su} = -\frac{kXS}{(k_s+S)} \qquad (2.8)$$

In bacterial systems used for wastewater treatment, the distribution of cell age is such that not all the cells in the system are in the log-growth phase. Consequently, the expression for the rate of growth must be corrected to account for the energy required for cell maintenance.

Other factors, such as death and predation, must also be considered. Usually, these factors are lumped together, and it is assumed that the decrease in cell mass caused by them is proportional to the concentration of organisms present. This decrease is often identified in the literature as the endogenous decay. The endogenous decay term can be formulated as follows:

$$r_d \text{ (Endogenous decay)} = -k_d X \qquad (2.9)$$

where k_d=endogenous decay coefficient, s^{-1}.

The corresponding expression for the net specific growth rate is given by Eq(2.10), which is the same as the expression proposed by Van U (1967):

$$\mu' = \mu_m \frac{S}{K_s + S} - k_d \quad (2.10)$$

where μ ' = net specific growth rate, s^{-1}.

The effect of endogenous respiration on the net bacterial yield are accounted for by defining an observed yield as follow:

$$Y_{OB} = \frac{r'_g}{r_{su}} \quad (2.11)$$

so another way to write eq(2.11) is:

$$\mu' = Y_{OB}\mu_m \frac{S}{K_s + S} - k_d \quad (2.12)$$

The temperature effect of the biological reaction-rate constant is very important in assessing the overall efficiency of a biological treatment process. Temperature not only influences the metabolism activities of the microbial population but also has an effect on such factors as gas-transfer rates and settling characteristics of the biological solids.

The effect of temperature on the reaction rate of a biological process is usually expressed in the form expressed by eq(2.11), [75], [78].

2.1.2 Tertiary treatment or chemical treatment

The incapacity of conventional biological wastewater treatment to remove effectively many industrial recalcitrant and/or toxic pollutants, evidences that efficient tertiary treatment, *chemical oxidation*, systems are needed. It can be used as pre-treatment step to the biological treatment.

Chemical oxidation is an important and effective method to degrade organic compounds. The objective of degradation is mineralisation i.e. conversion of the target compound to carbon dioxide, water and –depending on the nature of the compound – inorganic ions like e.g. nitrate, chloride, sulphate and phosphate. In practice, complete mineralisation is normally not request, except for extremely dangerous materials. In many cases it is both justified and efficient to partially degrade the target compound in order to enable further degradation by

microbiological treatment. For that case, the chemical oxidation step is needed to destroy persistent molecular structures, to remove high ecotoxicity and enhance water solubility.

Classical and conventional oxidation technologies include the application of oxidants for either disinfection of pathogenic contamination or the oxidation of pollutants, [78]. The classical oxidation treatment consists of adding to the water that contain contaminants and an oxidizing agent.

The most commonly employed oxidising agents are:

- *Chloride*: represent strong and cheap oxidizing agent that can be used efficiently for waste treatment. The disadvantage is the low selectivity, the case that imply high dosage and chloro-organics by-products formation;
- *Oxygen*: is considered as a moderate oxidising agent;
- *Hydrogen peroxide*: is one of the most proposed oxidizing agent, it can be applied alone or with catalyst, but sometimes the peroxide alone do not work with some organic matter. It is recommended for a large variety of systems;
- *Ozone*: it is strong oxidising agent and it has the same advantage as oxygen and hydrogen peroxide. The disadvantage it is the difficulty and the instability of gas controlling;
- *Potassium permanganate*: is a very strong oxidant able to react with many functional groups inorganic or organic substances such secondary alcohols, chlorinated solvents, aldehydes, sulphides, etc. $KMnO_4$ is decomposed in water resulting in formation of manganese dioxide and gaseous oxygen.

There is another group of chemical-oxidative processes, called Advanced Oxidation Processes (AOPs), [94]. AOPs are characterised by the generation of hydroxyl radicals. It is therefore able to oxidise and mineralise almost every organic molecule yielding CO_2 and inorganic ions. Different techniques exist to generate hydroxyl radicals. The most important groups of AOPs are:

- Direct photolysis of oxidants (H_2O_2, O_3) or water with high energy UV radiation;
- Fenton and Fenton-like processes with transition metals;
- Cavitations technique (hydrodynamic and ultrasound).

Commonly applied treatment methods for COD removal from wastewater consisted of integrated processes involving various combinations of chemical and biological methods [2]. In the first process the toxic and /or recalcitrant compounds would be eliminated until the point where no longer inhibition due to their toxicity and/or non-biodegradability. In order to be able to combine chemical process outlet with biological process, it is necessary to determine the variation in biodegradability as a function of the chemical reaction and in the last years the study has been increased in this area.

2.2 Selection of pollutants

2.2.1 Polycyclic aromatics hydrocarbons PAHs

2.2.1.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are most commonly found environmental pollutants that belong to hydrophobic organic compounds (HOCs) based on their properties.

The fate of PAHs in nature is of great environmental and human health concern due to their carcinogenic, mutagenic and teratogenic properties [89], [101], [123] as well as their high concentration and frequency found in the environment [58], [70], [106]. Some PAHs metabolites bind to protein, DNA, and RNA, and adducted compounds may cause damage to cell and cause carcinogenic effects [49].

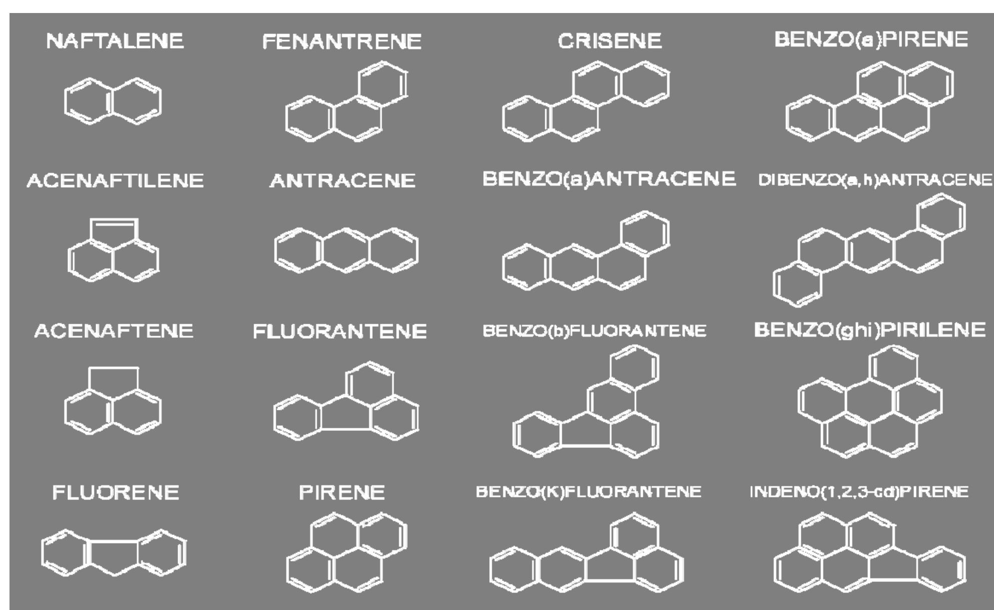

Figure 2.5 *The 16 PAHs classified priority pollutants by EPA.*

These facts make PAHs priority pollutants needed to be controlled [61], [119]. Sixteen PAHs are listed and regulated by Environment Protection Agency EPA, as priority pollutants due their toxicity and mutagenicity as shown in the figure 2.5.

During the last three centuries, a relationship between higher incidence of cancer in urban and industrial areas than in rural areas, and the exposure of humans to PAHs, have prompted considerable research on the sources, occurrence, bioaccumulation, metabolism, and disposition of these pollutants in aquatic and terrestrial ecosystems [8], [9], [99].

2.2.1.2 PAHs in the environment

Occurrence of PAHs in the environment is due to both natural and anthropogenic processes [76]. Natural PAHs come from petrogenic and phytogenic sources [83], [85]. However, most PAHs in the environment possessing potential hazard to human health are anthropogenically produced.

PAHs are major components of crude oil, coal tar, creosote and wastes from the combustion of fossil fuel, coal gasification and liquefaction, and incineration of industrial wastes [49], [123]. Several theories on PAH pyrosynthesis have been proposed and extensive reviews on this subject are available [4].

PAHs enter the environment from a multiplicity of sources which include: wet and dry deposition, chronic leakage of sewage or industrial effluents, accidental discharges during the transport, use and disposal of petroleum products, or from natural sources such as oil seeps and surface water run-off from forest and prairie fire sites [40], [42], [56], [68], [70], [78], [86].

The possible fates of PAHs in the environment include volatilisation, photooxidation, chemical oxidation, bioaccumulation, adsorption to soil particles or sediments, and leaching and microbial degradation. PAHs are highly persistent in the environment.

2.2.1.3 Chemical–physical properties of PAHs

PAHs are nonpolar hydrophobic organic compounds and rapidly become associated with soil particles or sediments, where they may become buried and persist for long periods. They contain carbon and hydrogen with fused benzene rings in linear, angular or cluster arrangements. PAHs may also contain alkyl and nitro substituents and can be considered heterocyclic molecules with the substitution of an aromatic ring carbon with nitrogen, oxygen or sulphur.

Generally, PAHs of higher molecular weight, containing four or more fused rings, pose the greatest hazard to both environment and human health (USA EPA, 1982).

This is a consequence of the resistance of PAHs to decomposition processes [127], their high affinity for organic matter [69] and their low water solubility [92]. Table 2.2 shows the properties of some polycyclic aromatic hydrocarbon PAHs. As shown in Table 2.2 and figure 2.5, the solubilities of PAHs decrease as the number of benzene rings increases. Even though PAHs have low solubility in water, their dissolution can contaminate large amounts of ground water for long periods [74].

Their lipophilicity, environmental persistence, and genotoxicity increase as the molecular size of PAHs increases up to 4 or 5 fused benzene rings, and their toxicological concern shifts towards chronic toxicity, primarily carcinogenesis [56], [80].

PAHs	Molecular mass [Da]	Vapor pressure [Pa]	Log octanol-water partition coefficient	Solubility [mg/L]	Ionization potential [Ev]
Naphthalene	128.18	12.0	3.58	30	8.13
Acenaphthene	154.20	4.02	3.92	3.6	7.86
Acenaphthylene	155.20	3.87	3.90	3.88	8.22
Phenanthrene	178.24	0.0161	4.46-4.63	1	7.91
Anthracene	178.24	0.001	4.45	0.015	7.43
Benzo[a]anthracene	228.30	20.0×10^{-5}	5.9	0.01	7.6
Fluorene	166.23	0.13	4.18	2.0	7.89
Fluoranthene	202.26	0.001	5.22	0.25	7.95
Pyrene	202.26	0.0006	5.88-6.7	0.1-0.12	7.44
Benzo[a]pyrene	252.32	7.0×10^{-7}	5.79-6.4	0.001-0.006	7.7

Table 2.2 *Properties of some polycyclic aromatic hydrocarbons PAHs.*

2.2.1.4 Bacterial degradation of PAHs

The microbial degradation of PAHs has been known to be strongly correlated with PAHs water solubility, rather than with the degree of condensation of the PAHs: cluster against linear arrangement of the same number of rings [123]. Initial activation and oxidation of PAHs involves enzymes, called oxygenases. Oxygenases produced by microorganisms catalyze an oxygen-fixing reaction. The two groups of oxygenases (monooxygenases and dioxygenases) are both used for microbial degradation [75].

In order to degrade PAHs, initial ring oxidation is required and it is the rate-limiting step for PAH degradation. For ring oxidation, bacteria produce enzyme dioxygenases which can induce the incorporation of two oxygen atoms into the substrate to form dioxethanes. Oxygen incorporated dioxethanes are then sequentially oxidized to *cis*-didihydroxy products and then eventually to catechol [123].

During the past decade, a number of PAH degrading bacteria have been isolated and characterized for their ability to degrade different PAHs [16], [18], [38]. PAHs are degraded by bacteria through two degradation mechanisms. First is mineralisation and the other is co-metabolism. In mineralisation, bacteria can use some PAHs as their sole carbon and energy source.

Most studies have been conducted with two to four ring PAHs, and a few studies have been reported on bacterial degradation of PAHs with five or more

rings, in both environmental samples and pure culture [60], [97]. In the case of high molecular weight PAHs, most research has been focused on the five-ring molecule, benzo[a]pyrene [81]. For these high molecular weight PAHs, it has been reported previously that co-metabolism is the major degradation mechanism [60].

Aerobic biodegradation has been extensively studied during the past few decades and is currently regarded as the most common form of bioremediation applied to PAH contaminated soil. It is generally believed that molecular oxygen is required for PAH bioremediation. However, recent studies show that low molecular weight PAHs (mostly naphthalene, some phenanthrene) can be degraded with the use of nitrate, and possibly sulfate, as the electron acceptors [95]. It has also been reported that the presence of alternative electron acceptors, in addition to oxygen, can also stimulate enhanced hydrocarbon degradation [123].

2.2.2 Metalworking Fluids

2.2.2.1 Introduction

Metalworking fluids (MWFs) are part of a more general product family called lubricants and are widely used by manufacturing industries, including the automotive industry, for the lubrication and cooling of metal tools during machining processes [12]. They remove small metal chips, reduce the friction between work pieces, optimise tool life and provide protection against corrosion as well as improving the finished quality of the manufactured tools [52].

There are many varieties of metalworking fluids and lubricants used in metal fabrication. Initially it is necessary to determine whether the product itself is classified as a hazardous waste.

Metalworking fluids may also become hazardous during use because they ‘pick up’ other waste materials. Therefore, the chemical component of the wastes reflect not only the *original makeup* of the process fluid, but also the operation and conditions of their use. Many metalworking fluid wastes contain higher percentages of lubricating oil and suspended solids (dirt), and metal fines than they do metalworking fluid. If working with metals other than carbon steel, there is a possibility that *heavy metals* (such as cadmium, copper, chromium, lead, mercury, nickel, silver, zinc) in the fluid waste will result in it being classified as

hazardous waste.

In the UK industry alone, over 400,000 m^3 of spent MWFs are produced annually and the disposal costs are estimated to range between £8 and £16 million per year [20].

2.2.2.2 History of metalworking fluids

Metal working fluids (MWF) have been employed in industry since the Egyptians, for cooling and lubrication, during cutting and grinding of metal.

Towards the industrial revolution, scientific studies on friction started to appear and the impact of friction on moving parts and metalworking processes started to be appreciated.

The use of MWF was concomitant with the development of machine tools and the need to accelerate manufacturing processes. The need of cooling and lubricating to enhance productivity appeared. Nevertheless, [77] reports that four elements added to the industrial revolution and the invention of almost all machine tools, led to the development of modern metalworking fluids.

The first element is the discovery of petroleum that had a profound impact on the compounding of metalworking fluids. Mineral oil then replaced vegetable and animal based lubricants.

The second element is the introduction of better alloys for making harder tools and therefore working at faster speeds and higher pressure, increased the need to use good quality MWF.

The third element is the introduction of electrical power source that initiated the design for more powerful and sophisticated machinery. This new development increases stress between tools and work piece and the need for removing metal parts efficiently led to an even greater need to develop metalworking fluids.

The fourth essential element was the growth of industrial chemistry and petrochemistry that allows the manufacture of modern metalworking fluids, leading to the creation of soluble oil, emulsions, sulphurised, chlorinated compounds and additives.

Semi-synthetic and synthetic oils were introduced in 1940's [73]. The history of development that can be found in [77], including 94 references and shows that this development is totally interdependent on the development of machinery and interconnection of sciences as industrial chemistry, friction, and

lubrication.

Recently, metalworking fluids are also developed to take into account workers health, working comfort, environmental and disposal concerns.

2.2.2.3 Chemistry of Metalworking Fluids

MWFs can be classified in two main categories : oil based and water based. Oil based MWFs are further divided into straight oils and soluble oils. The water based MWFs can also be divided into synthetic and semi-synthetic MWFs In the table 2.3 are summarised a detailed classification and composition of MWFs[10], [11].

They usually contain additional Extreme Pressure (EP) additives (organic or inorganic additives) to improve performance and lengthen the life of the fluid and can represent less than 0.1% up to 30% of their organic contents.

EP formulations contain chlorinated, sulfurised, or phosphorus-type extreme pressure ingredients.

The additives of MWFs and their main contribution to MWFs properties were reviewed by [1].

In the table 2.4 are listed the constituents of a generic water soluble MWFs formulation.

The MWFs can be gas (mainly air), liquids or solids such as organic or inorganic involving surface coating technology. More descriptions can be found in specialized literature such as [59].

Type		%Petroleum oil in concentrate	Water content	Compostion
Oil based	**Straight oil**	100 %	Little or no water	Can contain animal, marine and vegetable oil. Many oils do not contain additives.
	Soluble oil	50-90%	Oil in water emulsion	Contain once processed or reprocessed mineral oil. Chelating agents are added to maintain the emulsion as well as biocides to avoid biological degradation of emulsifiers.
Water based	**Synthetic**	0%	Mixture of highly soluble organics	Contain various compounds such as hydrocarbon based polyalphaoleins, polyethylene/polypropylene glycols or ester oils as their basis.
	Semi-synthetic	2-50%	Combination of soluble and synthetic oils	Typically contain 2 to 50% of mineral oil and also compounds used in synthetic MWFs.

Tabel 2.3 *Classification and composition of MWFs adapted from [1].*

Component	Chemistry	Function
Basic oil	Paraffinic hydrocarbon	Lubrication and some corrosion protection
Lubricity additive	Phosphate ester	Provides extra lubricity
Corrosion additive	Triethanolamine/amine borate	Resists the onset of corrosion
Emulsifier	Tall oil	Stabilizes the base oil as an emulsion
Antifoam additive	Siloxanes	Reduces the formation of foam in the diluted fluid
biocides	Formaldehyde condensate	Preservation of the dilute fluid
Coupling agent	Glycol ether	Holds all the additives together in the concentrated form

Tabel 2.4 *Example of the individual components sometimes used to formulate a MWFs adapted from [110], [117],[118].*

In addition to these compounds, spent MWFs contain contaminants such as particulate or dissolved metals, cleaning agents as well as microbial contaminants responsible of their rancid odours.

A full range of MWF exist in the market with various chemical compositions.

The properties of MWFs are important when choosing which MWFs to use for a specific application. More details on metalworking fluid chemistry, formulation and their application can be found in literature such as [107].

2.2.2.4 Biodegradation of Metal Working Fluids

The limitation in biodegrading MWFs resides in the fact that oil is insoluble in water and tends to make the organic fraction unavailable to the microorganisms. In the particular case of water-based MWF, the organic compounds are dissolved or emulsified. Therefore, their constituents are more readily available to microorganisms.

It is important to know the composition of MWFs to assess and optimise the effectiveness of treatment options with an emphasis on biological processes the efficiency of which is very dependant on the toxicity of the waste.

The organics contained in MWFs are susceptible to biodegradation [1].

The biological processes are increasingly adopted as an alternative treatment method of spent MWFs with the potential of substantial cost savings [62], [63], [66], [67],[110], [111]. For efficient treatment, the MWFs wastes should be highly biodegradable and non toxic to micro-organisms.

The potential of treating a simulated MWFs waste made of eight cutting fluids had evaluated by [62], [63], [66] in aerobic and anaerobic systems and they obtained for the combined anaerobic/aerobic process, 65% COD removal occurred anaerobically and 23% aerobically. They also reported that almost complete degradation of organics containing nitrogen in MWFs such as alkanolamines and amino acids were possible anaerobically or aerobically.

Activated carbon as a polishing method for treating MWFs wastes where MWFs were absorbed on activated carbon was studied by [52].

[84] used a bench scale aerobic sequencing batch reactor (SBR) for the treatment of an industrial water based MWF with an initial COD of 20,200 mg/L and reported 87% COD removal which is similar to the value reported by [66].

The efficiency of biodegradation processes though depends on the chemical composition of the individual wastes under investigation [112] and it is known that polycyclic aromatic and quaternary carbon atoms hydrocarbons are more bioresistant than other simpler chemical structures [1], [13], [122].

The possible methods for the treatment of a spent MWF ultrafiltration permeate with a COD of 1390 mg/L were screened by [79]. They considered chemical oxidation with chemicals such as *sodium dichromate* or *potassium permanganate* among other oxidants, chemical oxidation with UV/H_2O_2 or UV/O_3 known as advanced oxidation processes, biological treatment and evaporation.

Advanced oxidation processes (AOPs) have been investigated for the treatment of industrial wastewaters [104] and could be an alternative for the treatment of spent MWFs. However the literature is limited on this subject. Due to the presence of non biodegradable carbon in MWFs, AOPs could be used after biological treatment for the removal of residual recalcitrant carbon or before biological processes to increase the biodegradability or reduce the toxicity of the wastes.

Chapter

3

Mobility agents used to increase the bioavailability of ROCs

A brief summary of the various mobility agents and their role to increase the bioavailability of ROCs are presented in this chapter.

3.1 Organic solvents

3.1.1 Characteristics of solvents

Organic solvents are chemical class of compounds that are used routinely in commercial industries. They are liquids which have the ability to dissolve, suspend or extract other materials. They make it possible to process, apply, clean or separate materials. They share a common structure, low molecular weight, lipophilicity and volatility. They may be grouped further into aliphatic-chain compounds, such n-hexane and aromatic compounds such as benzene. Alcohols, ketones, are substitutions for a hydrogen group. Organic solvents are useful because they can dissolve oils, fats, resins, rubber, and plastic.

They can be classified into two categories : *polar* and *non-polar* solvents. The physical properties of solvents used in this project are shown in table 3.1.

Solvent		Chemical formula	Boiling point [ºC]	Dielectric constant	Density [g/mL]
Non-polar	**Hexane**	C_6H_{14}	69	1.88	0.655
Polar	**Acetone**	C_3H_6O	56	21	0.786
	Methanol	CH_3OH	65	33	0.791

Table 3.1 *Physical properties of some organic solvents.*

General health hazards associated with organic solvent exposure include toxicity to the nervous system, cancer, damage of internal organs like liver and kidneys, brain, and dermatitis.

3.1.2 Remediation application of organic solvents

The organic solvents are able to enhance the bioavailability and biodegradation of hydrophobic organic compounds (HOCs) like PAHs [116], [6].

The first solvents used were almost certainly hydrocarbons, such as ethanol from wine spirits.

Ancient Syrians found that heating wood sealed away from the air resulted in

a variety of tars and liquids. One of the liquids, now called methanol by chemists, proved to be a useful solvent and fuel.

Over the past three decades, the solvents industry has implemented measures to address concerns about environmental performance, especially related to air quality. In fact, air quality in Europe has continuously improved during this period.

Most organic solvents rapidly biodegrade in the soil or wastewater, i.e., they do not bio-accumulate or persist in the environment, and have relatively low ecotoxicity [37],[91], [122].

Most bacteria and their enzymes are destroyed or inactivated in the presence of organic solvents. Organic solvent tolerant bacteria are a relatively novel group of extremophilic microorganisms that combat these destructive effects and thrive in the presence of high concentrations of organic solvents as a result of various adaptations. These bacteria are being explored for their potential in industrial and environmental biotechnology, since their enzymes retain activity in the presence of toxic solvents. Bacterial strains reported to grow on and utilize saturated concentrations of organic solvents such as toluene can revolutionize the removal of such pollutants. It is now known that enzymes display striking new properties in the presence of organic solvents [126].

3.2 Surfactants

3.2.1 Characteristics of surfactants

Surfactants are a group of surface active agents and they are amphipathic molecules consisting of a hydrophilic polar head moiety and a hydrophobic nonpolar tail moiety.

The hydrophobic parts have little affinity for polar solvents, while the hydrophilic parts have strong affinity for polar solvents such as water and prevent the surfactant molecules from being completely separated from the polar solvent. Therefore, the co-existence of hydrophobic and hydrophilic parts makes a surfactant have high surface activity.

The most common hydrophobic parts of synthetic surfactants are the alkyl chain of fatty acids, paraffins, olefins, alkylbenzenes, alcohols, alkylphenols, or polyoxypropylene; the hydrophilic moiety can be a sulphonate, sulphate, carboxylate (anionic), quaternary ammonium (cationic), sucrose, polypeptide, or

polyoxyethylene (nonionic).

Surfactants can also be of natural (biosurfactants) and anthropogenic (synthetic surfactants) occurrence. Many microorganisms can produce their own surfactants i.e. biosurfactants. However, only anthropogenic or synthetic surfactants are of environmental and health concern since they are widely used in daily life and in almost all industrial sectors.

According to the nature of the hydrophilic moieties of the surfactant molecule, a surfactant can be classified by the ionic charge associated with their polar moiety : *nonionic - no charge*, *anionic – negative charge*, *cationic – positive charge*, and *zwitterionic* (*amphoteric*)*- positive and negative charge*.

Of the various types, the anionic surfactants are commercially and quantitatively the most important [76].

The major classes of biosurfactants include glycolipids, phospholipids, fatty acids, lipopeptides/lipoproteins, and biopolymeric surfactants.

At low concentration, surfactants are fully soluble in water but when the concentration is increased, in order to decrease the free energy of the system, hydrophobic groups begin to aggregate and build up the core of a cluster. This small aggregate is called a "*surfactant micelle*" and the formation of micelles is called micellization. The critical micellization concentration (CMC) is the lowest concentration at which micelles begin to form. The CMC becomes different according to temperature, surfactant type and structures.

Different shapes and sizes of micelles may be present depending on the surfactant structure and type. Surfactant micelles have received much attention because micelles are able to solubilise hydrophobic organic compounds which have low water solubility through incorporation of them into the hydrocarbon-like core of the micelle.

This process is called 'micellar solubilisation'. Micellar solubilisation of hydrophobic organic compounds can be described as the partitioning of hydrophobic organic compounds between the micelle cores of surfactant and surrounding water.

The activity of surfactants to the aqueous phase can be expressed in quantitative terms with the parameter HLB (Hydrophile Lypophile Balance):

$$HLB = 0.2\frac{MW_{hydrophilic\ part}}{MW_T}$$

3.2.2 Remediation application of surfactants

The surfactants are believed to be able to enhance the solubility and bioavailability of hydrophobic organic compounds (HOCs) [120], [125]. Therefore, there is currently a growing interest on the behaviour of surfactants in the environment and their influences on solubility, adsorption/desorption, migration, bioavailability, degradation of HOCs [46], [54], [96], [98], [120].

The bioavailability increased with surfactant addition can be attributed at two main mechanisms:

- First, the surfactants can reduce the interfacial tension between the aqueous phase and the non-aqueous phase liquid (NAPL). The presence of the surfactant can disperse the NAPL and increase the contact area which can give enhanced bioavailability for microorganisms;
- Second, the surfactants can increase the aqueous solubility of less soluble organic compounds significantly so can provide more available substrate for microorganisms.

Some researchers [103] proposed three possible mechanisms to explain the enhanced biodegradation of PAH by the surfactant addition and they are:

- The first is that bacteria might be able to utilize micelle portioned PAHs directly through the cell membrane;
- The second is that surfactant can increase mass transfer to the aqueous phase, and bacteria might subsequently use the aqueous phase PAHs;
- The third is that the surfactant might change the hydrophobicity of the cell in order to enhance the direct cell attachment to PAHs or NAPL.

There have been many positive reports about the increased bioavailability by surfactant addition [15], [41], [64].

When microorganisms could use dissolved substrate only, non-ionic surfactants were reported to enhance the bioavailability of PAHs [45] therefore the non-ionic surfactants (e.g., Tween 80, Triton X100) increased bioavailability of PAHs by the *Pseudomonas saccharophila* strain is showed by [15].

The similar results indicating that Triton X-100 did not inhibit phenanthrene mineralization at concentrations above the critical micelle concentration.

Some researchers [45] reported that a fraction of the micellar phase

phenanthrene was directly bioavailable, and concluded that the bioavailability fraction of the micellar phase substrate was independent of the biomass concentration and was a function of the surfactant concentration, the polyoxyethylene surfactant chain length and the surface characteristics of the biomass.

Even though many researchers reported that surfactants have also shown other adverse effects:

- First, due to the increased mobility of PAHs by surfactant addition, fairly immobile PAHs can begin to move and to spread through the subsurface aquifer. Therefore, the coupled transport of PAHs with surfactants can cause contamination of larger amounts of groundwater than normal treatment would have. Also, the surfactant itself could spread and contaminate the groundwater;
- Second, surfactants can cause toxic effects on PAH degrading bacteria. Nonionic surfactants are reported to be less toxic than ionic surfactants [120]. In 1975 [50] explained the toxic effect of surfactants on bacteria through two possible mechanisms. They reported that the surfactant might disturb cellular membranes by interaction with lipid components, or surfactant molecules can react with proteins in the bacteria which are essential to the functioning of the cell;
- The third adverse surfactant effect on PAH degradation is depletion of oxygen by the surfactant degrading bacteria, and preferential surfactant utilization by PAH degraders[50], [65];
- Fourth, the surfactant can inhibit bacteria adhesion to PAH contaminated soils or NAPLs, which would induce a decrease in the bioavailability of PAHs [11], [108].

Recent studies have reported that some bacteria can utilize PAHs directly without being dissolved into the aqueous phase [21], [23], [57].

Some researchers reported that bacteria overcome the limitation of bioavailability of PAHs by direct attachment and biofilm formation on the compounds in order to increase the PAH diffusion rate into the cell and facilitate the degradation rate of the slightly water soluble compounds [43], [44], [93]. A study conducted by [22] observed direct biofilm growth on pyrene and

phenanthrene crystals without any other available carbon source. These results indicated that bacteria can attach on soil phase PAHs and form a biofilm in order to utilize the solid substrate and overcome mass transfer limitations of the poorly soluble PAHs. In that case, the addition of surfactant might inhibit bacterial adhesion and biofilm formation on the PAHs and eventually decrease the PAH degradation rate.

The overall impact of surfactant addition on PAH biodegradation depends on how the PAH-utilizing bacteria take up or respond to the basic diffusion pathways of PAHs in soil, in addition to the toxicity or utilization of surfactants [120].

Currently, bacterial responses to synthetic surfactants have not shown robust results, even though much research has been conducted.

Overall, when using surfactants for bioremediation, evaluating the effects of surfactants on the bioavailability of PAHs is crucial for surfactant-enhanced PAH bioremediation. In addition, desirable surfactant characteristics such as effectiveness (solubility at ground water temperatures, low CMC, low adsorption to soil, cost), biodegradability and lower toxicity to humans, animals and plants, need to be considered [45], [120].

Surfactants have received attention as environmental and human health concerns not only due to their direct toxicity [17], [102] but also to their impacts on environmental processes and on the fate and behaviour of other contaminants to a great extent [46].

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Section II

Experimental section

Chapter 4

Materials and methods

In this chapter are generally summarized the materials and analytical methods employed. Particularly details for each experiment are given in the next chapter.

4.1 Materials

4.1.1 Bacteria used for biological treatment

The microorganisms used for synthetic PHE wastewater are different from those used in real MWFs wastewater.

The microorganisms used for synthetic wastewater were a mixed bacterial culture Bulab 5733 and Bulab 5738 containing many different kinds of bacterial strains including *Rhodococcus, Aeromonas, Streptomyces, Bacillus, Pseudomonas.* These bacteria were selected, adapted, increased in pure culture, collected and preserved by freeze drying before being mixed in the final formulation. In addition to bacteria, the mixed bacterial culture contains amylase, cellulose and lipase in the form of free enzymes and these have an important role in the initial phase of bacterial growth.

After being rehydrated, the microorganisms become metabolically active in a short time and then compete with each others and form an active biomass. The strains are suitable for the formation of flakes and produce extracellular biopolymers useful for the development and maintenance of a good glomerular structure.

They are capable to degrade different substrate and are suitable for the biodegradation of constituents of very different nature, almost everywhere present in wastewater.

The role played by each of these strains is determined by the mechanism of natural selection of the population.

The mixed bacterial culture appears as a light brown granular powder containing lyophilized microorganism on a substrate of bran cereal and freeze dried enzymes figure 4.1 and the optimal conditions of their efficiency are indicated in the table 4.1.

Figure 4.1 *Bacterial mixed cultures Bulab 5733* & *5738.*

Parameter	Minimum	Optimal	Maximum
pH	5	7	9
O2dissolved [mg/L]	1	2	-
C/N/P	100/5/1	100/7/1	100/10/1
T[°C]	7.5	25	45

Tabel 4.1 *Optimal conditions for the growth of mixed culture Bulab 5733* &*5738.*

The microorganisms used for MWFs wastewater were a mixed pure culture or consortium consisted of five different bacteria, *Agrobacterium radiobacter, abbrev. form :AG RAO*, *Comamonas testosterone, abbrev. form : COM TEST, Methylobacterium mesophilicum, abbrev. form : METH MES, Microbacterium esteraromaticum, abbrev. form : M EST, Microbacterium saperdae*, *abbrev. form: MIC SAP* and were isolated from MWFs wastewater as described by [117], [118].

4.1.2 Bacterial liquid cultures

The culture medium used was minimal salts medium, MSM, for synthetic

wastewater, Tryptic Soy Broth ,TSB, and MSM for MWFs wastewater. The MSM is a synthetic solution obtained by dissolving seven salts in deionised water in sufficient concentration to ensure the ideal conditions for microbial growth (macro and micro elements) and especially the right amount of nitrogen and phosphorus.

The composition of the minimal salts medium referred to an organic load of 1000 mg/L glucose is shown in the table 4.2.

Salts	Concentration [mg/L]
$MgSO_4$	100
$(NH_4)_2SO_4$	500
$FeSO_4$	0,5
$CaSO_4$	30
Na_2HPO_4	873
KH_2PO_4	527
Deionised water	to 1L

Tabel 4.2 *Composition of minimum salts medium, MSM.*

The two salts, (Na_2HPO_4, KH_2PO_4) and their concentrations were chosen to ensure pH close to neutrality. For each growth test, the MSM has been prepared taking into account the concentration of organics in it that had to be dissolved in order to maintain the recommended ratio between carbon, nitrogen and phosphorus.

The phosphate buffered saline solution PBS, was used for washing the cell biomass because it contains isotonic concentration of ions to prevent bacterial cells bursting from osmotic pressure which may be caused in deionised water.

Culture medium used for MWFs was employed at 10% w/v Tryptic Soy Liquid Broth with the following preparation:

-To 500 ml deionised water, 1.5 g of Tryptic Soy Broth (Oxoid Limited, Basingstoke, Hampshire UK) was added by stirring and transferred to 250 mL bottle flasks and autoclaved.

4.1.3 Reactors used for aerobically microbial growth

All the operations for growth and acclimatization of microbial culture were

carried out in batch reactors of two types : Erlenmeyer baffled bottom flasks, working volume 50 mL, aerated on a rotary shaker at a temperature of 25ºC (figure 4.2. A) and laboratory fermenter LKB 1601 Ultroferm Fermentation System provided with control of agitation and temperature, working volume 3 L, temperature 25 ºC, figure 4.2 B and bench top fermenter provided with control of agitation and temperature, working volume 1.5 L, figure 4.2 C.

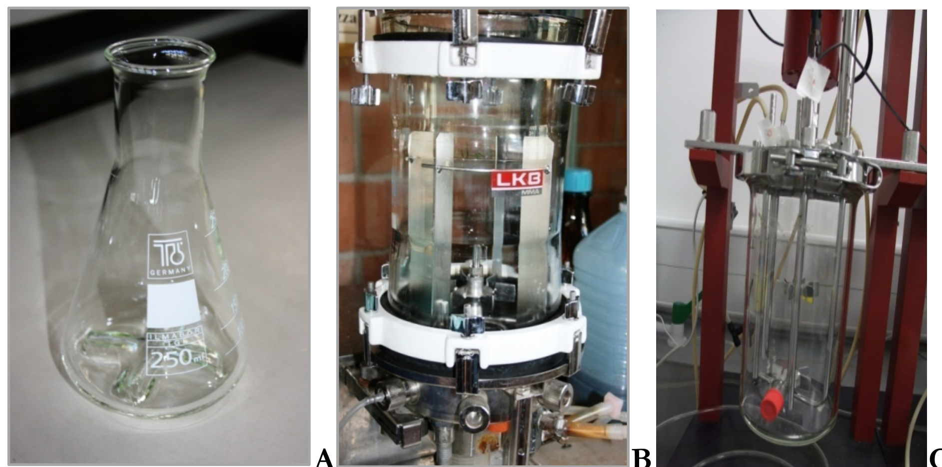


Figure 4.2 **A**-*Erlenmeyer baffled bottom flasks*; **B**-*Fermentatore LKB 1601 Ultroferm Fermentation System;* **C**-*bench top fermenter*.

4.1.4 Bacterial growth on plates

A loopful of the pure bacterial culture was inoculated aseptically by streaking onto sterile nutrient agar plate and incubated in incubator at 30ºC for 24 hours. Preparation of 10% w/v Tryptic Soy Broth plus Agar for plates are described following:

-To 500 ml deionised water, 1.5 g of Tryptic Soy Broth (Oxoid Limited, Basingstoke, Hampshire UK) was added by stirring. Then 6 g of purified agar Technical 3 (Oxoid Limited, Basingstoke, Hampshire UK) was added by mixing and sterilized by autoclaving, allowed to cool to 55ºC, then poured onto media plates.

4.1.5 Bacterial growth on liquid broth

A loopful of bacterial culture growth into Tryptic Soy Broth plusAgar (TSB-Agar) plate was inoculated aseptically into Erlenmeyer flask (250 mL) containing fresh sterile TSB 10% v/v (100 mL).

The five strains were inoculated separately into 250 mL conical flask. The individual cultures were incubated at 28ºC in an orbital shaker for 24 hr at 180 rpm. Upon growth, the bacterial culture was used in the subsequent experiments.

The cell suspension were removed and centrifuged at 4000 rpm for 20 minutes. After that the cell biomass was washed with buffer solution PBS and re-centrifuged at 4000 rpm for 20 minutes. This operation was repeated thrice in order to eliminate the excess of TSB 10% . The biomass was mixed together and suspended in MWFs wastewater at 10% (v/v) inoculums into the bioreactors.

4.1.6 Model pollutants

The polycyclic aromatic hydrocarbon used in this research was Phenanthrene (PHE) and it was obtained from Merck.

4.1.6.1 Phenanthrene

Phenanthrene is one of the polycyclic aromatic hydrocarbons and it was selected as a model pollutants in this research project. It is composed of three fused benzene rings ($C_{14}H_{10}$), with five possible resonance structure and is isomer with anthracene. Phenanthrene is more stable than its linear isomer anthracene. The chemical structure of phenanthrene is shown in figure 4.3.

Phenanthrene derived from coal tar melts and it is insoluble in water but is soluble in most organic solvents such as toluene, carbon tetrachloride, ether, chloroform, acetic acid and benzene.

It is white crystalline substance with a bluish fluorescence. It is used in the synthesis of dyes, explosives and drugs. The principal characteristics are shown in table 4.3.

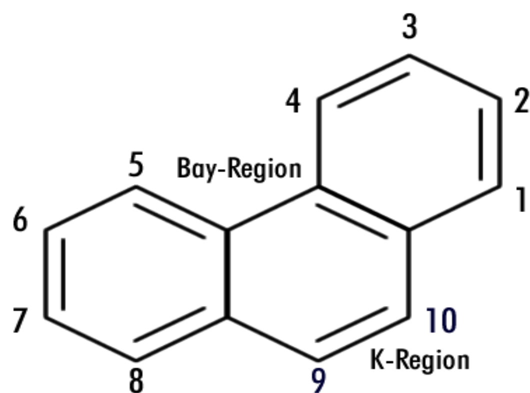
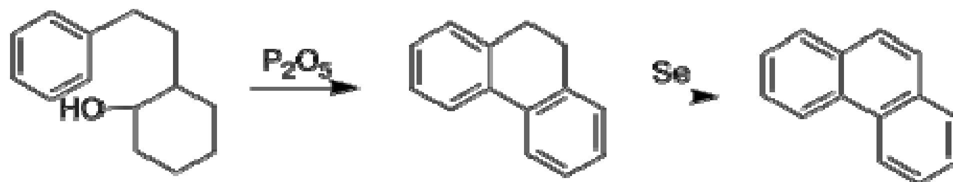


Figure 4.3 *Chemical structure of Phenanthrene.*

A classical phenanthrene synthesis is the *Bardhan-Sengupta Phenanthrene Synthesis* (1932). The first step is simply an electrophilic aromatic substitution reaction, which is allowed when the diphosphorous pentoxide makes the alcohol a better leaving group. In the second step of this reaction 9,10-dihydrophenanthrene is oxidized with elemental selenium.



Phenanthrene	
IUPAC name	Phenanthrene
CAS number:	85-01-8
Molecular formula	$C_{14}H_{10}$
Molecular weight	178.23 g/mol
Boiling point	99 °C
Solubility in water	insoluble
Solvent solubility	Soluble in benzene, carbon tetrachloride, diethyl ether, ethanol ,acetone, hexane.

Tabel 4.3 *Characteristics of Phenanthrene.*

In the case of PHE the reagents applied in the degradation experiments were also applied for standard preparation.

4.1.6.2 Metalworking fluids

The Metal Working Fluids, MWFs used in the second research part was provided by Microbial Solution, Oxford, UK.

4.1.7 Reagents applied in experiments and analysis

All experiments were performed in distilled water matrix. Ultrapure water was obtained from a Millipore Milli-Q® system. Milli-Q® water was applied for preparation of all analytical standards and for eluent preparation and sample dilution in high performance liquid chromatography.

4.1.7.1 Organic solvents

The organic solvents used were Methanol (METH, 99.8%, Sigma-Aldrich,), Acetone (98%) and n-Hexane(96%) were obtained from Merck and their characteristics are presented in the following tables, table 4.4 to 4.6.

Methanol	
IUPAC name	Methanol
Others names	Hydroxymethane; methyl alcohol, wood alcohol
CAS number:	67-56-1
Molecular formula	CH_4O
Molecular weight	32.04 g/mol
Boiling point	64.7°C
Solubility in water	miscible
EU classification	Flammable, toxic.

Tabel 4.4 *Characteristics of Methanol.*

Acetone	
IUPAC name	Propanone
Others names	Dimetilchetone, β-chetopropano, acetone
CAS number:	67-64-1
Molecular formula	C_3H_6O
Molecular weight	58.08 g/mol
Boiling point	56.53 °C
Solubility in water	miscible
EU classification	Flammable, irritant.

Tabel 4.5 *Characteristics of Acetone.*

n-Hexane	
IUPAC name	Hexane
Others names	n-hexane
CAS number:	110-54-3
Molecular formula	C_6H_{14}
Molecular weight	86.18 g/mol
Boiling point	69 °C
Solubility in water	13 mg/L at 20°C
EU classification	Flammable, harmful, dangerous for the environment.

Tabel 4.6 *Characteristics of n-Hexane.*

All solvents applied in chromatography were HPLC grade.

4.1.7.2 Oxidising agents

The oxidising agents used were potassium permanganate and hydrogen peroxide obtained from Merck and their characteristics are presented in the following tables, table 4.7 and 4.8.

Potassium permanaganate	
IUPAC name	Potassium manganate VII
Other names	Potassium permanganate, Chameleon minerals, Condy's crystals
CAS number	7722-64-7
Molecular formula	$KMnO_4$
Density	2.703 g/cm^3
Melting point	240 ºC decomp.
Solubility in water	6.38 g/100 mL (20 ºC)
EU classification	Oxidant, harmful, dangerous for environment.

Table 4.7 *Characteristics of Potasium permanagante.*

Hydrogen peroxide	
IUPAC name	Dihydrogen dioxide
Other names	dioxidane
CAS number	7722-84-1
Molecular formula	H_2O_2
Density	1.463 g/ cm^3
Boiling point	150 ºC
Solubility in water	miscible
EU classification	Oxidant, corrosive and harmful.

Table 4.8 *Characteristics of Hydrogen peroxide.*

4.1.7.3 Surfactants

The surfactants employed Tween 80, Triton X100 were obtained from Across.

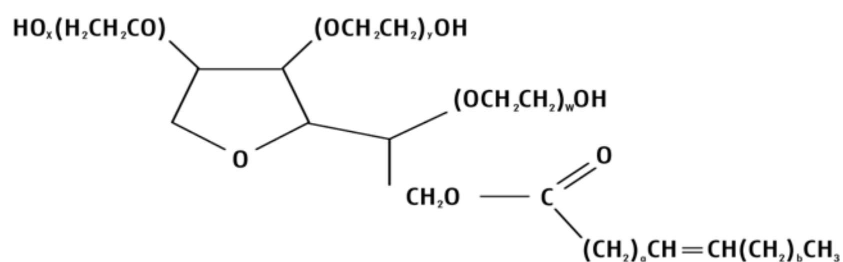


Figure 4.4 *Chemical structure of Tween 80.*

TWEEN 80	
IUPAC name	Polyoxyethylene (20) sorbitan monooleate
Other names	(x)-sorbitan mono-9-octadecenoate poly(oxy-1,2-ethanediyl) Tween 80
CAS number	9005-65-6
Molecular formula	$C_{64}H_{124}O_{26}$
Density	1.06-1.09 g/mL, oily liquid
Boiling point	>100°C
Solubility in water	Very soluble

Tabel 4.9 *Characteristics of Tween 80.*

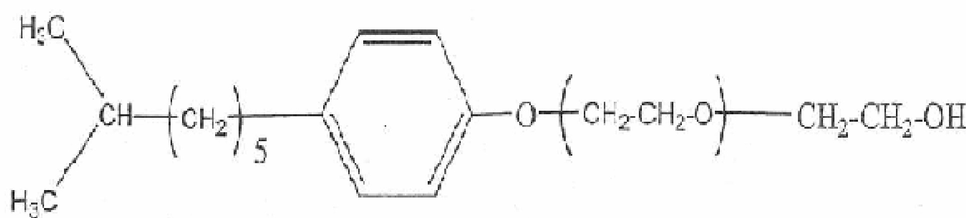


Figure 4.5 *Chemical structure of Triton X100.*

TRITON X 100	
IUPAC name	Triton X100
Other names	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether 4-octylphenol polyethoxylate
CAS number	9002-93-1
Molecular formula	$C_{14}H_{22}O(C_2H_4O)_n$, (n= 9-10)
Density	1.07 g/cm^3
Boiling point	>200°C
Solubility in water	soluble

Table 4.10 *Characteristics of Triton X100.*

All the glassware and other apparatus were carried out by autoclaving at 121°C, 103 kPa for 15 minutes.

Autoclaving is a standard laboratory technique which was used repeatedly throughout this project. It is an essential step in experiments involving bacteria to avoid contamination. Any biological waste produced was also autoclaved

prior to disposal.

4.2 Methods

4.2.1 Measure of microbial growth

The growth of a bacterial population is measured in time following the variation of cells number or cell mass.

There are several methods to count the cell number or estimate the cell mass; the choice of methods to use depends of the type of microorganism and the problem will be addressed. In these trials were monitored the variation of cell mass in time. The two techniques for measuring cell biomass are:

- *Optical Density* – OD, measurement of optical density of the samples;
- *Dry Weight* – DW, determining the dry weight of cell mass on a fixed volume of suspension. It is a direct method of measuring the cell mass.

The measurement of optical density is based on the determination of the amount of light scattered by a suspension of cells using a spectrophotometer.

This measure is based on the fact that when the particles are uniformly suspended in a liquid, their power to disperse the light beam through the suspension reducing the amount of the light transmitted and gives a measure of cell density. The turbidity of a sample is dependent on cell size, their shape and color, the range of refractive indices in the liquid and the wavelength of light.

Whether all these parameters are kept constant, the optical density is proportional to cell density.

These measurements were made using a spectrophotometer Schimadzu UV-1801 at a wavelength of 600 nm, figure 4.6 B.

The determination of the dry weight is a direct method of measuring cell and it included the following steps:

-from the batch reactors is collected a microbial suspension volume at a pre-determinated time in order to verify the growth. These samples are placed in the freeze in order to stop the microbial growth and consumption of substrates;

-once the wet, each samples are filtered through a filtration unit (fig. 4.6 A) with cellulose acetate membrane filters with a diameter of 45 mm and pore size diameter 0.45 μm;

-the membrane filters have been dried before and after use in an oven (fig. 4.6 C) at a temperature of 105°C for one hour and placed into desiccators (fig. 4.6 D) for 30 minutes. This procedure is repeated until reaching the stability of the weight;
-according to the weight difference of membrane filters before and after filtration it is assessed the concentration of biomass in terms of g/L for each sample.

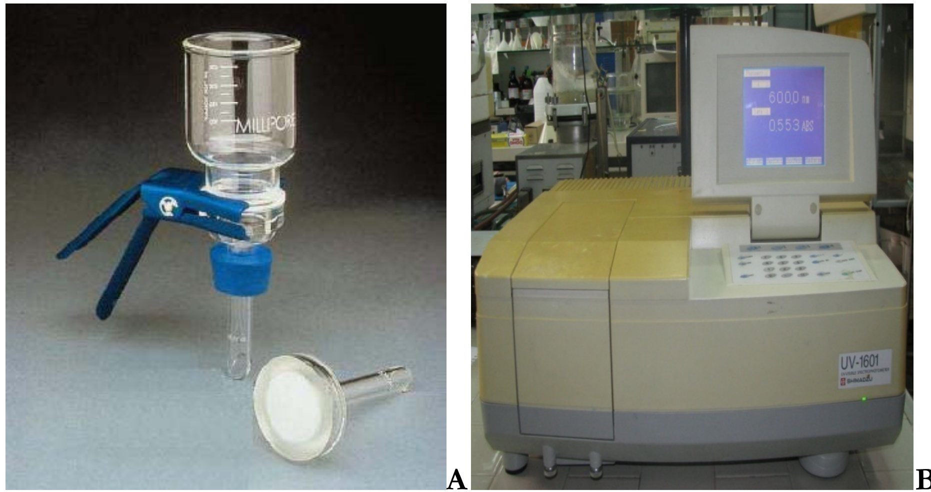


Figure 4.6 A-*Filtration unit;* **B**-*Spectrophotometer Schimadzu UV-1801.*

Figure 4.7 A- *Oven*; **B**-*Dessicator.*

4.2.2 Procedure for rehydration microbial

The microorganisms were supplied freeze dried on a support of bran base, so before they could be used in experiments they were first rehydrated and then acclimated on easily biodegradation substrate.

The procedure for the rehydration consist on mixing 10 g of bacterial mixed culture and add 90 mL of minimal salts medium in a beaker. After that the suspension was placed for agitation by magnetic stirrer for one hour at 30ºC.

Then the mixture was left to settle for 30 hour because in this way we separate the bran from the liquid phase which containing the microorganisms. The liquid phase was then centrifuged for 15 minutes at 6000 rpm. After centrifugation, the solid phase were taken up with medium and were inoculated in Erlenmeyer flasks and/or bench fermenter for the next phase, the acclimatisation.

4.2.3 Procedure for microbial acclimatisation

The phase of acclimatisation preceded each experiment. This procedure has served both to have a sufficient amount of biomass and to use active and microorganisms able for degrading substrates for growth in the solution in which they were placed.

The carbon source used during the growth was glucose, GLU and tryptic soy broth,TSB.

We conducted three cycles of acclimatisation before any aerobic test when GLU was the carbon substrate (table 4.11) and only one cycle when TSB was a carbon substrate (table 4.12): the first, lasting 14 hours on a concentration of 1000 mg /L of GLU, the second lasting 10 hours more in 1000 mg /L of GLU, the third lasting 12 hours, at a concentration of 2000 mg /L of GLU.

Normally the first and the second cycles of acclimatisation were carried out in the Erlenmeyer flask and the third round in the fermenter in order to have a greater amount of biomass in view of the planned test. It is operated in such way as to ensure that each microorganisms were subjected to the same type of acclimation (on time and quantity), that for each growth test the microorganisms had undergone the same pre-treatment and therefore the experiments were comparable.

Microorganisms centrifuged were washed with PBS solution and suspended in a solution containing glucose GLU, and/or tryptic soy broth TSB up to a value of optical density of 0.1.

The solution was so divided in many bioreactors, each with a volume of 100 mL. After the first cycle, lasting 14 hours, the broth was centrifuged-washed-re-centrifuged and the microorganisms have been placed in a new fresh solution containing the substance selected for the acclimatisation, to start a new cycle of growth, only for glucose. At each stage the concentration of microorganisms increased.

Cycle	Glucose [mg/L]
1st	1000
2nd	1000
3rd	2000

Tabel 4.11 *Glucose cycles of acclimatisation of microorganisms.*

Cycle	Tryptic soy broth [mg/L]
1st	1500

Tabel 4.12 *Tryptic soy broth cycle of acclimatisation of microorganisms.*

At the end of the third cycle, lasting 12 hours, the microorganisms were collected at their stationary phase, centrifuged-washed (with PBS)-re-centrifuged figure 4.8. and after inoculated in synthetic wastewater.

Figure 4.8 *Centrifuges A* & *B.*

4.2.4 Determination of the bacterial growth rate

The rate of bacterial growth in the various tests was calculated as follows:

-it was reported in a Cartesian graph the curve of bacterial growth given by the value of optical density versus time. In this curve are considered the points concerning the exponential phase of bacterial growth reported in a semi logarithmic diagram.

It is derived by interpolating the lines whose slope is precisely the rate of bacterial growth, figure 4.9.

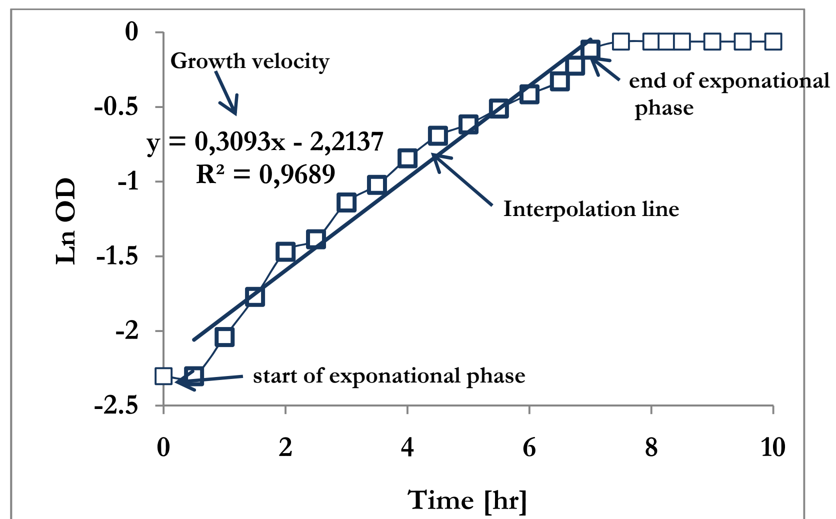


Figure 4.9 *The calculation of bacterial growth rate.*

4.3 Analytical Techniques

4.3.1 Contaminant concentration by HPLC-UV

4.3.1.1 Equipment description and measurement theory

To follow the degradation of the studied model pollutants in High Performance Liquid Chromatography with UV detection (HPLC-UV) was used, whenever the pollutant allowed it, i.e. the molecule absorbs light with a wavelength greater at 254 nm (in our case) sufficiently and can be separated by

the chromatographic system.

The chromatographic system employed in this system consisted of a chromatograph of the Waters Associates series containing a vacuum solvent degassing system I-Line AF, a pump model 600A Waters, an autosampler model 717 Plus Waters and a UV detector system model 2487 Waters.

The chromatographic column used for separation was a C18 reversed phase column Chrompack type Chromsphere (length 15cm and diameter 4.6 mm), figure 4.10.

Figure 4.10 *HPLC laboratory system.*

The whole system control and the data evaluation are conducted via a PC interface with dedicated software.

In HPLC a mobile phase is pumped through the system under laminar conditions, so that vertical mixing is negligible. The pressure drop along the chromatographic column is high due to the small pore size of the chromatographic column (usually in the range of 50-200 bars depending on the mobile phase's viscosity and the column properties).

The equilibrium solution in stationary and mobile phase dependent on the analyte properties constitutes the separation principle of the method. Organic substances are dissolved into the stationary phase and re-dissolved again into the mobile phase as a function of their affinity to the stationary phase.

In reversed-phase chromatography hydrophobic substances are retained stronger than hydrophilic substances, i.e. they move slower through the

chromatographic column and hence are detected later. The ultrapure mobile phase is usually a mixture of an organic solvent (normally acetonitrile (ACN) or methanol) and water. As the percentage of the organic solvent in the mobile phase increases, the analytes become increasingly dissolved in the mobile phase and they migrate faster through the chromatographic system. To provide good separation results the contaminants to be detected should be uncharged inside the chromatographic system.

Consequently, in the case of weak acids or bases, the pH of the mobile phase is adjusted accordingly (e.g. acidic to detect weak acids). Depending on the difficulty of the separation problem, there are two different elution methods; isocratic elution (no change of mobile phase during the analysis) is preferred for simple separation problems, whereas gradient elution (change of mobile phase composition during the analysis) is applied to more complex problems, e.g. if several contaminants have to be analysed simultaneously.

After passing the chromatographic column the contaminant generates a signal at the UV/Vis detector passing the flow-through cell in relation with the contaminants absorptive properties and its concentration. The analogue signal is digitized and recorded against time by the software generating peaks with Gaussian form. For quantification there exists a linear relationship between the peak area and the contaminant concentration in the sample, which is obtained by calibration with standard solutions of the analyte figure 4.11 and 4.12.

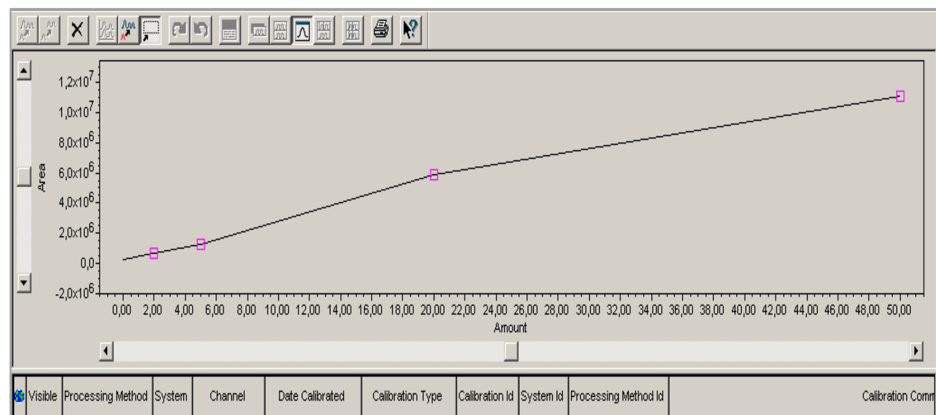


Figure 4.11 *Calibration curve with standards solutions of analyte, PHE 0-50 mg/L.*

4.3.1.2 Procedure

In this work sample filtration through 0.45 µm pore size PTFE syringe-

driven filters (Millipore Millex® GN) was applied. The filtered sample is then injected into the HPLC-UV system and analysed.

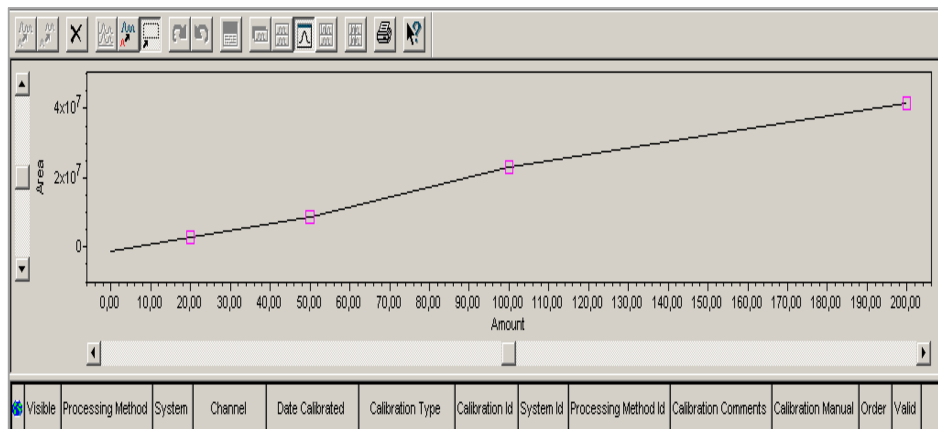


Figure 4.12 *Calibration curve with standards solutions of analyte, PHE 50-200 mg/L.*

As the mobile phase was used for analysis a mixture of 80% methanol acidified with 0.1% phosphoric acid and the remaining 20% ultrapure water acidified with phosphoric acid also at 0.1%.

The mobile phase flow inside of column was 1.20 mL/min, the wavelength used was 254 nm and the injections of different samples was 20 mL ,isocratic conditions and the retention time for PHE was 13.056 min as shows in the chromatogram figure 4.13.

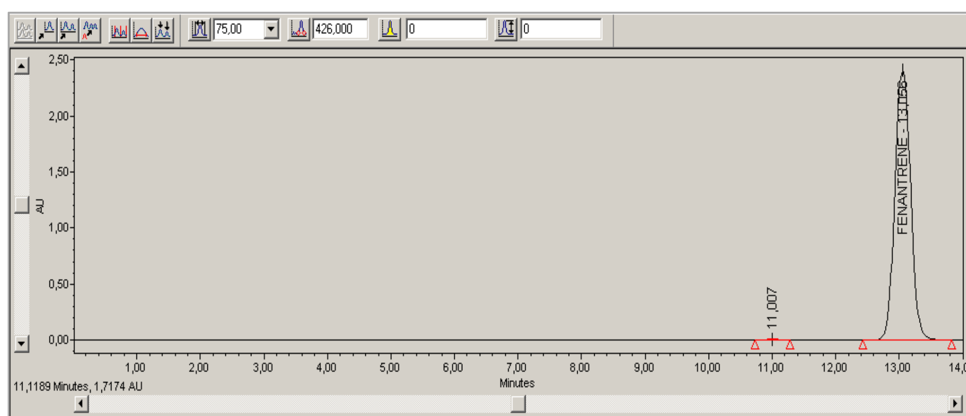


Figure 4.13 *HPLC chromatogram for a sample which contain 400 mg/L PHE.*

4.3.2 Total organic carbon TOC

A different way to measure oxidation progress of an organic compound is the determination of the carbon content of the oxidation product mixture.

The total carbon content TC is defined as the sum of the total organic carbon TOC (hydrocarbons) and total inorganic carbon TIC (carbon dioxide, bicarbonate, carbonate).

Due to oxidation, the carbon skeleton of an organic compound is gradually chopped into shorter carbon chain molecules containing oxygen-based functional groups (aliphatic, aldehydes and carboxylic acids). The last member in the oxidation sequence is formic acid, which upon oxidation yields the unstable carbonic acid. The TOC level of the oxidation product mixture decreases by release of carbon dioxide (mineralization) and volatile or gaseous intermediates.

The measurement of TOC does not reveal information about the chemical or toxicological properties of the sample. However, if the total carbon content equals the total inorganic carbon content, the organic has been completely mineralized and the remaining toxicity is only due to inorganic ions originating from covalently bonded elements.

Samples collected for measurement of TOC were analyzed , and these were collected using a 5 mL plastic syringe to withdraw samples from the batch reactors, and then the samples were filtered through a syringe filter 25 mm diameter and 0.45µm pore size. Filtered samples were collected in 5 mL glass vials and sealed with parafilm prior to analysis.

TOC measurements were performed using a Shimadzu Scientific Instruments Inc. model TOC-V_{CPH} analyser (Japan) figure 4.14.

The equipment was calibrated following standard method for water and wastewater 5310B, one stock solution was prepared following the same method for organic carbon. The calibration curve was prepared for total organic carbon TOC covering a range of 0-1000 mg/L.

Standards solutions were prepared pipetting the appropriate volumes of organic carbon stock solution (potassium phthalate) into 100 mL volumetric flask and then filling with distillate water to create the various dilutions.

The values of each point in the standard curves corresponded to the average of three injection, and the least squares option was selected for linear fit analysis. Calibration curves were checked at least once per month using standard solutions prepared from fresh stock solutions.

Figure 4.14 *TOC - V analyzer.*

4.3.3 Chemical Oxygen Demand (COD)

Chemical oxygen demand was measured following the USEPA approved reactor digestion method for reporting wastewater analysis (HACH method 8000, 0-1500 mg/L range).

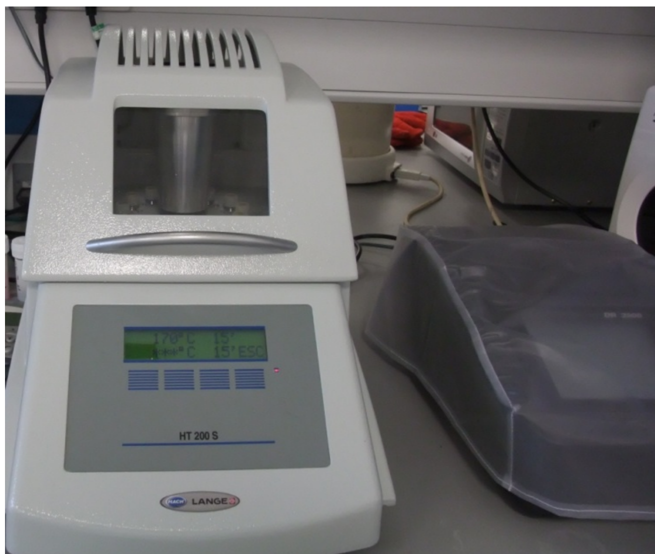


Figure 4.15 *COD – HT 200S reactor* & *HACH 2000 spectrophotometer analyzer.*

The method is an adaptation of Standards Methods for Examination of Water and Wastewater 5220D, APHA, 1998.

Samples were filtered through a filter paper with 0.45µm pore size. From this, 1 mL and/or 0.5 mL were introduced into the COD vial and then was put into COD HT200 reactor and heated for 2 hours at 150ºC. COD concentrations were measured in HACH 2000 spectrophotometer, figure 4.15. COD concentrations in samples were then calculated using the appropriate dilution factor.

Chapter

5

Results and Discussion

In this chapter are given all the experimental results achieved during the last three years in this dissertation work and consequently the discussion . There are also given some particular details for each experiments.

Part I

This part deals with the solubilisation and biodegradation of *Phenanthrene PHE* in synthetic aqueous wastes

5.1 Solubilisation of Phenanthrene in presence of mobility agents – organic solvents

5.1.1 Introduction

The enhanced solubility of solid phenanthrene PHE, in the presence of Methanol 99%, Acetone and n-Hexane was tested.

It was possible to verify the solubilisation of PHE in function of high concentration of organic solvents.

5.1.2 Experimental set-up and results

The solubilisation of three tests are adopted the following concentrations of organic solvents : Methanol 99%, 50%, 25%, 12.5%; n-Hexane 96% and Acetone 98%, 50%, 25%, 12.5%. PHE was used in excess for all tests.

For the third solvent was considered only the higher concentration because in contact with solid PHE and minimal salts medium, MSM, it formed a bi-phasic system.

The solubilisation experiments were carried out as follows:

Methanol [%]	Phenathrene in excess [mg/L]	Phenathrene solubilised [mg/L]
99	400	316
50	400	176
25	400	97
12.5	400	52

Table 5.1 *Solubilisation results of phenathrene using Methanol obtained from HPLC.*

n-Hexane [%]	Phenathrene in excess [mg/L]	Phenathrene solubilised [mg/L]
96	400	356

Table 5.2 *Solubilisation results of phenathrene using n-Hexane obtained from HPLC.*

Acetone [%]	Phenathrene in excess [mg/L]	Phenathrene solubilised [mg/L]
98	400	384
50	400	221
25	400	137
12.5	400	93

Table 5.3 *Solubilisation results of phenathrene using Acetone obtained from HPLC.*

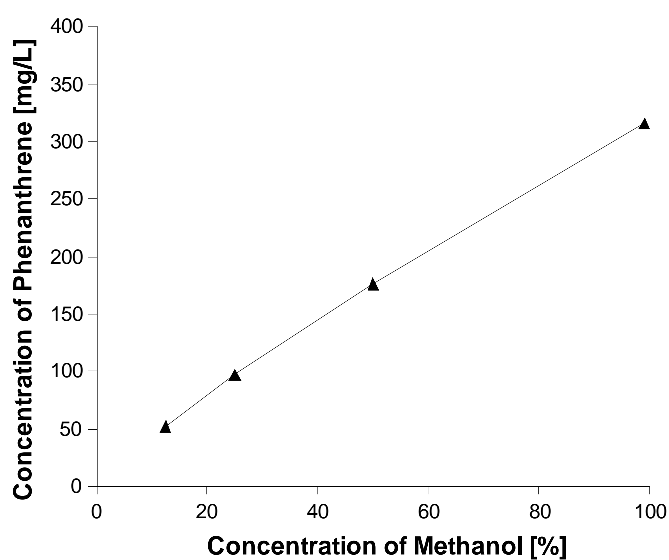


Figure 5.1 *Solubilisation curve of PHE using Methanol.*

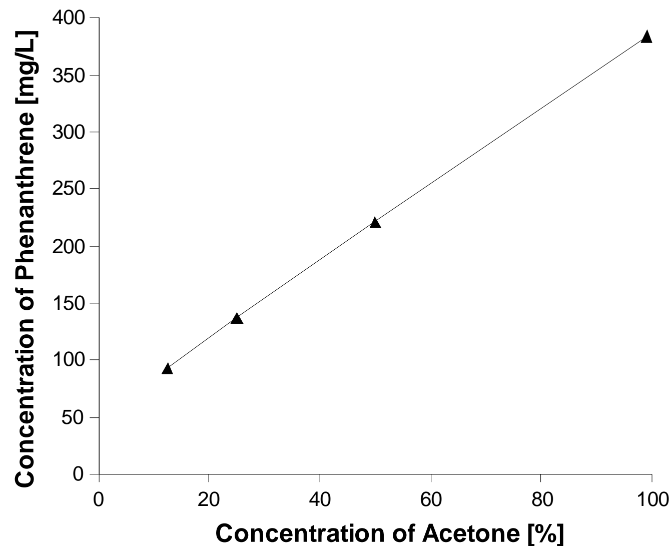


Figure 5.2 *Solubilisation curve of PHE using Acetone.*

Analysis of the data reported in tables and figures describe that:

- The solubility of solid PHE increase linearly with the concentration of organic solvents and began immediately but the solvents are characterised by a very high vapour pressure that determines immediately evaporation.

5.2 Solubilisation of Phenanthrene in presence of mobility agents – surfactants

5.2.1 Introduction

The enhanced solubility of solid phenanthrene PHE, in the presence of non-ionic surfactants Tween 80 or Triton X100 are tested.

The solubilisation experiments are carried out as follows:

- Solubilisation of PHE in MSM in presence of non-ionic surfactant Tween 80;
- Solubilisation of PHE in deionised water in presence of non-ionic surfactant Tween 80;

- Solubilisation of PHE in MSM in presence of non-ionic surfactant Triton X100;
- Solubilisation of PHE in deionised water in presence of non-ionic surfactant Triton X100.

In this way it was possible to verify the solubilisation of PHE in function of the concentration of salts presents in the MSM.

In addition, in each of these tests are used different concentration (increasing concentrations) of Tween 80 or Triton X100 in order to value the solubility of PHE in function of the amount of surfactant used.

5.2.2 Experimental set-up

In the solubilisation tests the following concentrations of surfactants are adopted (Tween 80 or Triton X100): 0.05, 0.1, 0.2, and 0.5%. The values are considered immediately above the critical concentration, CMC, of surfactants Tween 80 or Triton X100 because at that concentration the surfactants begin to form micelles and they are able to demonstrate their effects like emulsifiers and solubilizer.

In every test were applied the following steps:

- Tween 80 or Triton X100 once weighed in glass ships, was placed in 100 ml volumetric flasks;
- In the flasks was added the MSM or deionised water depending on the test and the solution was stirred for 15 minutes using magnetic stirred: Tween 80 or Triton X100 are soluble in MSM and deionised water but dissolution required that time for completion;
- After the solubilisation of Tween 80 or Triton X100 completed, 50 ml of solution were poured into 250 ml Erlenmeyer flasks. Inside the Erlenmeyer flasks was added an amount of PHE in excess 400 mg/L. The flasks were sealed (to prevent the possible evaporation of the solution) using parafilm and completely wrapped in aluminium foil to prevent light penetration. This is a precautionary measure to be adopted because PHE are very sensitive to light and may undergo changes as a result of photochemical reactions;

- The flasks were placed in an oscillator shaker for 48 hours at a temperature of 25°C and speed of 175 rpm. After 48 hours of stirring, the contents of the flask was centrifuged for 20 minutes at 6000 rpm and the liquid phase was filtered with a 0.45µm filter paper to remove the residual crystals of PHE. Then, 5 ml of solution was taken and analyzed with the HPLC.

5.2.3 Results

5.2.3.1 Solubilisation of phenanthrene in minimal salts medium in presence of non-ionic surfactant Tween 80

The results obtained from HPLC for the first test carried out using Tween 80 and MSM for the solubilisation of PHE can be summarized in the following table:

Tween 80 [%]	Phenathrene in excess [mg/L]	Phenathrene solubilised [mg/L]
0.05	400	0.43
0.1	400	10.89
0.2	400	21.11
0.5	400	102.82

Tabel 5.4 *Solubilisation results of phenathrene using Tween 80 at different concentrations and MSM obtained from HPLC.*

The results on the table 5.4, the chromatogram obtained by HPLC analysis in figure 5.3 and the solubilisation curve of PHE in MSM at different concentration of Tween 80, obtained by plotting the variation of PHE concentration in solution depending by the concentration of surfactant used.

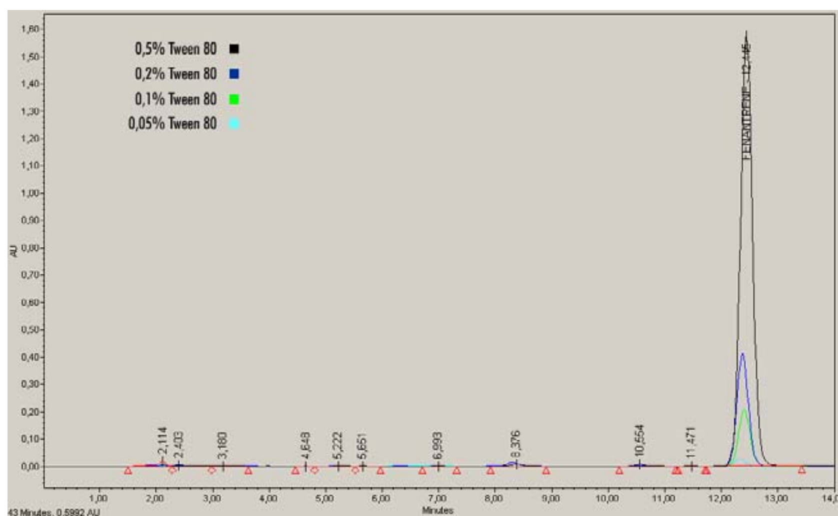


Figure 5.3 *Solubilisation chromatogram of phenanthrene using Tween 80 at different concentrations and MSM.*

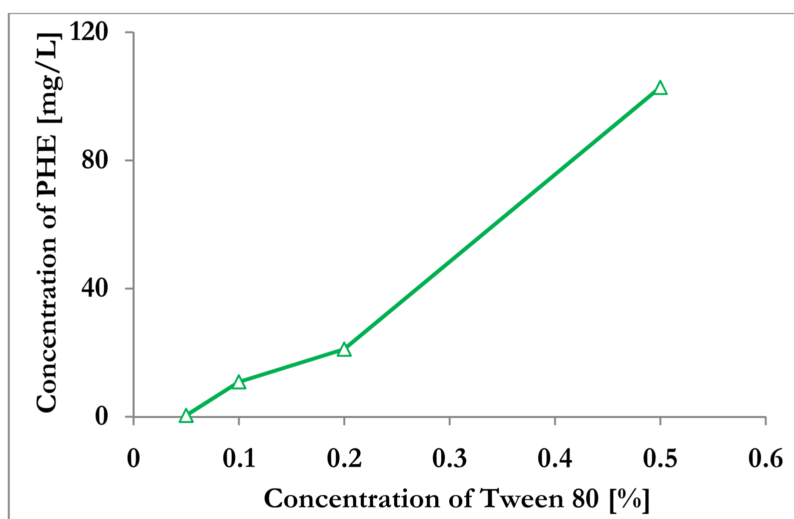


Figure 5.4 *Solubilisation curve of phenanthrene using Tween 80 at different concentrations and MSM.*

5.2.3.2 Solubilisation of phenanthrene in deionised water in presence of non-ionic surfactant Tween 80

The results obtained from HPLC analysis relative to solubilisation of PHE using Tween 80 and deionised water are shown in the table 5.5:

Tween 80 [%]	Phenanthrene in excess [mg/L]	Phenanthrene solubilised [mg/L]
0.05	400	18.03
0.1	400	44.90
0.2	400	82.47
0.5	400	183.78

Tabel 5.5 *Solubilisation results of phenathrene using Tween 80 at different concentrations and deionised water obtained from HPLC.*

In the figure 5.5 and 5.6 it is reported the chromatogram obtained by HPLC analysis and the solubilisation curve of PHE using Tween 80 at different concentrations and deionised water.

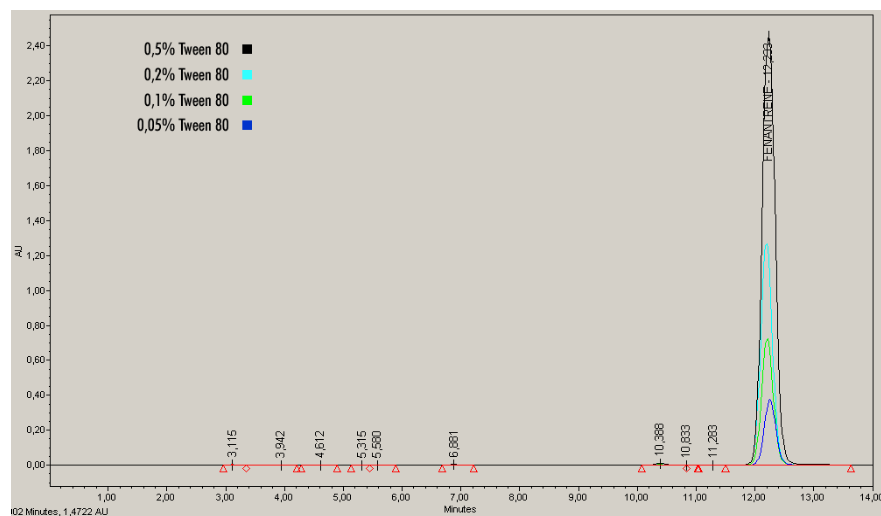


Figure 5.5 *Solubilisation chromatogram of phenanthrene using Tween 80at different concentrations and deionised water.*

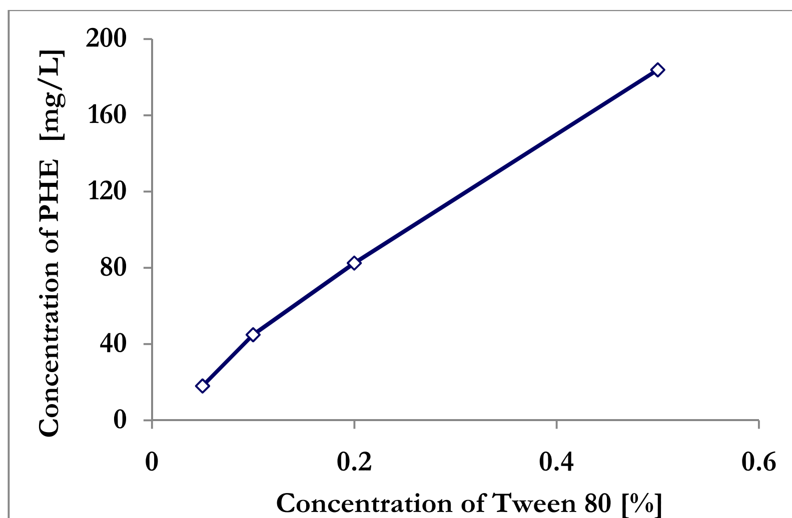


Figure 5.6 *Solubilisation curve of phenanthrene using Tween 80 at different concentrations and deionised water.*

5.2.3.3 Solubilisation of phenanthrene in minimal salts medium in presence of non-ionic surfactant Triton X100

The results obtained from HPLC for the third test carried out using the solubilisation of PHE in Triton X100 and MSM can be summarized in the following table (Table 5.6):

Triton X100 [%]	Phenathrene in excess [mg/L]	Phenathrene solubilised [mg/L]
0.05	400	2.57
0.1	400	26.58
0.2	400	46.58
0.5	400	107.77

Tabel 5.6 *Solubilisation results of phenathrene using Triton X100 at different concentrations and minimal salts medium obtained from HPLC.*

The results on the table 5.6, the chromatogram obtained by HPLC analysis in figure 5.7 and the solubilisation curve of PHE in MSM at different concentration of Triton X100 figure 5.8, obtained by plotting the variation of PHE concentration in solution depending on the concentration of surfactant

used.

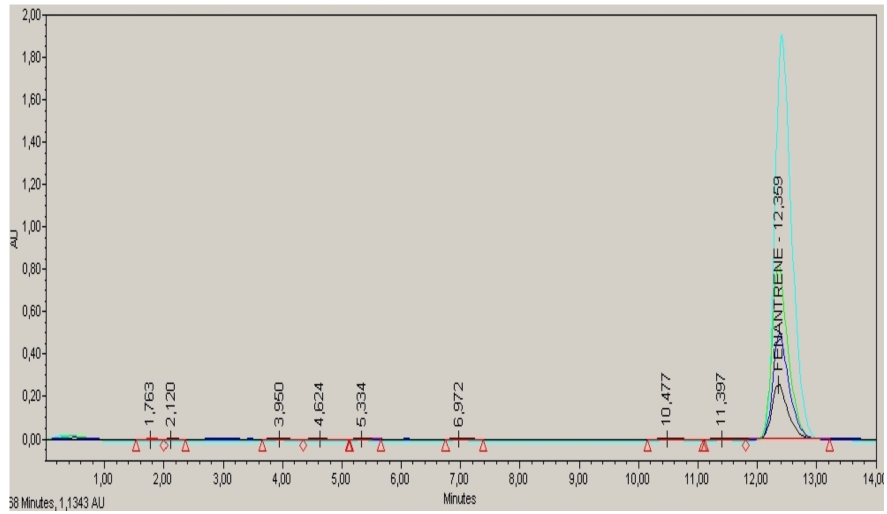


Figure 5.7*Solubilisation chromatogram of phenanthrene using Triton X 100 at different concentrations and minimal salts medium.*

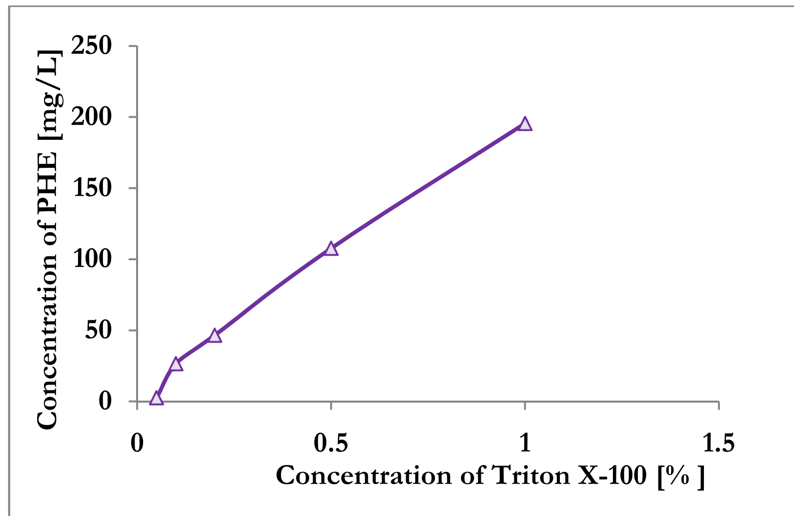


Figure 5.8 *Solubilisation curve of phenanthrene using Triton X100 at different concentrations and minimal salts medium.*

5.2.3.4 Solubilisation of phenanthrene in deionised water in presence of non-ionic surfactant Triton X100

The results obtained from HPLC analysis relative to solubilisation of PHE using Triton X100 and deionised water are shown in the table 5.7:

Triton X100 [%]	Phenanthrene in excess [mg/L]	Phenanthrene solubilised [mg/L]
0.05	400	16.79
0.1	400	32.56
0.2	400	53.6
0.5	400	130.06

Tabel 5.7 *Solubilisation results of phenathrene using Triton X100 at different concentrations and deionised water obtained from HPLC.*

In the figure 5.9 and 5.10 it is reported the chromatogram obtained by HPLC analysis and the solubilisation curve of PHE using Triton X100 at different concentrations and deionised water.

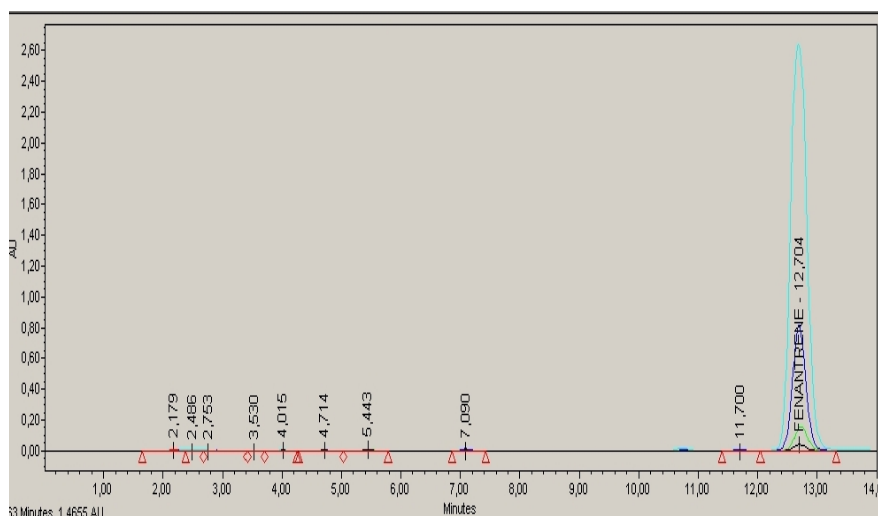


Figure 5.9 *Solubilisation chromatogram of phenanthrene using Triton X100 at different concentrations and deionised water.*

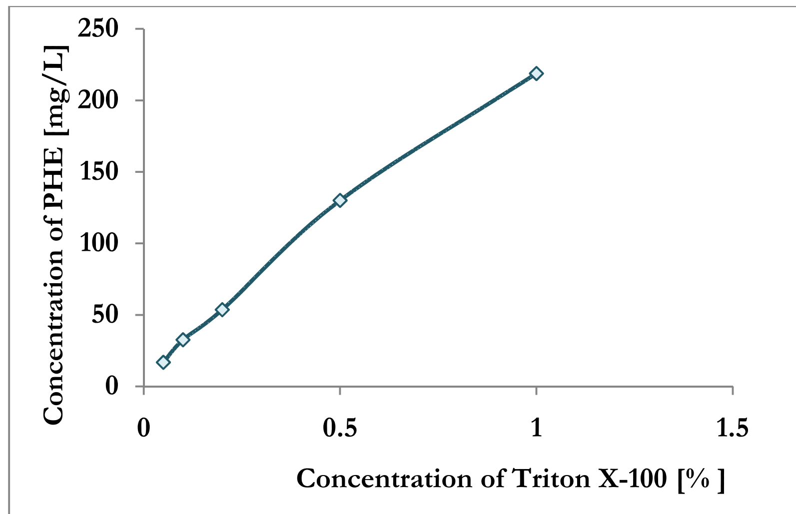


Figure 5.10 *Solubilisation curve of phenanthrene using Triton X100 at different concentrations and deionised water.*

5.2.3.5 Summary of results

The results of the fourth tests are summarized in the table 5.8 and table 5.9 which also shows the percentage yield of solubilisation of PHE as a function of concentrations of Tween 80 and Triton X100 used:

	1stTest Minimal salts medium		2nd Test Deionised water	
Tween 80 [%]	**PHE [mg/L]**	**PHE [% solublised]**	**PHE [mg/L]**	**PHE [% solubilised]**
0.05	0.43	0.11	18.03	4.51
0.1	10.89	2.72	44.90	11.23
0.2	21.11	5.28	82.47	20.62
0.5	102.82	25.71	183.78	45.95

Tabel 5.8 *Solubilisation results of phenanthrene in Tween 80 using MSM and deionised water.*

	3rdTest Minimal salts medium		4th Test Deionised water	
TritonX100 [%]	**PHE [mg/L]**	**PHE [% solubilised]**	**PHE [mg/L]**	**PHE [% solubilised]**
0.05	2.57	0.64	16.79	4.20
0.1	26.58	6.65	32.56	8.14
0.2	46.58	11.64	53.6	13.40
0.5	107.77	26.94	130.06	32.51

Tabel 5.9 *Solubilisation results of phenanthrene in Triton X100 using MSM and deionised water.*

Below are given the solubility curves (Fig. 5.11, Fig. 5.13) and percentage of solubilisation (Fig. 5.12, Fig. 5.14) of PHE as a function of the concentrations of surfactant and salts content in the MSM.

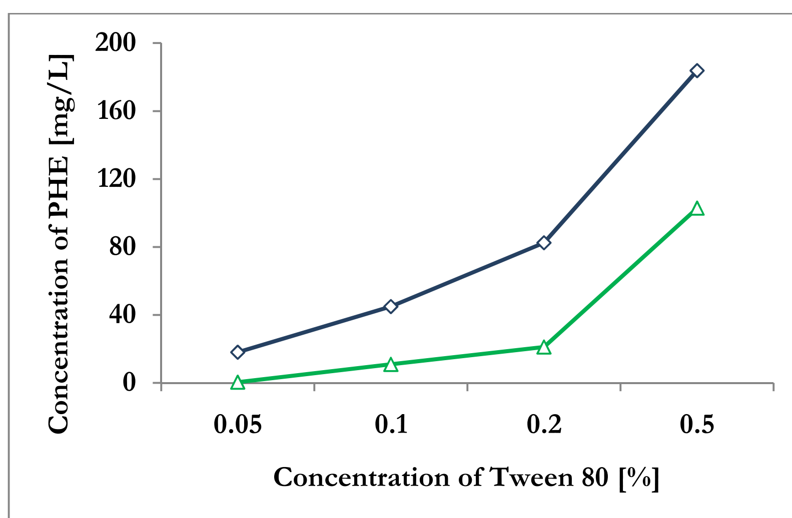


Figure 5.11 *Solubilisation curve of phenanthrene in Tween 80 employed the two tests:* ◇ *Deionised water;* △ *Minimal salts medium.*

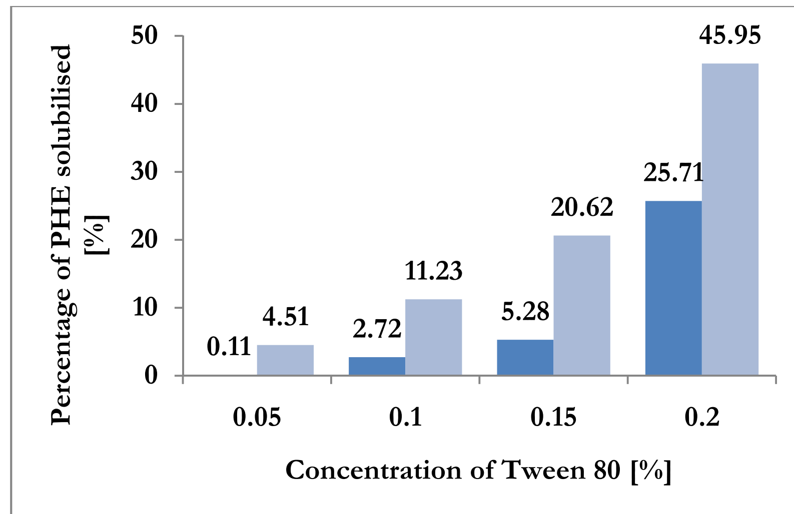


Figure 5.12 *Percentage of phenanthrene solubilised as a function of the concentration of Tween 80 and salts presents in minimal salts medium:* ■ *Minimal salts medium;* ■ *Deionised water.*

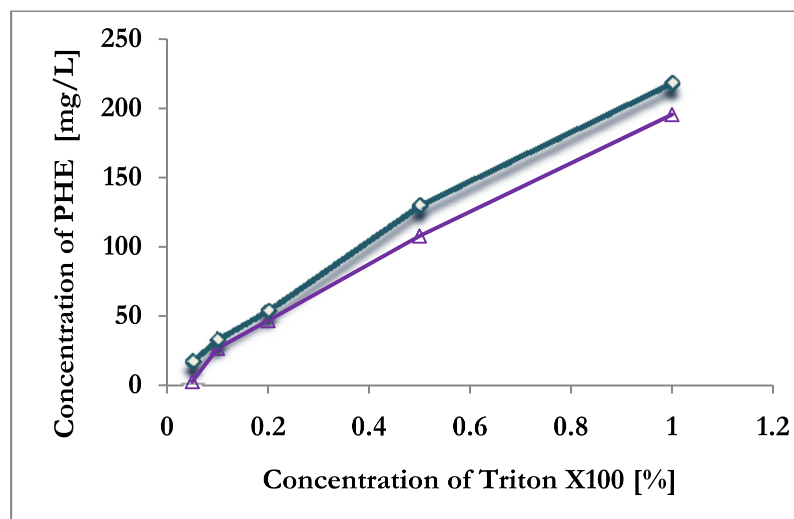


Figure 5.13 *Solubilisation curve of phenanthrene in Triton X100 employed the two tests:* ◇ *Deionised water;* △ *Minimal salts medium.*

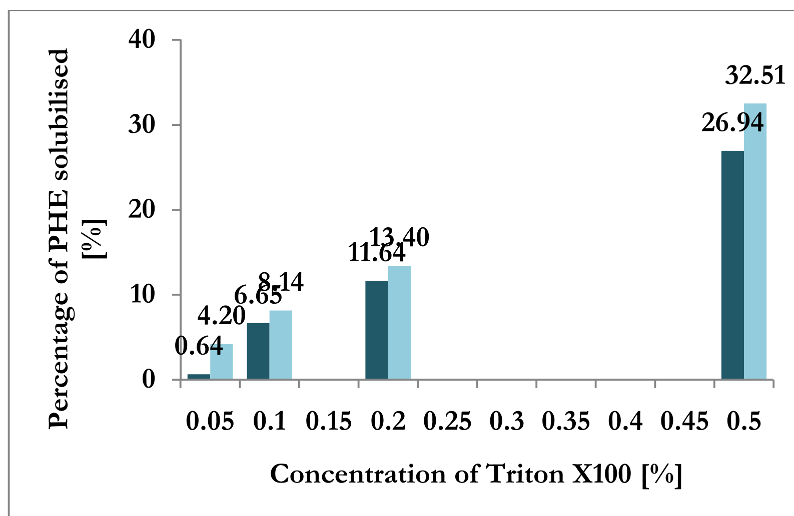


Figure 5.14 *Percentage of phenanthrene solubilised as a function of the concentration of Triton X100 and salts presents in minimal salts medium:* ■ *Minimal salts medium;* ■ *Deionised water.*

Analysis of the data reported in tables and figures describe that:

- As in [16] and [45] the solubility of solid PHE increases with the surfactants addition.
- The solubility of solid PHE in addition in MSM and deionised water increase approximately linearly with the concentration of surfactants and began after 48 hours.

5.3 Effects of organic solvents and surfactants on bacterial growth

5.3.1 Introduction

This chapter describes two series of growth tests of microorganisms, whose objective were to:

- Check the biocompatibility of organic solvents and surfactants against mixed bacterial cultures used: they must ensure in practice that they are

not toxic to microorganisms and do not inhibit the growth;

- Check the bioavailability of organic solvents and surfactants towards mixed bacterial cultures used, verify whether they are used by microorganisms as a carbon source.

These are the two fundamental characteristics that the surfactants must have when used with the aim of increasing the bioavailability and biodegradation of hydrophobic organic substrate. To check these characteristics were conducted in two parallel series of tests carried out in Erlenmeyer flask batch reactors with microorganisms acclimated to glucose:

1. ***Biocompatibility tests***: evidence of growth of microorganisms on glucose and mobility agent to verify precisely the biocompatibility of the organic solvents and surfactant against microorganisms;
2. ***Bioavailability tests***: arranged in the same conditions and following the same procedures of the previous tests of biocompatibility, the only difference was in this case we have not performed the addition of glucose because the purpose of these tests is to verify if the organic solvents and mobility agents can be a carbon source by microorganisms, that is making them bioavailable.

Each test was conducted using the same concentration of organic solvents and different concentrations of Tween 80 and Triton X100, still above the critical micellar concentration value, in order to monitor changes in growth conditions to vary the content of the surfactant. It has also set up a positive control C (+), under the same conditions, consisting of a crop with the same amount of inoculation of the flasks with glucose, in which no organic solvent and/or no surfactant were added.

5.3.2 Experimental setup

In both sets of tests were considered the following concentrations of organic solvents 12.5% and the solubilisation was immediate, but for Tween 80 and Triton X100: 0.05, 0.1, 0.2, 0.5 and 1% was therefore followed the following steps:

- The Tween 80 and Triton X100 once weighed was placed in 100 ml volumetric flasks and after was added MSM. The solution was stirred for 15 minutes using magnetic stirred bar;

- After solubilisation of Tween 80 and Triton X100 completed, 50 ml of solution contained in each flask were transferred into 250 ml Erlenmeyer flasks. Then the flasks were inoculated with mixed microbial culture previously acclimated to glucose, GLU (following the steps listed in Chapter 4) until reaching an OD of 0.1;

- In the Erlenmeyer flask for biocompatibility tests were added GLU at concentration of 1000 mg/L;

- A total of 5 Erlenmeyer flasks are followed, one for each concentration of surfactant tested for each of the two tests and a positive control flask C (+) with only medium and GLU on the test biocompatibility;

- The flasks were then kept under agitation into shaker at 175 rpm and a thermostatic chamber at 25°C;

- The growth of biomass was carried out by monitoring the absorbance at 600 nm using a spectrophotometer.

5.3.3 Results

5.3.3.1 Results of biocompatibility

Below (Figure 5.15 and Fig. 5.17) report the growth curves in terms of optical density versus time for organic solvents and different concentrations of Tween 80 and Triton X100 at the same concentration of GLU 1000 mg/L. These curves are compared with the control C (+), produced under the same experimental conditions but in the absence of surfactant.

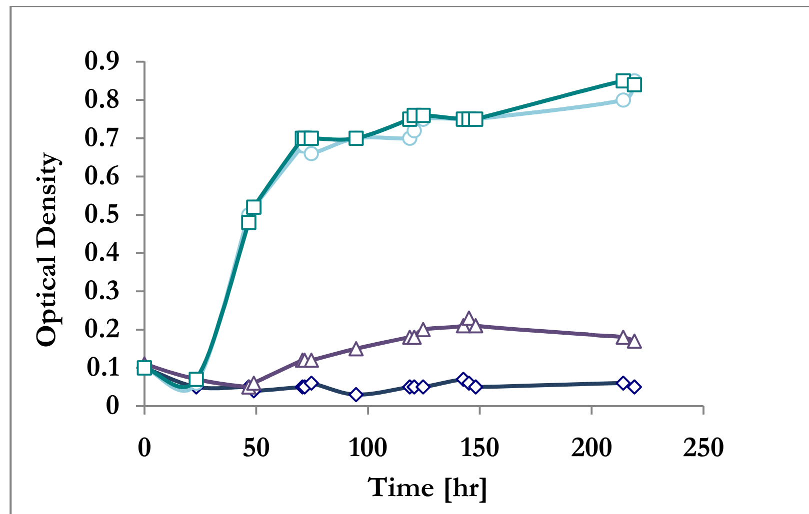


Figure 5.15 *Biocompatibility growth curves with Methanol, Acetone and n-Hexan in confront with the* (+)*control:*○*Glucose 0.1%-control* (+);□*Glucose 0.1+ Methanol 12.5%;*△*Glucose 0.1% + Acetone 12.5%;*◇*Glucose 0.1% + n-Hexane 12.5%.*

The growth curves for the same concentrations of organic solvents showed a trend qualitatively similar to that of the control sample in case of Methanol, but show a decrease in growth rate in case of Acetone and n-Hexane.

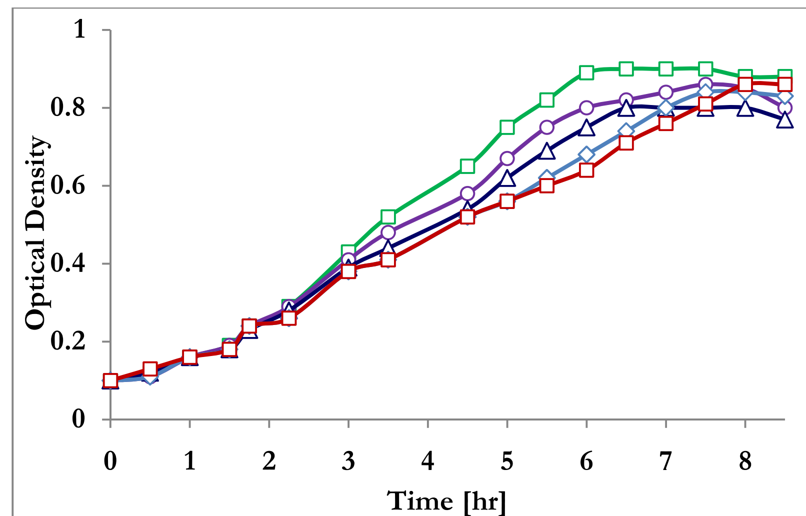


Figure 5.16 *Biocompatibility growth curves with Tween 80 in confront of* (+)*control* :□ *Glucose 0.1%-control* (+);○*Glucose 0.1% + Tween 80 0.05%;*△*Glucose 0.1% + Tween 80 0.1%;*◇*Glucose 0.1% + Tween 80 0.2%;*□*Glucose 0.1% + Tween 80 0.5%.*

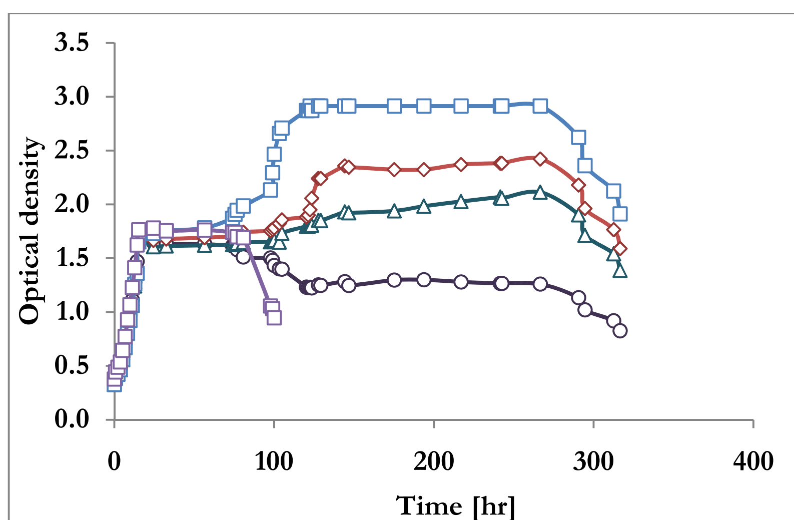


Figure 5.17 *Biocompatibility growth curves with Triton X100 in confront of (+)control :* □ *Glucose 0.1%-control;* ○ *Glucose 0.1% + Triton X100 0.05%;* △ *Glucose 0.1% + Triton X100 0.1%;* ◇ *Glucose 0.1% + Triton X100 0.2%;* □ *Glucose 0.1% + Triton X100 0.5%.*

The growth curves for different concentrations of Tween 80 showed a trend qualitatively similar to that of the control sample, but show a slight decrease in growth rate with increasing concentration of surfactant used, what emerges from the values of the growth rate calculated followed for each crop, for each initial concentration of Tween 80.

The growth curves for different concentrations of Triton X100 showed a trend qualitatively similar to that of the control sample, but after the glucose was used the curves showed an increase in optical density with the increasing of Triton X100 concentrations. A slight decrease in optical density was observed after the plateau for the 0.5% concentration of Triton X100.

Below, the figure 5.18 to figure 5.22 we report the graphs of all four results in semi-logarithmic scale with their interpolation lines and equations (the slope of these lines is precisely the growth rate).

The experimental points used to calculate the growth rate are shown in bold.

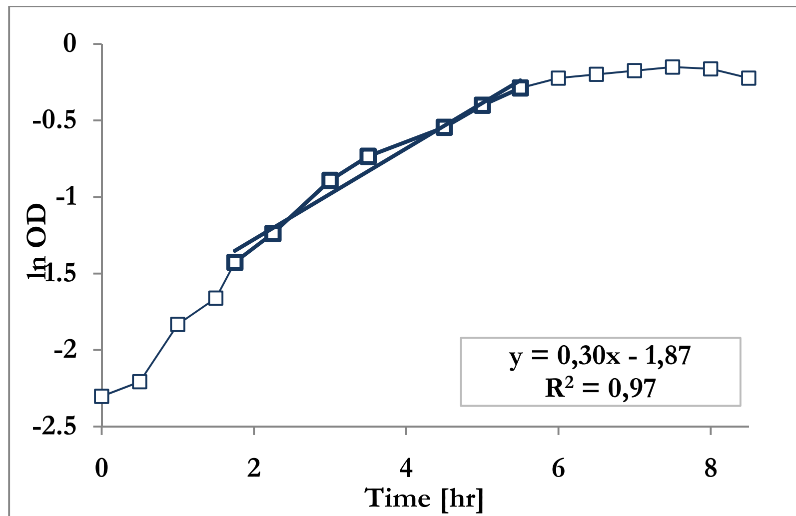


Figure 5.18 *Growth curve of microorganisms on Tween 80 0.05% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

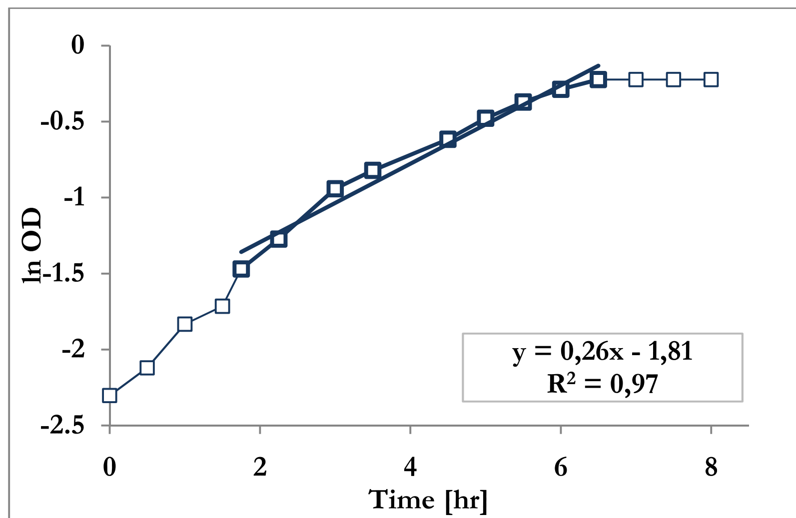


Figure 5.19 *Growth curve of microorganisms on Tween 80 0.1% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

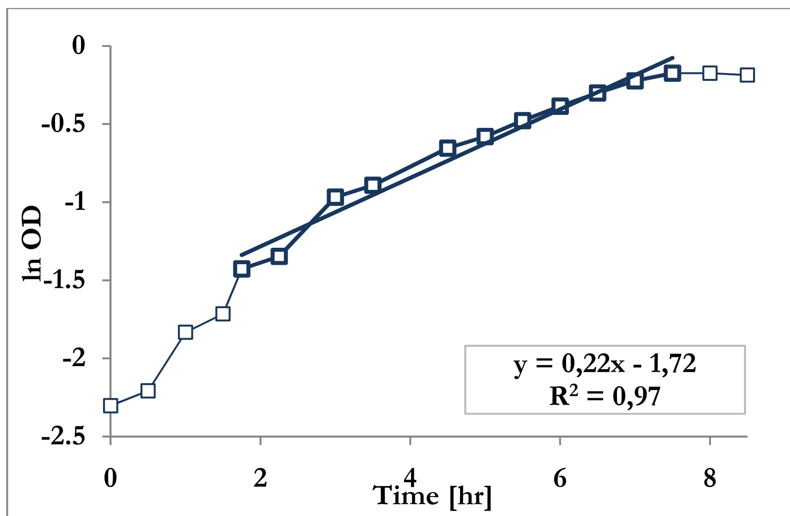


Figure 5.20 *Growth curve of microorganisms on Tween 80 0.2% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

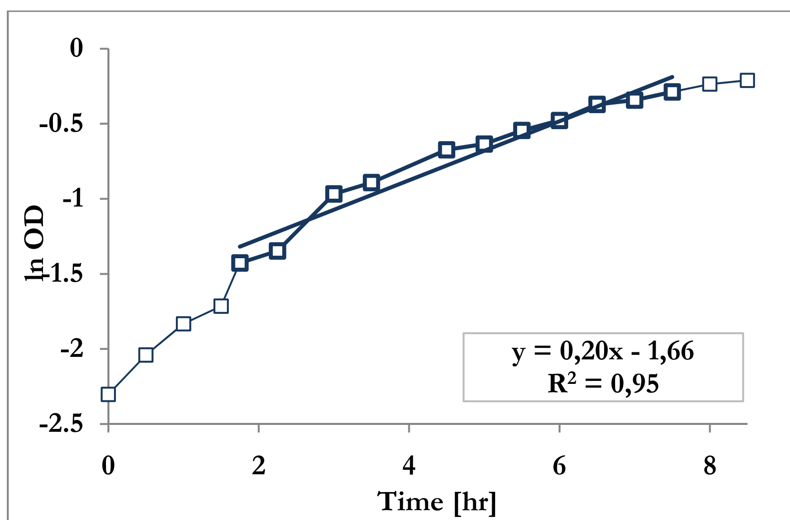


Figure 5.21 *Growth curve of microorganisms on Tween 80 0.5% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

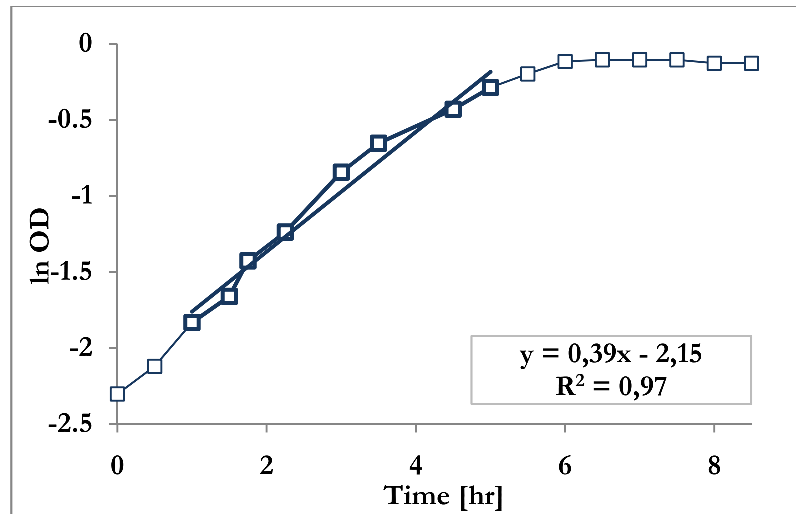


Figure 5.22 *Growth curve of microorganisms on Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

The table 5.10 and figure 5.23 show the values of growth rates obtained for various tests. These figures show a slight decrease in terms of growth rate with the increasing concentration of Tween 80.

Tween 80 [%]	Glucose [%]	Growth velocity [1/hr]
0	0.1	0.39
0.05	0.1	0.30
0.1	0.1	0.26
0.2	0.1	0.22
0.5	0.1	0.20

Table 5.10 *Growth rate of microorganisms at different concentrations of Tween 80.*

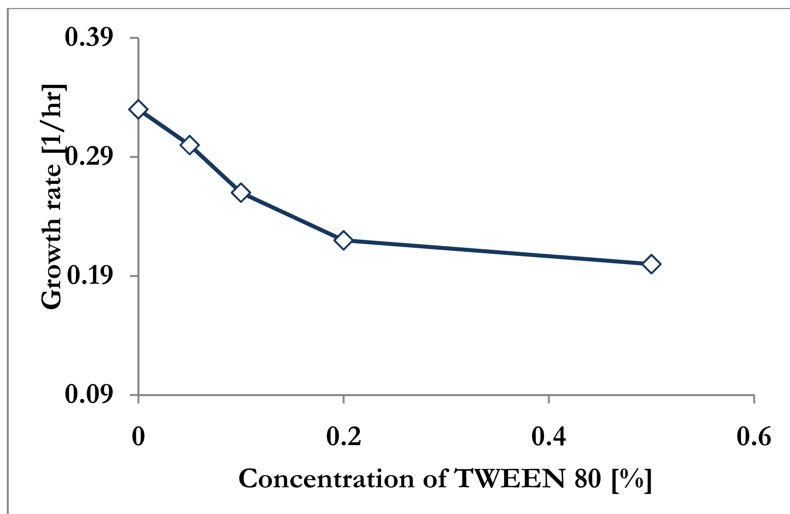


Figure 5.23 *Growth rate of microorganisms at different concentration of Tween 80.*

Below the figure 5.24 to figure 5.28 we report the graphs of all four results in semi-logarithmic scale with their interpolation lines and equations (the slope of these lines is precisely the growth rate) for Triton X100.

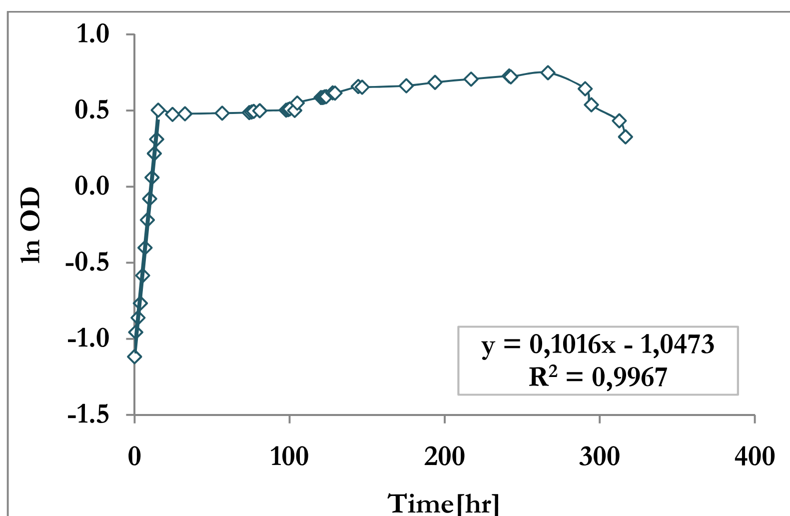


Figure 5.24 *Growth curve of microorganisms on Triton X100 0.05% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

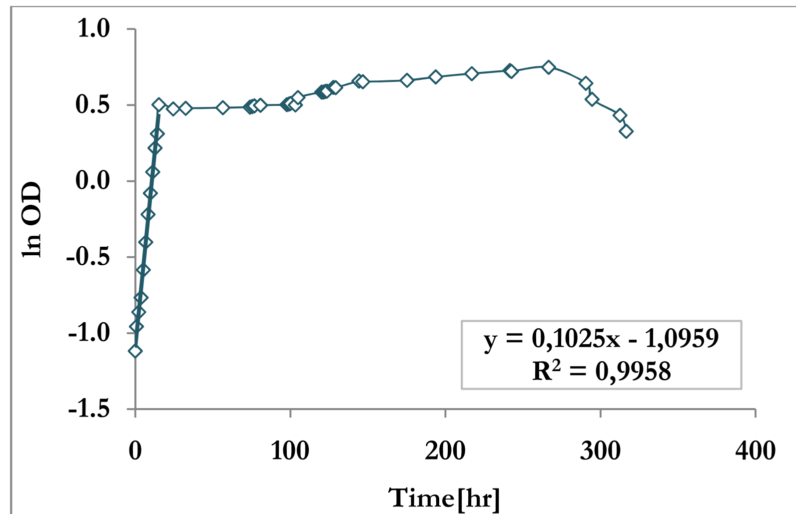


Figure 5.25 *Growth curve of microorganisms on Triton X100 0.1% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

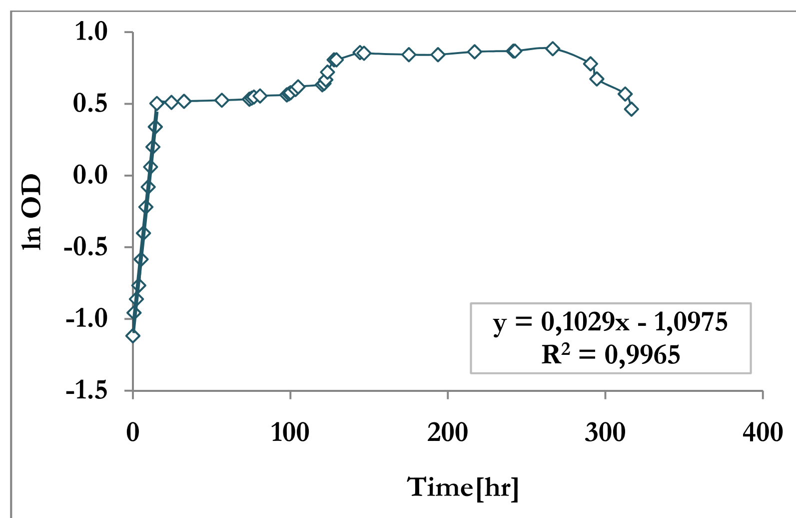


Figure 5.26 *Growth curve of microorganisms on Triton X100 0.2% and Glucose 0.1% in semi-logarithmic scale and the calculation of growth rate.*

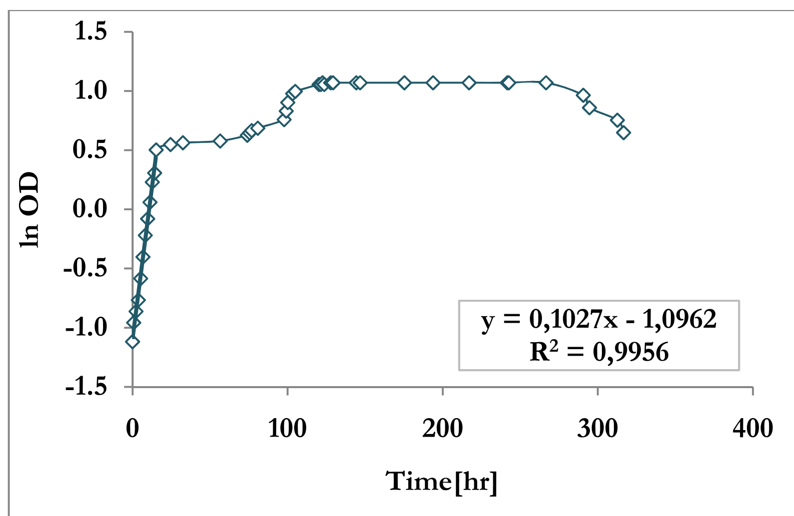


Figure 5.27 *Growth curve of microorganisms on Triton X100 0.5% and Glucose 0.1% in semi logarithmic scale and the calculation of growth rate.*

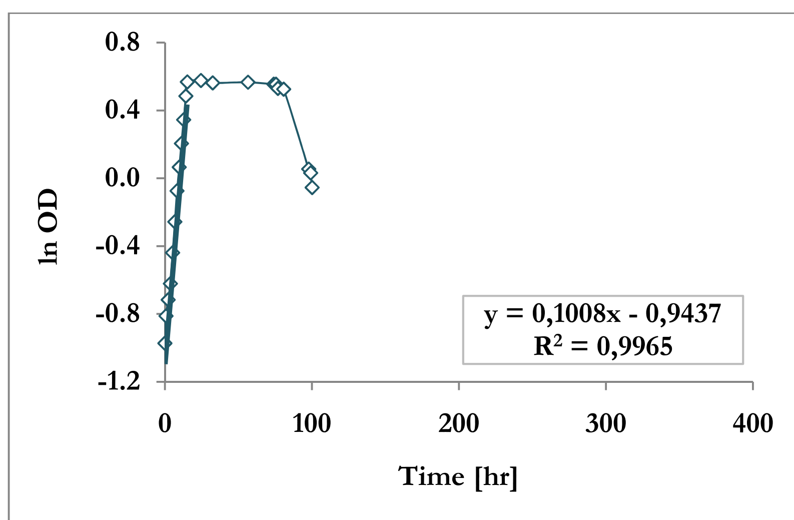


Figure 5.28 *Growth curve of microorganisms on Glucose 0.1% in semi logarithmic scale and the calculation of growth rate.*

The table 5.11 and figure 5.29 show the values of growth rates obtained for various tests. These figures show a high increase in terms of growth rate near the CMC, no lag phase and a slightly decrease at concentration of Triton X100

0.5%.

Triton X 100 [%]	Glucose [%]	Growth velocity [1/hr]
0	0.1	0.1008
0.05	0.1	0.1016
0.1	0.1	0.1025
0.2	0.1	0.1029
0.5	0.1	0.1027

Table 5.11 *Growth rate of microorganisms at different concentration of Triton X100.*

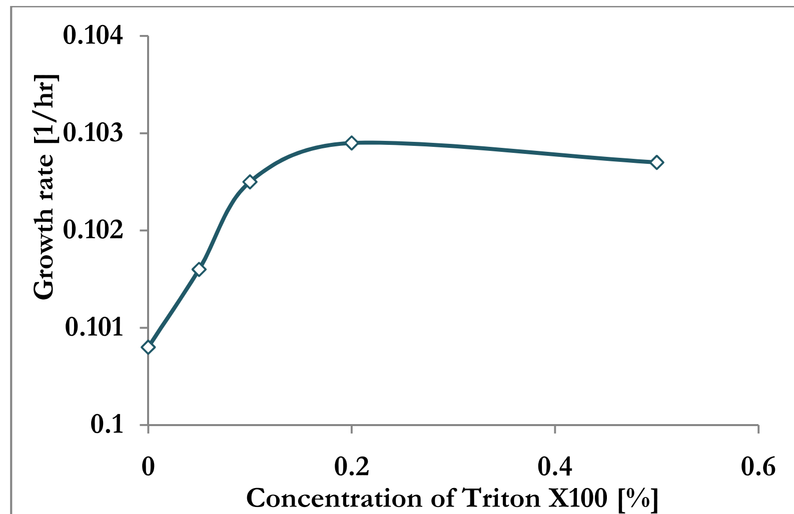


Figure 5.29 *Growth rate of microorganisms at different concentration of Triton X100.*

5.3.3.2 Results of bioavailability

The figures 5.30, 5.31 and 5.32 report the variation of optical density in time for the bioavailability tests in presence of organic solvents and surfactants as sole source of carbon and energy.

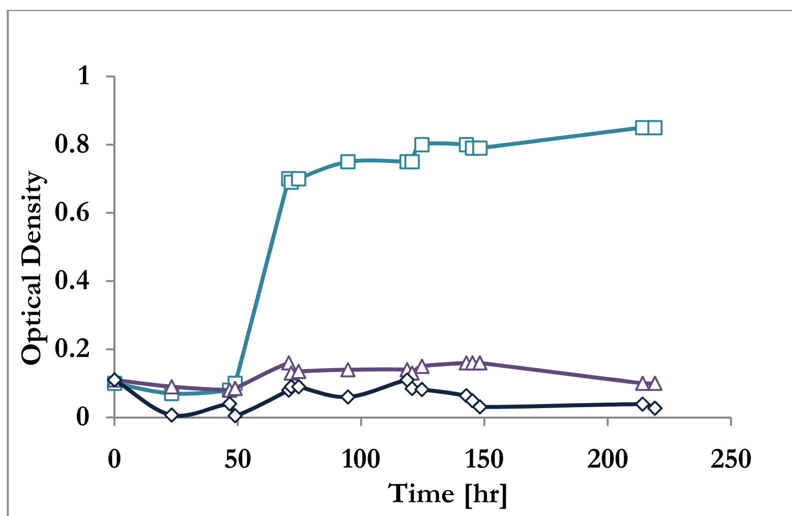


Figure 5.30 *Bioavailability curves of organic solvents: □Methanol 12.5 %;△Acetone 12.5%;◇n-Hexane 12.5%.*

The trend of optical density for the tests carried out at the same concentrations of organic solvents appear to be almost different and showing an increase of OD for Methanol 12.5% and a decrease of OD close to zero for Acetone 12.5% and n-Hexane, that is a fact of no growth and suggests in this concentration the Acetone and n-Hexane are not used by microorganisms as a source of carbon and energy.

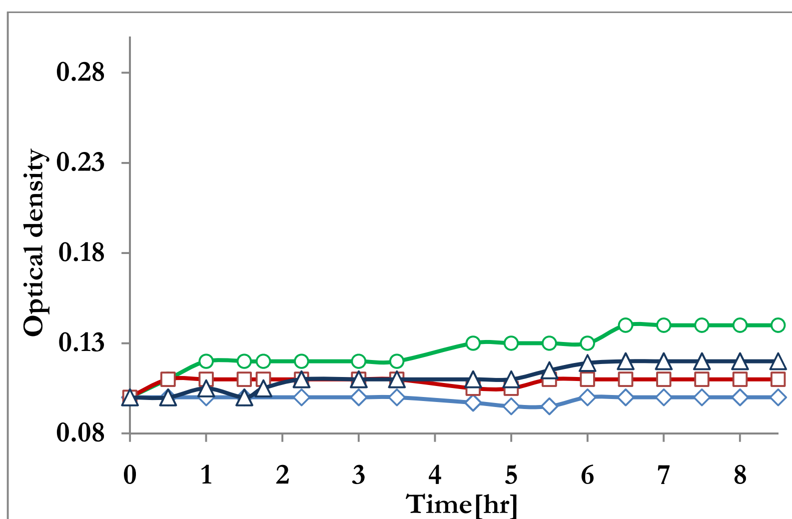


Figure 5.31 *Bioavailability curves at different concentration of Tween 80: ○Tween 80 0.05 %,◇Tween 80 0.1 %,□Tween 80 0.2 %;△Tween 80 0.5 %.*

The trend of optical density for the tests conducted at different concentrations of Tween 80 appear to be almost comparable and showing an increase of OD close to zero, that is a fact of no growth and suggests in this concentrations range Tween 80 is not used by microorganisms as a source of carbon and energy.

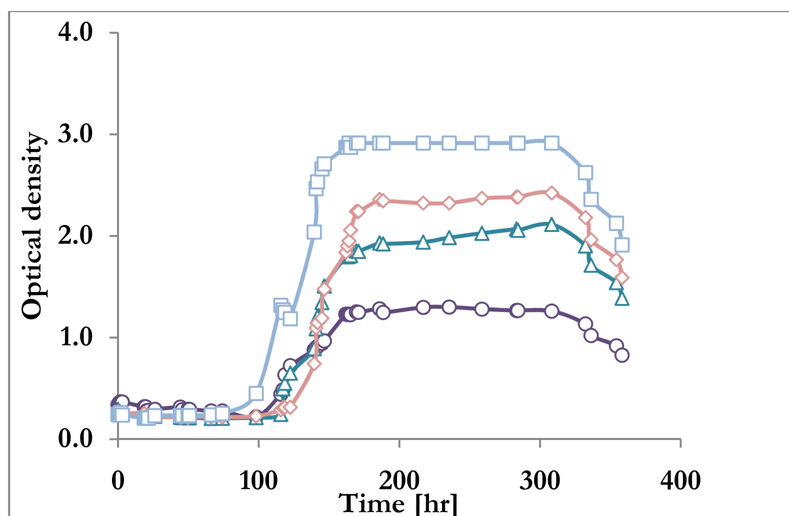


Figure 5.32 *Bioavailability curves at different concentration of Triton X100 %:* ○ *Triton X100 0.05%;* △ *Triton X100 0.1%;* ◇ *Triton X100 0.2%;* □ *Triton X100 0.5%.*

The measurements of optical density for the tests conducted at different concentrations of Triton X100 appear to be almost qualitatively comparable and showed an increase of OD after one hundred hours with the increasing concentrations of surfactant.

The maximum of optical density values was higher when Triton X100 concentrations increase. That is a fact of growth and suggests in this concentrations range Triton X100 is used by microorganisms as a source of carbon and energy.

5.3.3.3 Summary of biocompatibility and bioavailbility

Below (figure 5.33, 5.34 and 5.35) are reported for organic solvents and for each concentration of surfactants the comparison between the bioavailability growth curves (growth curves on GLU and /or organic solvents, Tween 80,

Triton X100) and those of biocompatibility curves (growth curves on only organic solvents, Tween 80 and/or Triton X100) and the growth curve relative to the control (growth on glucose).

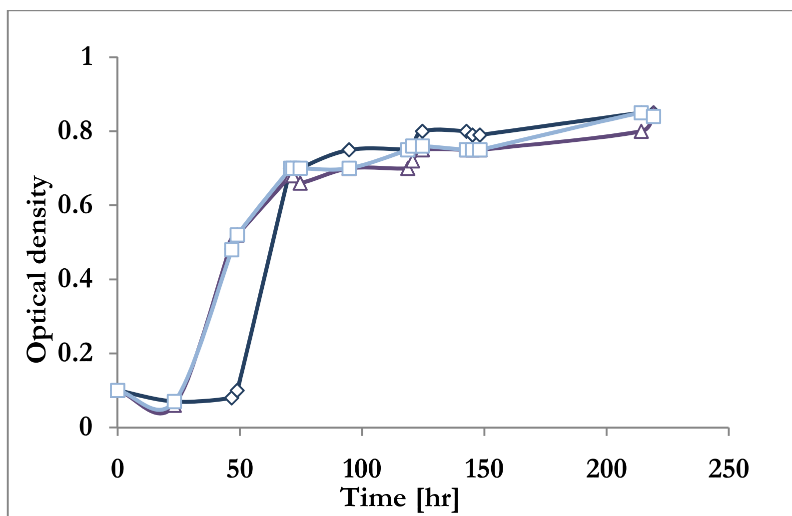


Figure 5.33 *Comparison between the biocompatibility and bioavailability tests refers as Methanol 12.5%: △Glucose 0.1% -control; □Glucose 0.1% + Methanol 12.5%;◇ Methanol 12.5%.*

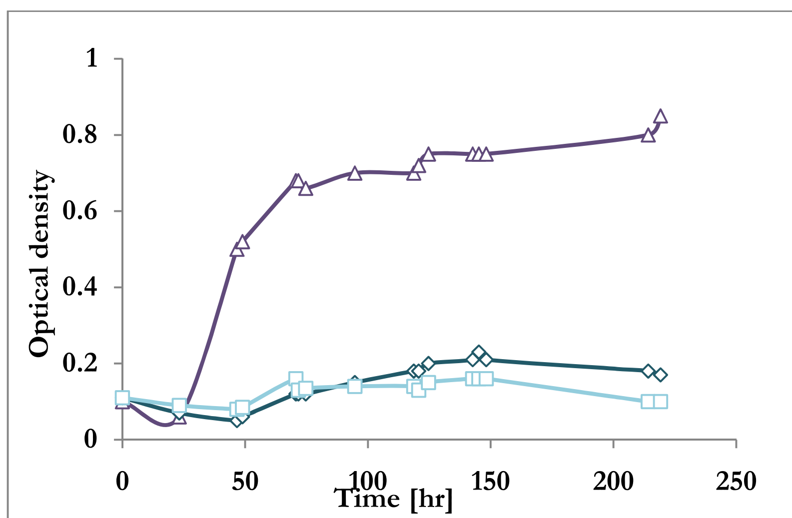


Figure 5.34 *Comparison between the biocompatibility and bioavailability tests refers as Acetone 12.5%: △Glucose 0.1%-control;□Glucose 0.1% + Acetone 12.5%;◇Acetone 12.5%.*

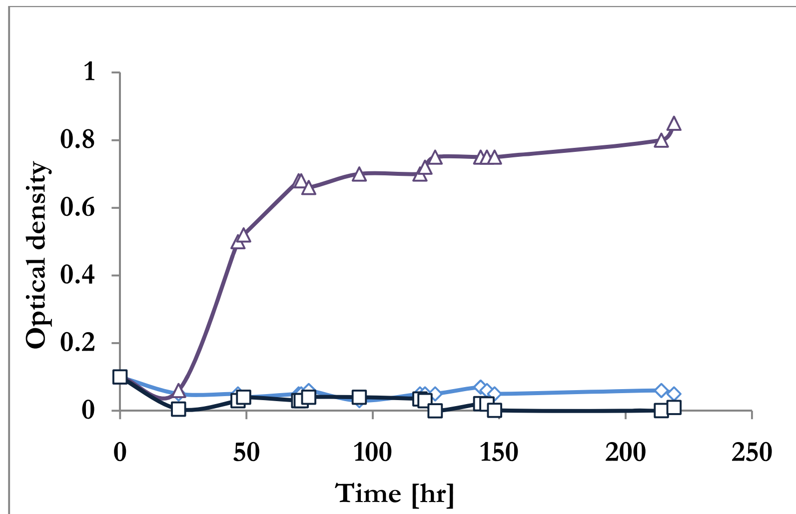


Figure 5.35 *Comparison between the biocompatibility and bioavailability tests refers as n-Hexane 12.5%: △Glucose 0.1%-control; □Glucose 0.1% + n-Hexane 12.5%;◇ n-Hexane 12.5%.*

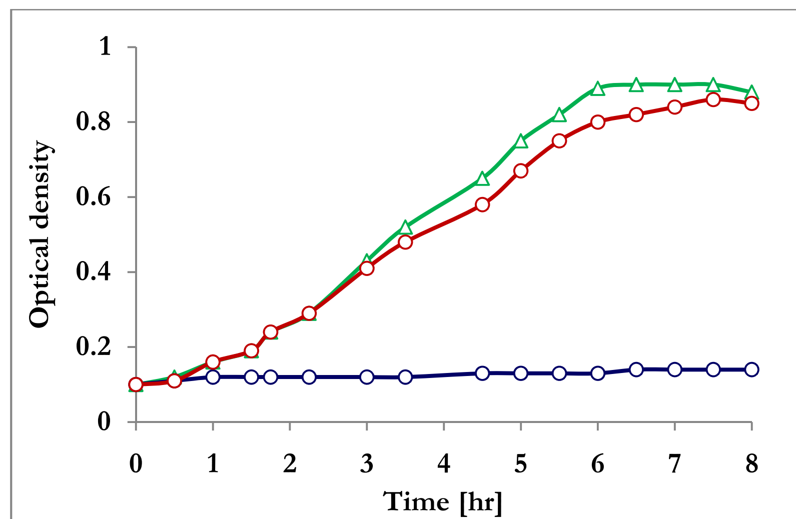


Figure 5.36 *Comparison between the biocompatibility and bioavailability tests refers as Tween 80 0.05%: △Glucose 0.1%-control,○Glucose 0.1% + Tween 80 0.05%,○Tween 80 0.05%.*

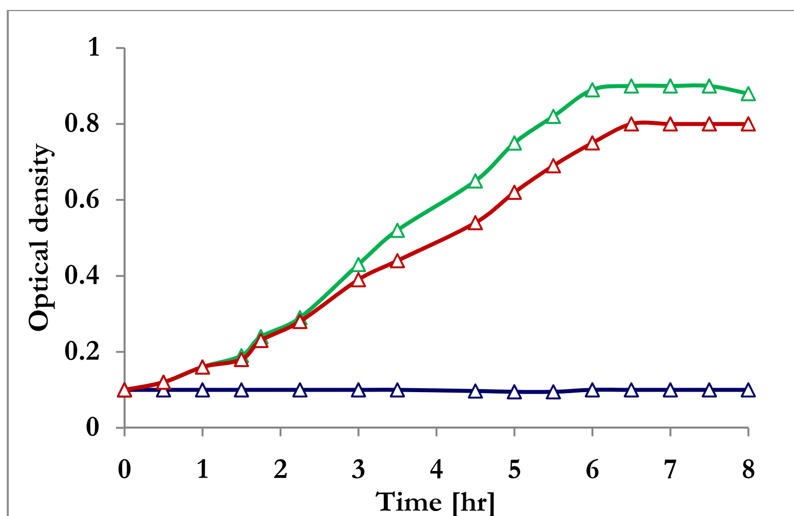


Figure 5.37 *Comparison between the biocompatibility and bioavailability tests refers as Tween 0.1%: △Glucose 0.1%-control;△Glucose 0.1% + Tween 80 0.1%;△Tween 80 0.1%.*

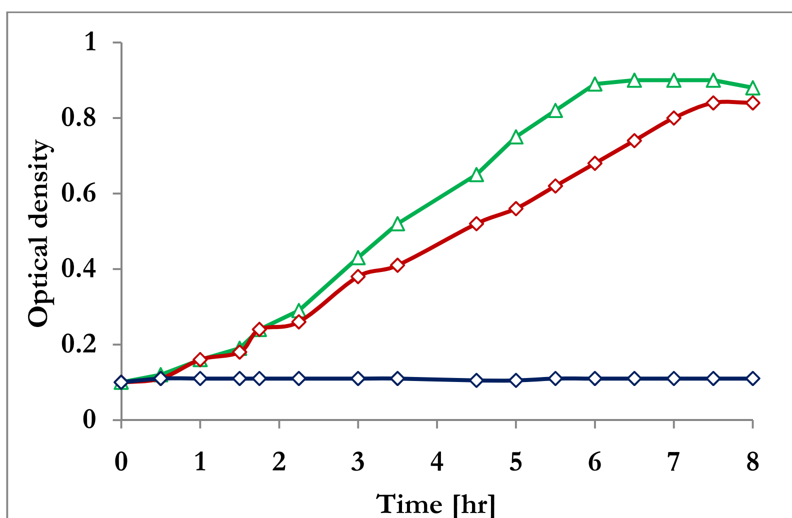


Figure 5.38 *Comparison between the biocompatibility and bioavailability tests refers as Tween 80 0.2%:△Glucose 0.1%-control;◇Glucose 0.1% + Tween 80 0.2%;◇Tween 80 0.2%.*

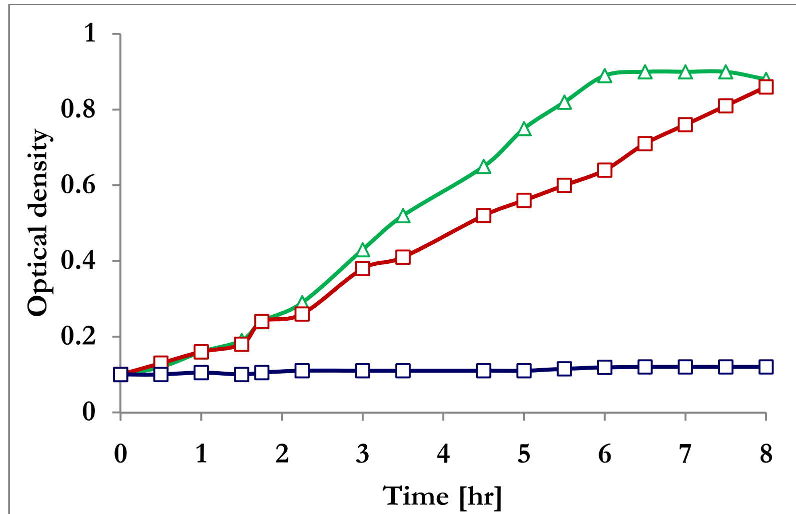


Figure 5.39 *Comparison between the biocompatibility and bioavailability tests refers as Tween 80 0.5%: △Glucose 0.1%-control;□Glucose 0.1% + Tween 80 0.5%;□Tween 80 0.5%.*

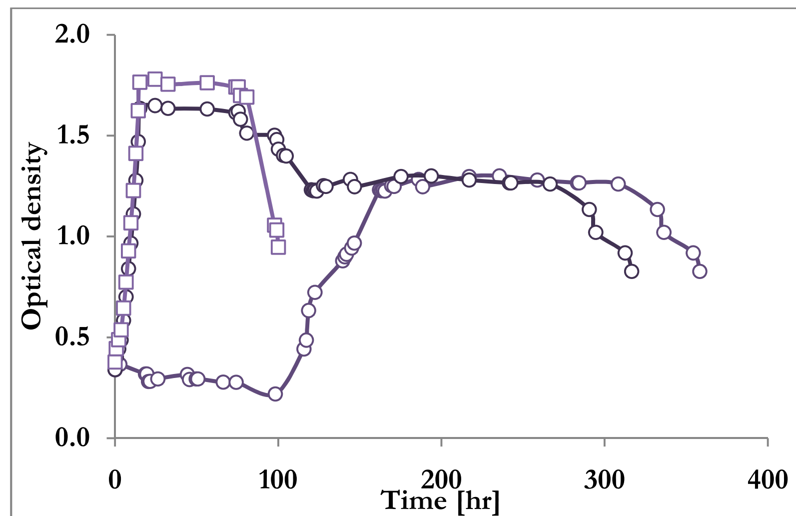


Figure 5.40 *Comparison between the biocompatibility and bioavailability tests refers as Triton X100 0.05%: □Glucose 0.1%-control;○Glucose 0.1% + Triton X100 0.05%;○ Triton X100 0.05%.*

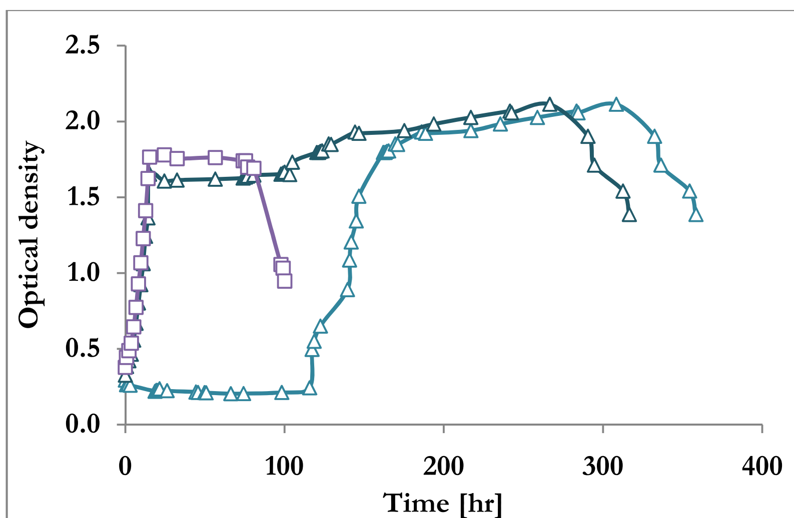


Figure 5.41 *Comparison between the biocompatibility and bioavailability tests refers as Triton X100 0.1%: □Glucose 0.1%-control; △Glucose 0.1% + Triton X100 0.1%; △ Triton X100 0.1%.*

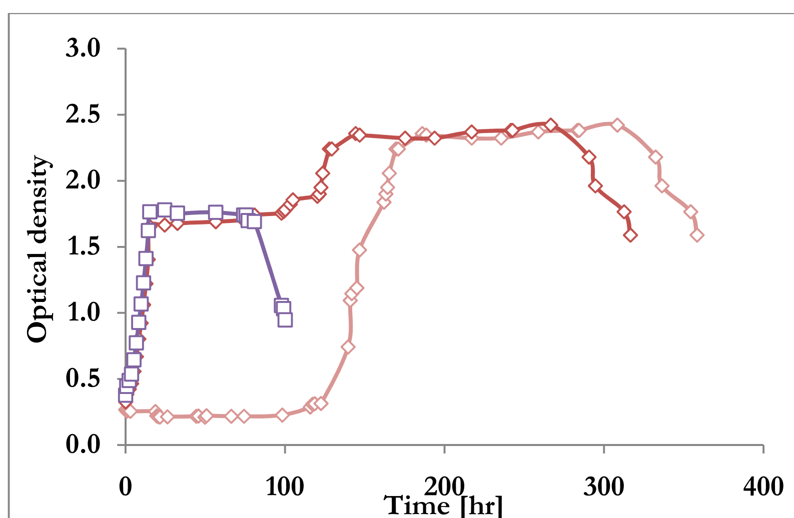


Figure 5.42 *Comparison between the biocompatibility and bioavailability tests refers as Triton X100 0.2%: □Glucose 0.1%-control; ◇Glucose 0.1% + Triton X100 0.2%; ◇ Triton X100 0.2%.*

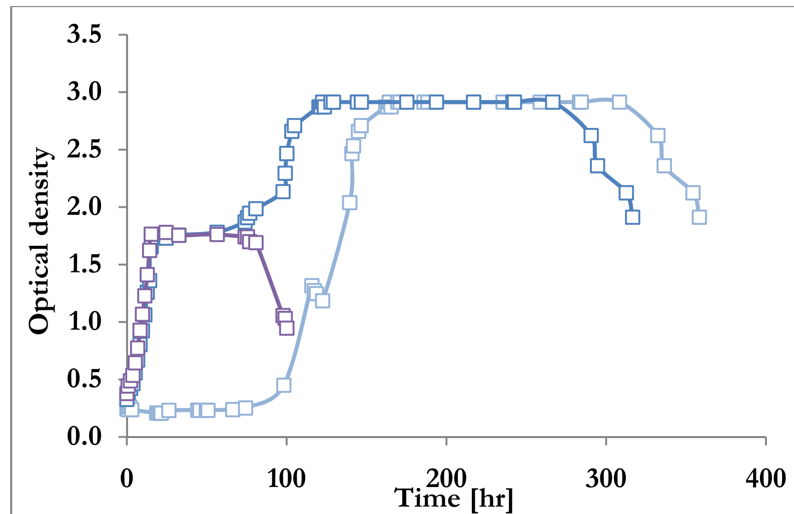


Figure 5.43 *Comparison between the biocompatibility and bioavailability tests refers as Triton X100 0.5%: ☐Glucose 0.1%-control; ☐Glucose 0.1% + Triton X100 0.5%; ☐ Triton X100 0.5%.*

5.3.3.4 Summary of results

The results of the biocompatibility and bioavailability tests referring to the bacterial mixed cultures Bulab 5738 & Bulab 5733, the constant concentration of organic solvents and the concentrations range of surfactants used (0.05-0.5%) described that:

- The mobility agents are not all biocompatible only Methanol and Triton X100 exhibit this property: in fact the growth is not inhibited in the their presence but the lag phase is longer for Methanol in confront with Triton X100 where it does not exist;

- Tween 80 is biocompatible and the growth start only after 1 hour. However, it has found a decrease in growth rate with increasing concentration of Tween 80, which may suggest that the surfactant exerts, at least at higher concentrations, a slight toxic action against microorganisms;

- Acetone and n-Hexane are not biocompatible;

- Methanol and Triton X100 are bioavailable for the microorganisms;

- The Tween 80, Acetone and n-Hexane are not bioavailable for microorganisms.

5.4 Biodegradation of phenanthrene in presence of Tween 80 and/or Triton X100

5.4.1 Introduction

This section describes the biological treatment applied for phenanthrene, PHE degradation using bacterial mixed cultures Bulab 5738 & Bulab 5733 previously acclimated to glucose, GLU and trypic soy broth ,TSB and was carried out in parallel with two types of tests:

- A test conducted in fermenter for a given concentration of PHE and Tween 80 or Triton X100;
- A series of tests performed in Erlenmeyer flasks. A constant concentration of PHE and different concentrations of Tween 80 or Triton X100.

5.4.2 Experimental setup

After the solubilisation tests described in the section 5.2, the solutions were then inoculated with bacterial mixed cultures previously acclimated to GLU and TSB until reaching an optical density of 0.1. The optical density was monitoring with a spectrophotometer (figure 4.6B in chapter 4) at 600 nm wavelength and the concentration of PHE was subsequently determined by HPLC analyses.

The abiotic controls were carried out under the same experimental conditions in parallel with the fermenter.

Fermenter		
Surfactant	**$[Phe]_t$ mg/L**	**$[Phe]_r$ mg/L**
Tween 80 [0.5%]	400	102.82
Triton X100 [0.5%]	400	107.77

Tabel 5.12 *Concentrations of PHE and surfactants used in the fermenter experiments.*

The concentration of PHE and surfactants used for Erlenmeyer batch reactors are the same obtained from the solubilisation tests, tables 5.4 and 5.6.

5.4.3 Summary of results

5.4.3.1 Fermenter batch reactors

The figures from 5.44 to figure 5.49 refer to PHE and Tween 80 and represent the curve of PHE concentrations analysed by HPLC for all the samples, the bacterial growth curve and the consumption of PHE, and the growth rate of bacteria on PHE during the experiment into fermenter.

5.4.3.1.1 Phenanthrene, Tween 80 and bacterial cultures acclimatised on GLU.

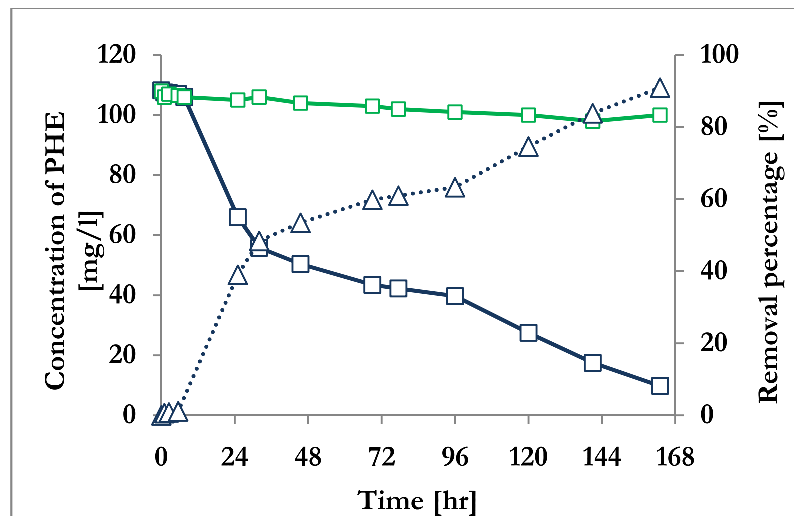


Figure 5.44 *The variation of PHE concentration and the removal percentage of PHE during the experiment using Tween 80 0.5%: □Concentration of PHE; △Efficiency; □ Abiotic control.*

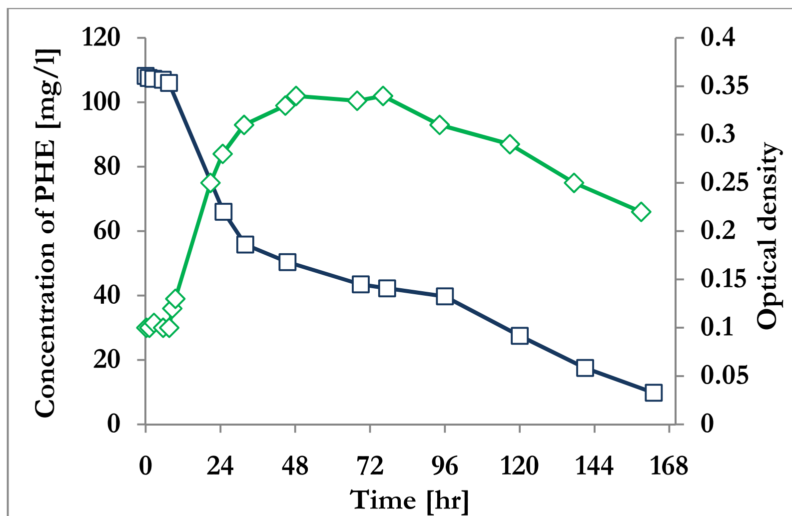


Figure 5.45 *The bacterial growth curve and the consumption of PHE in fermenter during the experiment using Tween 80 0.5%: □Concentration of PHE; ◇Optical density (OD).*

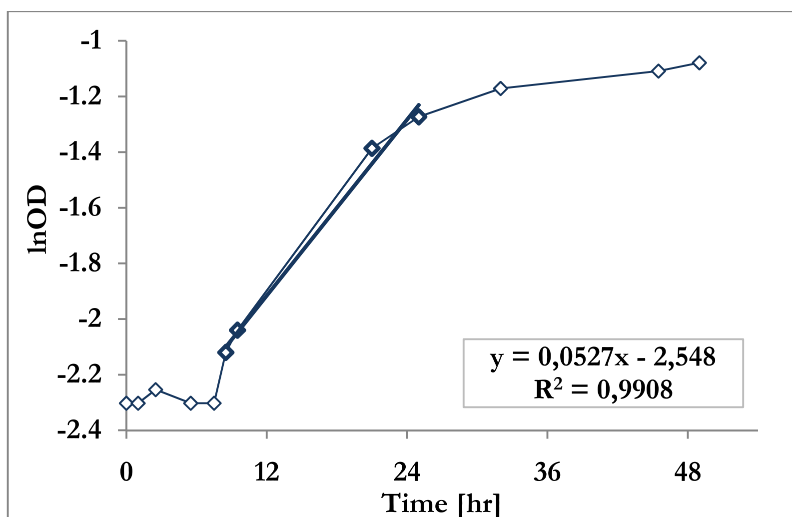


Figure 5.46 *The growth rate of microorganisms on PHE and Tween 80 0.5% in fermenter.*

5.4.3.1.2. Phenanthrene, Tween 80 and bacterial cultures acclimatised on TSB.

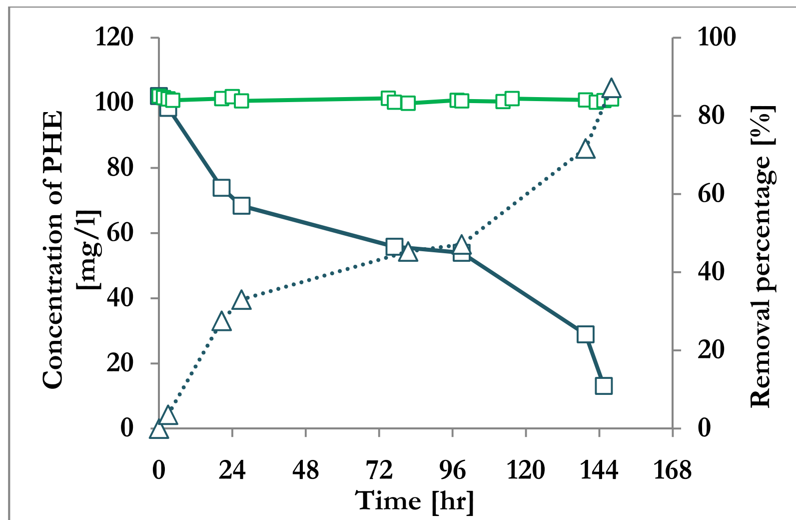


Figure 5.47 *The variation of PHE concentration and the removal percentage of PHE during the experiment using Tween 80 0.5%: □Concentration of PHE; △Efficiency; □ Abiotic control.*

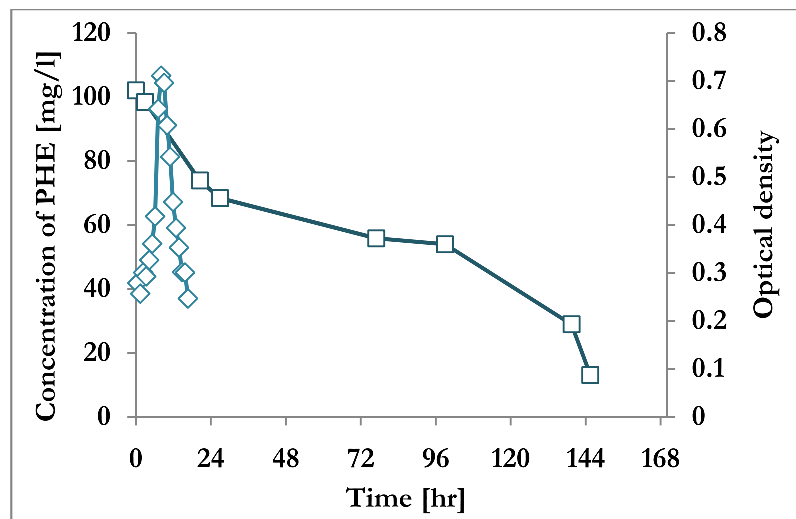


Figure 5.48 *The bacterial growth curve and the consumption of PHE in fermenter during the experiment using Tween 80 0.5%: □Concentration of PHE; ◇ Optical density OD.*

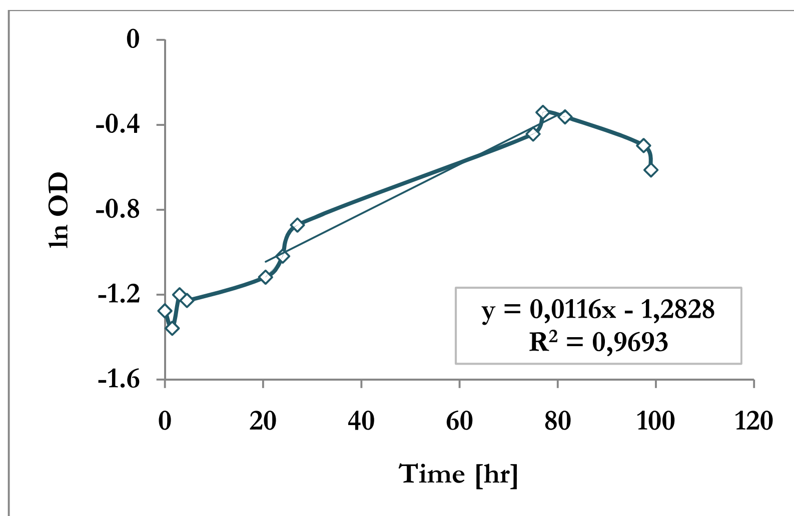


Figure 5.49 *The growth rate of bacteria on PHE and Tween 80 0.5% in fermenter.*

The figures from 5.50 to 5.55 refer to PHE and Triton X100 and represent the curves of PHE concentrations analysed by HPLC for all the samples, the growth of bacterial curve in terms of optical density and the consumption of PHE, and the growth rate of microorganisms on PHE and Triton X100 during the experiment into fermenter for both types of organic substrate used in the acclimatisation of microorganisms.

5.4.3.1.3 Phenanthrene, Triton X100 and bacterial cultures acclimatised on GLU in this case.

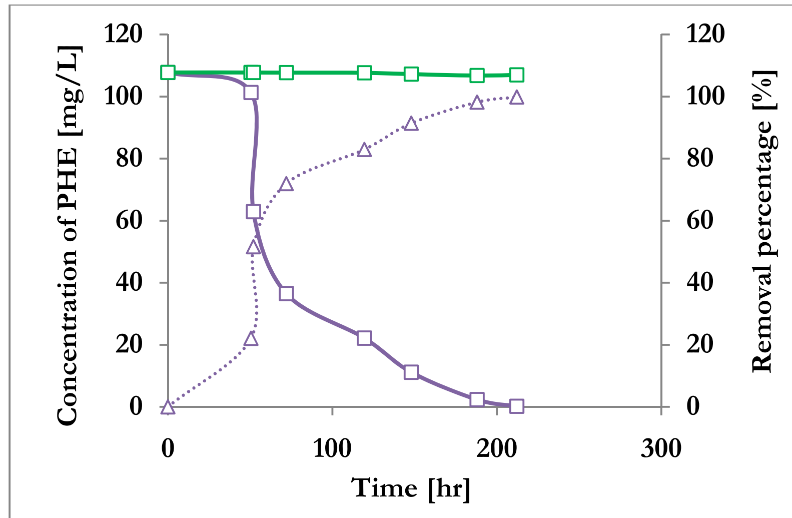


Figure 5.50 *The variation of PHE concentration and the removal percentage of PHE during the experiment using Triton X100 0.5%: □Concentration of PHE; △Removal percentage of PHE; □Abiotic control.*

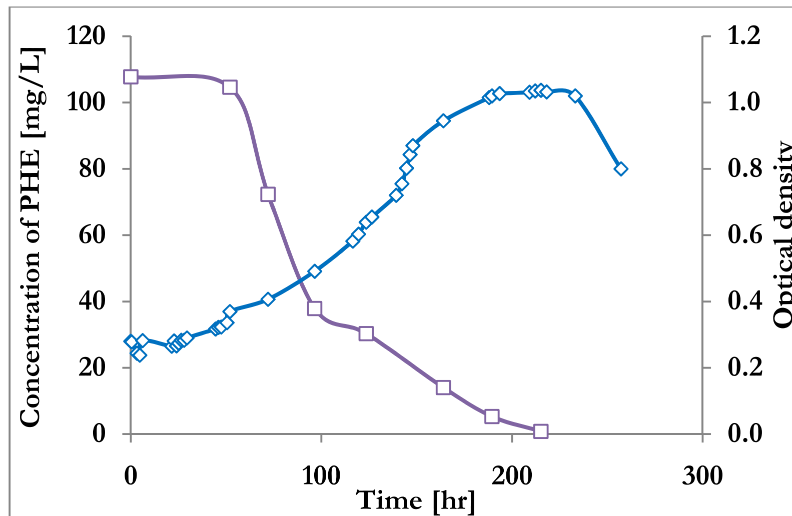


Figure 5.51 *The bacterial growth curve and the consumption of PHE into fermenter during the experiment using Triton X100 0.5%: □Concentration of PHE; ◇Optical density.*

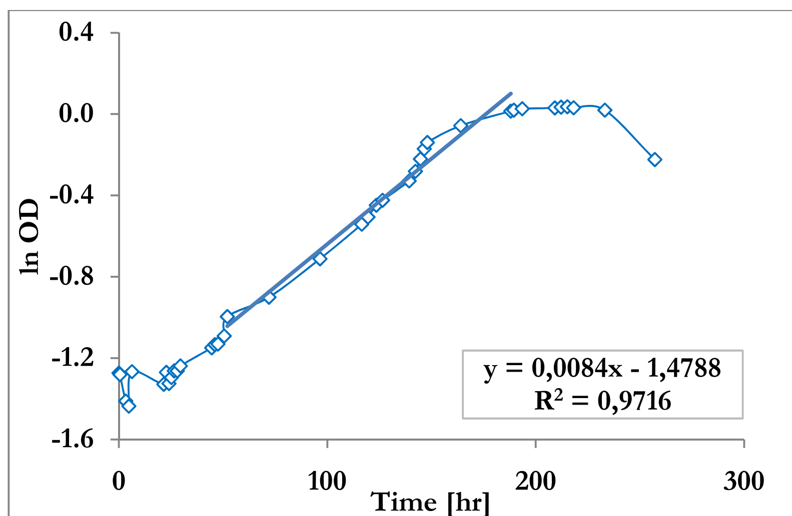


Figure 5.52 *The growth rate of microorganisms on PHE and Triton X100 0.5% in fermenter.*

5.4.3.1.4. Phenanthrene, Triton X100 and bacterial cultures acclimatised on TSB in this case.

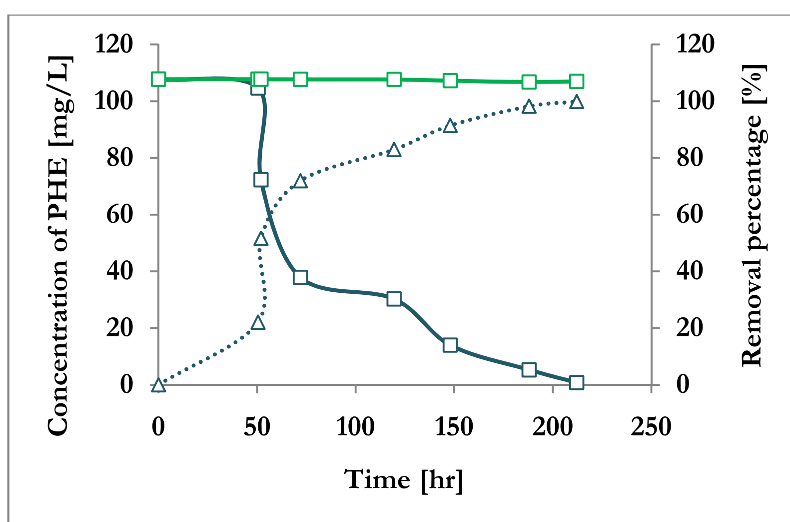


Figure 5.53 *The variation of PHE concentration and the removal percentage during the experiment using Triton X100 0.5%: □Concentration of PHE; △Efficiency; □Abiotic control.*

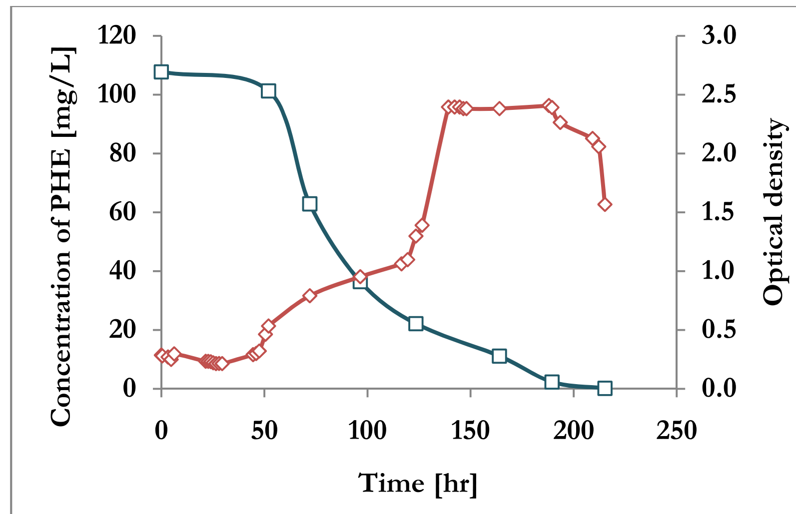


Figure 5.54 *The bacterial growth curve and the consumption of PHE into fermenter during the experiment using Triton X100 0.5%: □Concentration of PHE;◇ Optical density.*

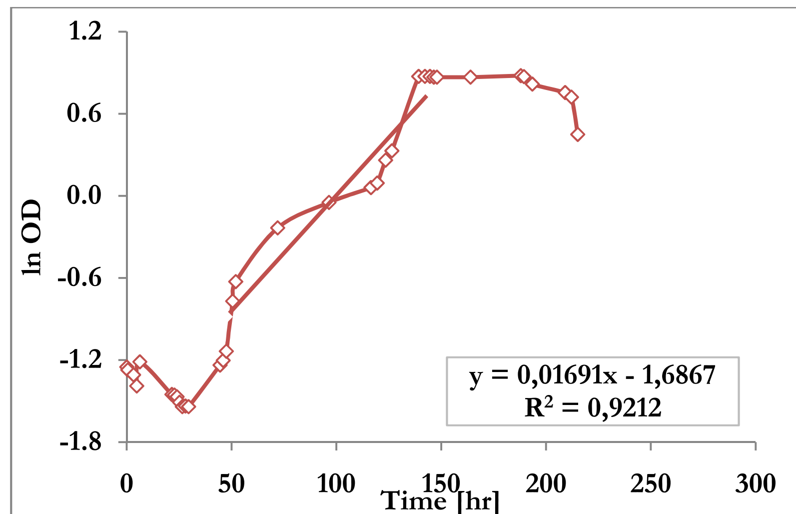


Figure 5.55 *The growth rate of microorganisms on PHE and Triton X100 0.5% in fermenter.*

The figure 5.57 represents a confront between the different substrate used for acclimatisation of bacterial cultures and their influence on the degradation of PHE.

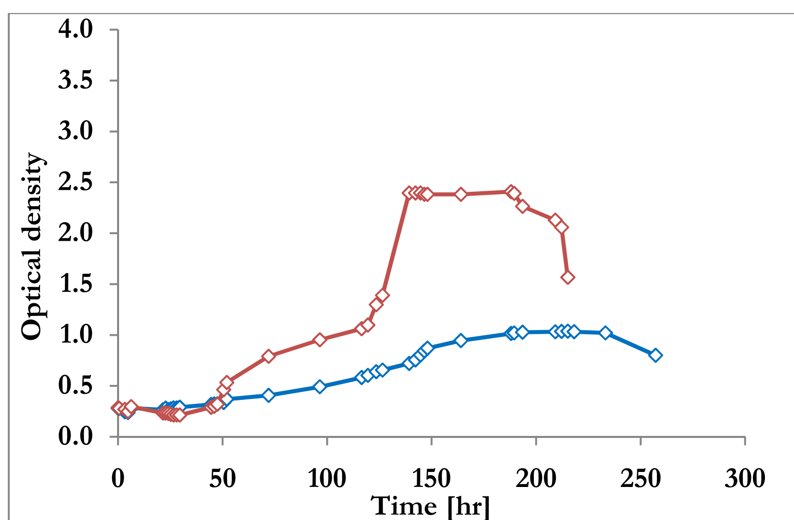


Figure 5.57 *The confront of bacterial growth curves of PHE and Triton X100 0.5% with bacterial cultures acclimatised on different organic substrates in fermenter: ◇ Optical density for bacteria acclimatized on GLU; ◇ Optical density for bacteria acclimatized on TSB.*

5.4.3.2. Erlenmeyer flask batch reactors

5.4.3.2.1 Phenanthrene PHE, Tween 80 and bacterial cultures acclimatised on glucose, GLU

$[PHE]_0$ mg/L	$[PHE]_8$ mg/L	$[PHE]_{24}$ mg/L	$[PHE]_{72}$ mg/L	$[PHE]_{163}$ mg/L
102.82	101.98	90.34	41.16	4.94
21.11	20.87	18.55	8.23	0.62
10.89	10.50	8.54	3.20	0.16
0.43	0.39	0.217	0	0

Table 5.13 *HPLC results refers of PHE in terms of mg/L during the experiments.*

The figure 5.58 shows the previous data of the table 5.13 and represents the consumption of PHE in terms of mg/L PHE, for glucose used as an organic substrate in the acclimatisation of microorganisms prior to degradation experiments.

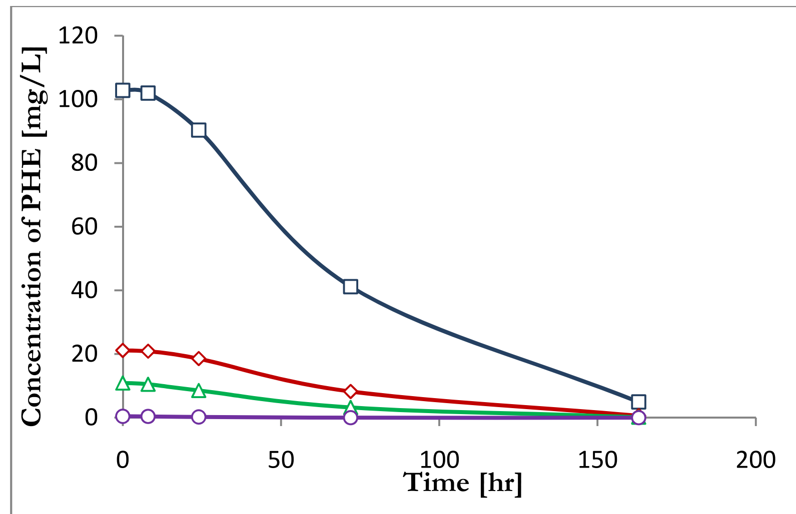


Figure 5.58 *Variation of PHE concentration in presence of different concentration of Tween 80 during the experiments:* □ *$[PHE]_0$ +Tween 80 0.5%;* ◇ *$[PHE]_0$ +Tween 80 0.2%;* △ *$[PHE]_0$ +Tween 80 0.1%,* ○ *$[PHE]_0$ +Tween 80 0.05%.*

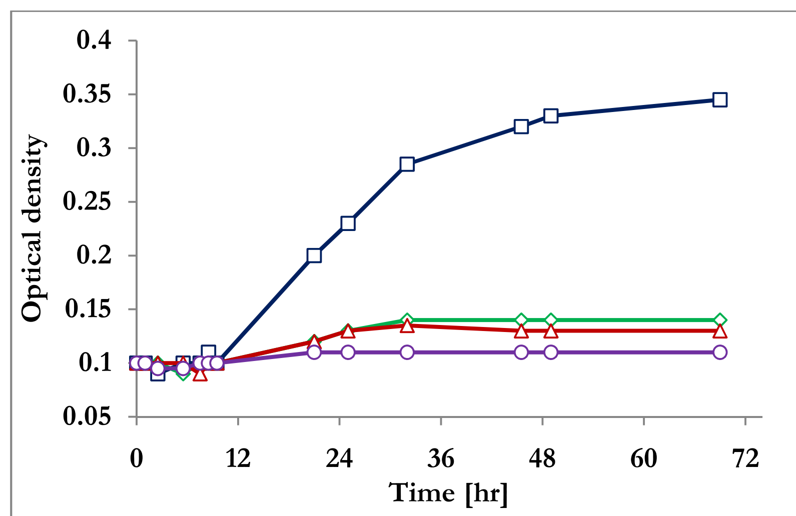


Figure 5.59 *Bacterial growth curves in presence of different concentrations of Tween 80 and $[PHE]_0$ during the experiments:* □ *$[PHE]_0$ +Tween 80 0.5%* ◇ *$[PHE]_0$ +Tween 80 0.2%;* △ *$[PHE]_0$ +Tween 80 0.1%,* ○ *$[PHE]_0$ +Tween 80 0.05%.*

The figure 5.59 show the variation of bacterial growth in terms of optical density in presence of different concentrations of Tween 80 and $[PHE]_0$ during

the experiment.

The figures from 5.60 to 5.63 report the graphs of all experiments at different concentrations of PHE and Tween 80, in semi logarithmic scale with their interpolation lines and equations (the slope of these lines is precisely the growth rate).

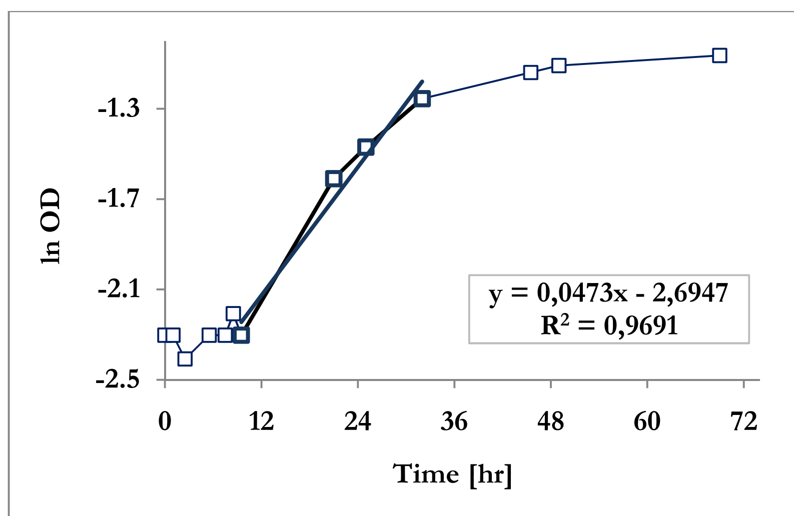


Figure 5.60 *Growth curve of microorganisms on [Phe]$_0$ and Tween 80 0.5% in semi-logarithmic scale and the calculation of growth rate.*

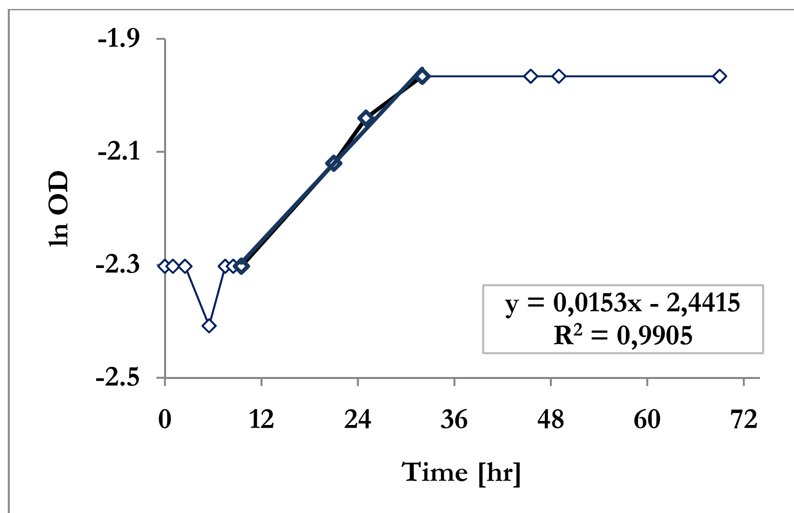


Figure 5.61 *Growth curve of bacteria on [Phe]$_0$ and Tween 80 0.2% in semi logarithmic scale and the calculation of growth rate.*

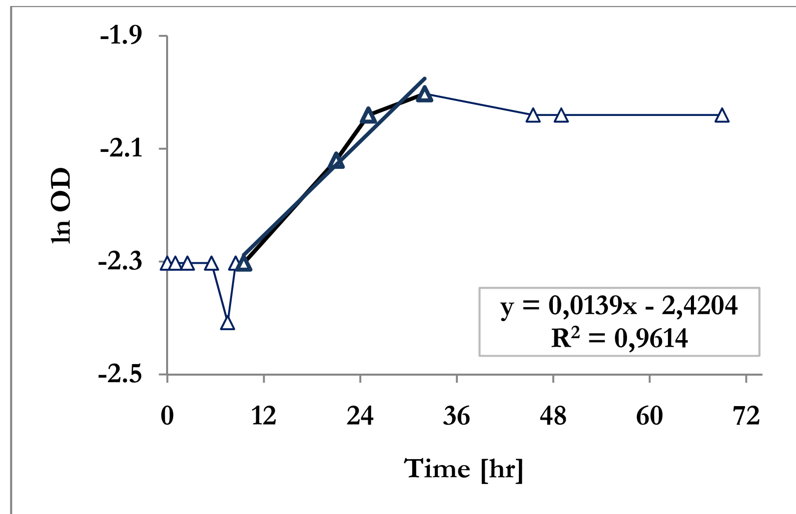


Figure 5.62 *Growth curve of bacteria on $[Phe]_0$ and Tween 80 0.1% in semi logarithmic scale and the calculation of growth rate.*

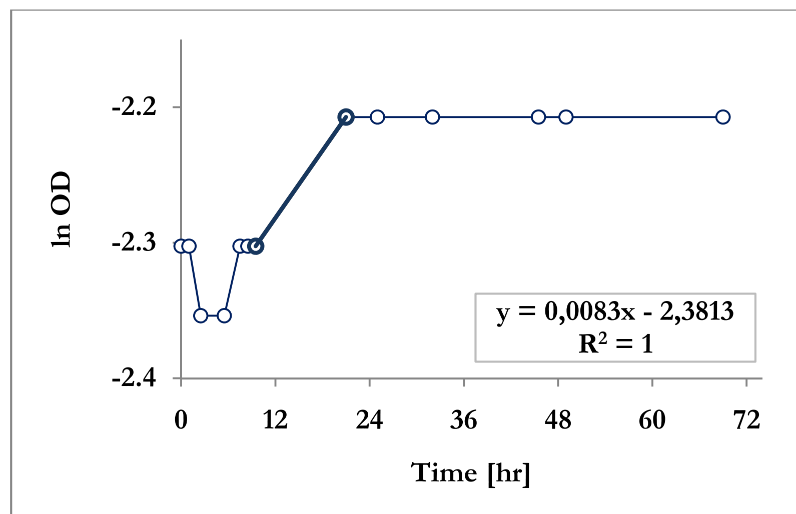


Figure 5.63 *Growth curve of bacteria on $[Phe]_0$ and Tween 80 0.05% in semi logarithmic scale and the calculation of growth rate.*

In table 5.14 are reported the previous kinetic results and showed the values of the rate growth depending on the concentration of PHE and Tween 80.

[Tween 80] %	$[PHE]_0$ mg/L	Growth velocity 1/hr
0.05	102.82	0.0083
0.1	21.11	0.0139
0.2	10.89	0.0153
0.5	0.43	0.0473

Table 5.14 *Growth rate of microorganisms at different concentrations of PHE and Tween 80.*

Figure 5.64 and 5.65 show how the growth rate of microorganisms increase with the increasing concentration of PHE and Tween 80 and the percentage removal of PHE was observed during the experiment.

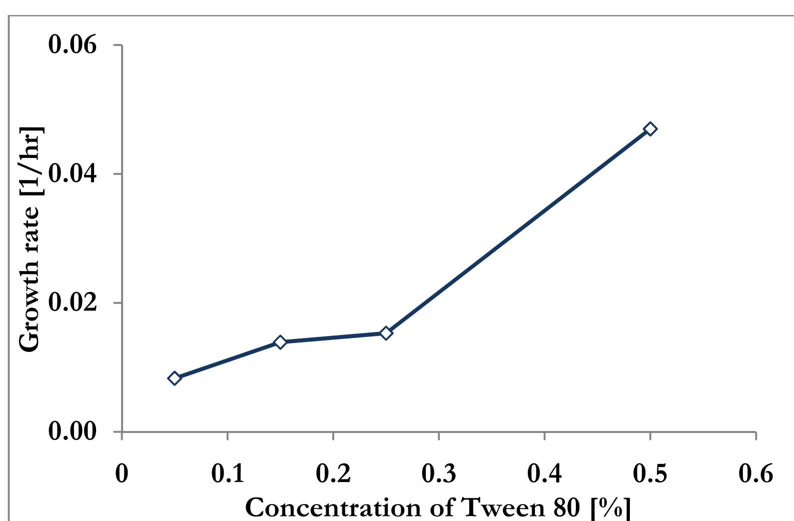


Figure 5.64 *Growth rate of microorganisms at different concentrations of PHE and Tween 80.*

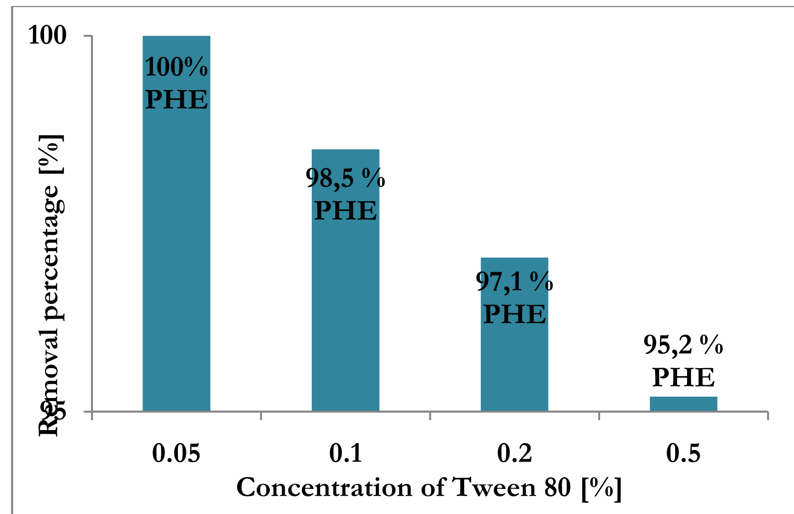


Figure 5.65 *Percentage removal of PHE at the end of experiments.*

The diagram confirms that concentrations of 0.05% Tween 80 allowing high rates of removal but the removal decreases with increasing concentrations of PHE and Tween 80.

5.4.3.2.2 Phenanthrene PHE, Tween 80 and bacterial cultures acclimatised on Tryptic soy broth, TSB

$[PHE]_0$ mg/L	$[PHE]_8$ mg/L	$[PHE]_{24}$ mg/L	$[PHE]_{72}$ mg/L	$[PHE]_{163}$ mg/L
102.82	90.67	63.34	35.78	1.94
21.11	18.93	11.08	6.79	0.37
10.89	8.67	7.17	5.71	0.16
0.43	0.35	0.22	0	0

Table 5.15 *HPLC results refers of PHE in terms of mg/L during the experiments.*

The figure 5.66 shows the previous data of the table 5.15 and represent the consumption of PHE in terms of concentration for tryptic soy broth used as an organic substrate in the acclimatisation of microorganisms prior to degradation experiments.

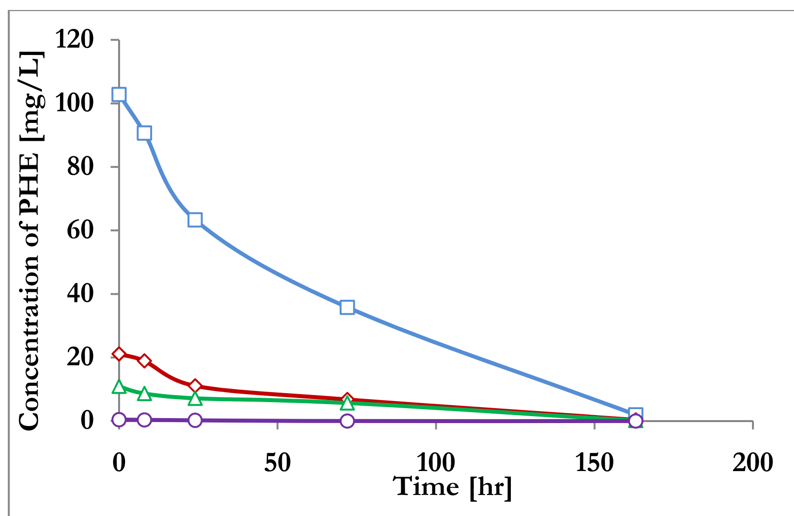


Figure 5.66 *Variation of PHE concentration in presence of different concentration of Tween 80 during the experiments:* □ *$[PHE]_0$ +Tween 80 0.5%;* ◇ *$[PHE]_0$ +Tween 80 0.2%;* △ *$[PHE]_0$ +Tween 80 0.1%;* ○ *$[PHE]_0$ +Tween 80 0.05%.*

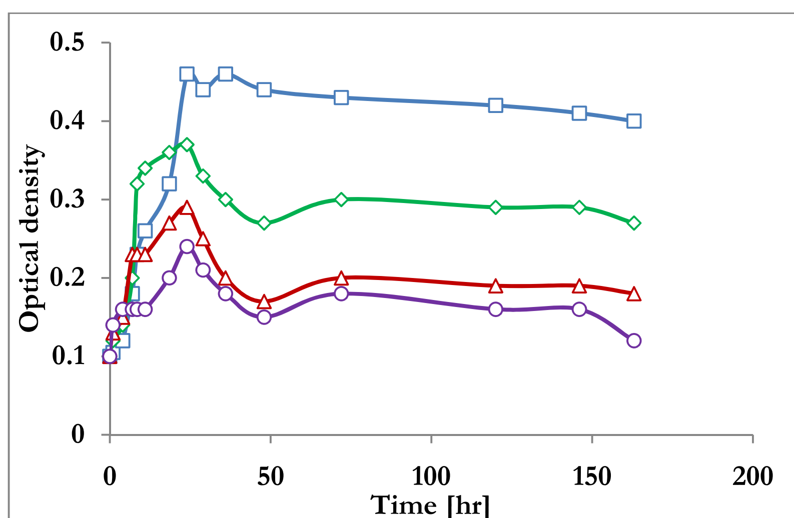


Figure 5.67 *Bacterial growth curves in presence of different concentrations of Tween 80 and $[PHE]_0$:* □ *$[PHE]_0$ +Tween 80 0.5%* ◇ *$[PHE]_0$ +Tween 80 0.2%;* △ *$[PHE]_0$ +Tween 80 0.1%;* ○ *$[PHE]_0$ +Tween 80 0.05%.*

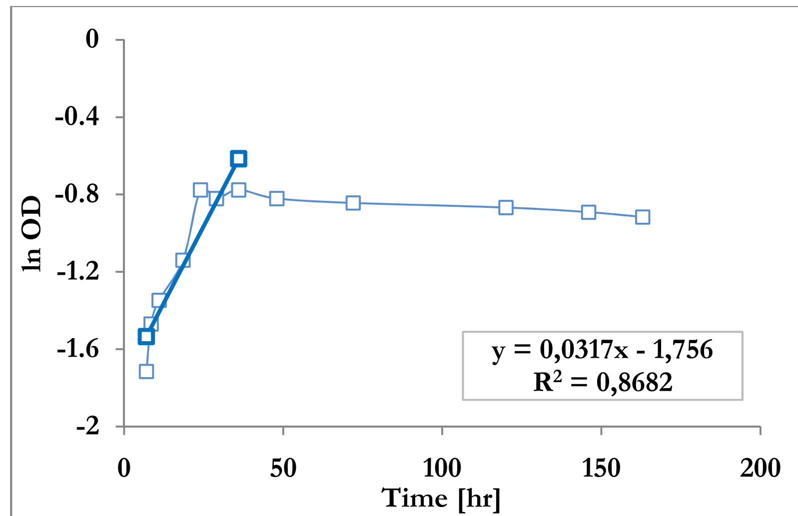


Figure 5.68 *Growth curve of bacteria on $[Phe]_0$ and Tween 80 0.5% in semi logarithmic scale and the calculation of growth rate.*

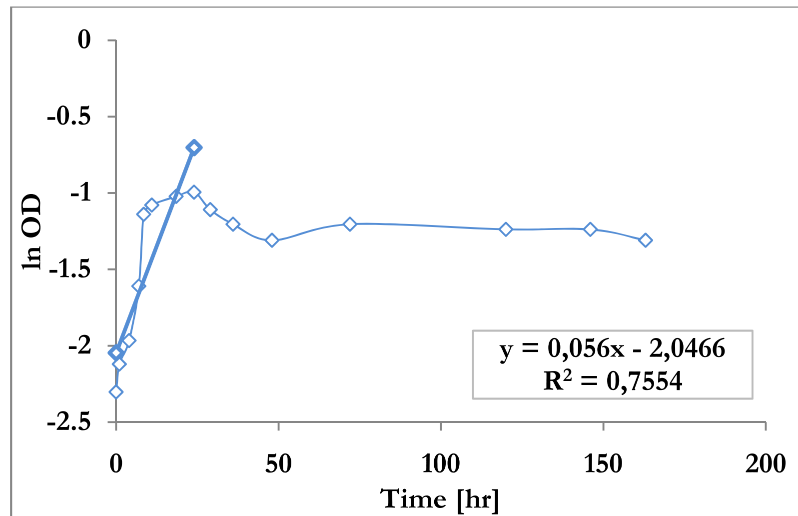


Figure 5.69 *Growth curve of bacteria on $[Phe]_0$ and Tween 80 0.2% in semi logarithmic scale and the calculation of growth rate.*

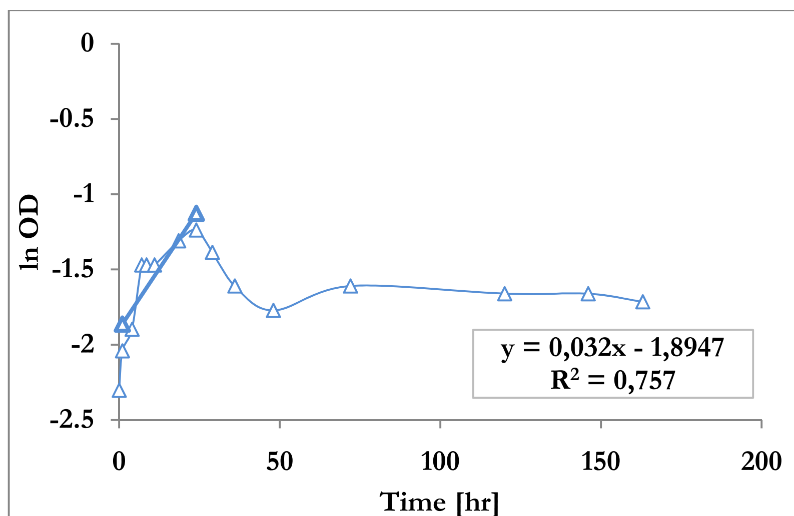


Figure 5.70 *Growth curve of bacteria on [Phe] and Tween 80 0.1% in semi logarithmic scale and the calculation of growth rate.*

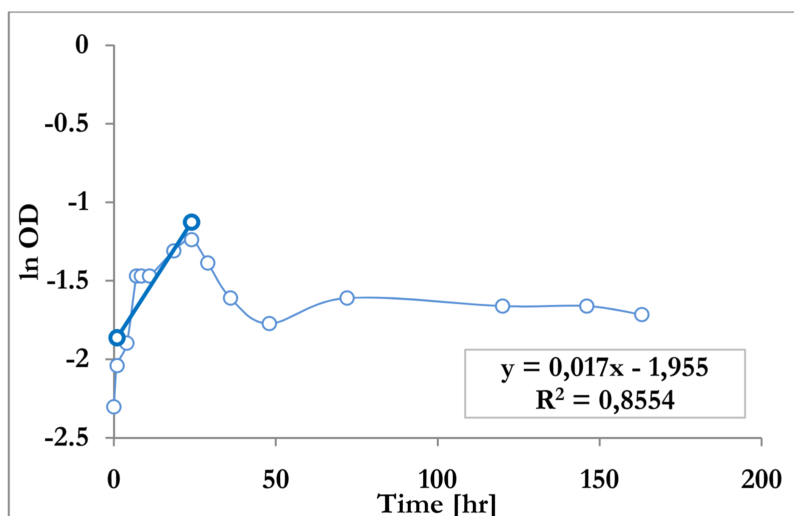


Figure 5.71 *Growth curve of bacteria on $[Phe]_0$ and Tween 80 0.05% in semi logarithmic scale and the calculation of growth rate.*

In table 5.16 are reported the previous kinetic results and showed the values of the rate growth depending on the concentration of PHE and Tween 80.

[Tween 80] %	$[PHE]_0$ mg/L	Growth velocity 1/hr
0.05	102.82	0.017
0.1	21.11	0.032
0.2	10.89	0.056
0.5	0.43	0.0317

Table 5.16 *Growth rate of microorganisms at different concentrations of PHE and Tween 80.*

Figure 5.72 and 5.73 show how the growth velocity of microorganisms increase until CMC of surfactant and decrease rapidly at 0.5% Tween 80. The percentage removal of PHE was observed during the experiment.

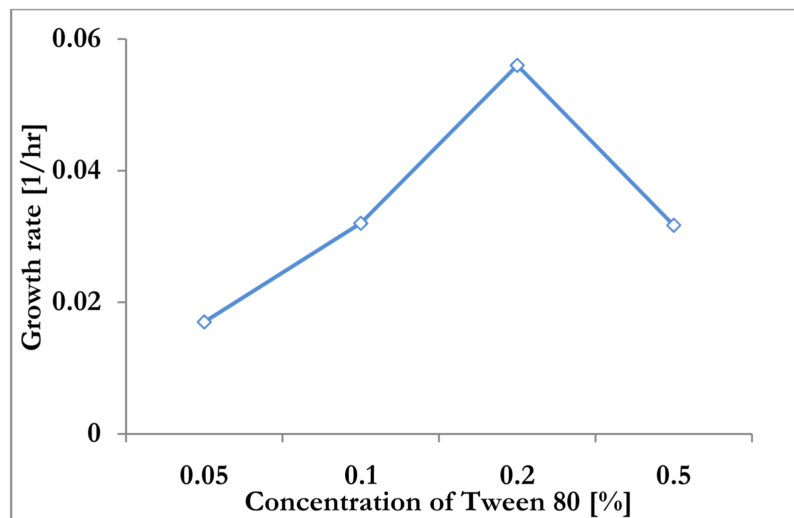


Figure 5.72 *Growth rate of microorganisms at different concentrations of PHE and Tween 80.*

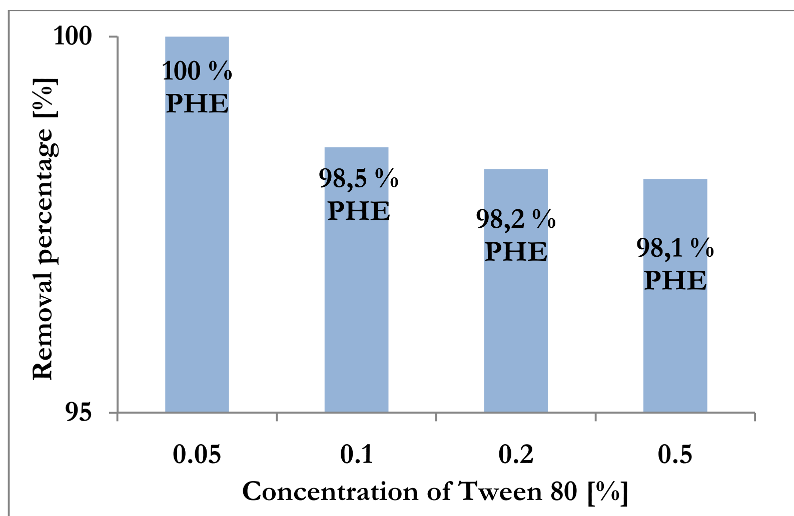


Figure 5.73 *Percentage removal of PHE at the end of experiments.*

5.4.3.2.3 Phenanthrene PHE, Triton X100 and bacterial cultures acclimatised on glucose, GLU.

$[PHE]_0$ mg/L	$[PHE]_{50}$ mg/L	$[PHE]_{100}$ mg/L	$[PHE]_{150}$ mg/L	$[PHE]_{257}$ mg/L
107.77	90.36	63.90	27.92	6.13
46.58	35.91	23.17	11.91	4.82
26.58	19.91	10.36	5.91	1.33
2.57	1.88	0.87	0	0

Table 5.17 *HPLC results refers of PHE in terms of mg/L during the experiments.*

Below the figure 5.74 shows the previous data of the table 5.17 and represents the consumption of PHE in terms of mg/L PHE for glucose used as an organic substrate in the acclimatization of microorganisms prior to degradation experiments.

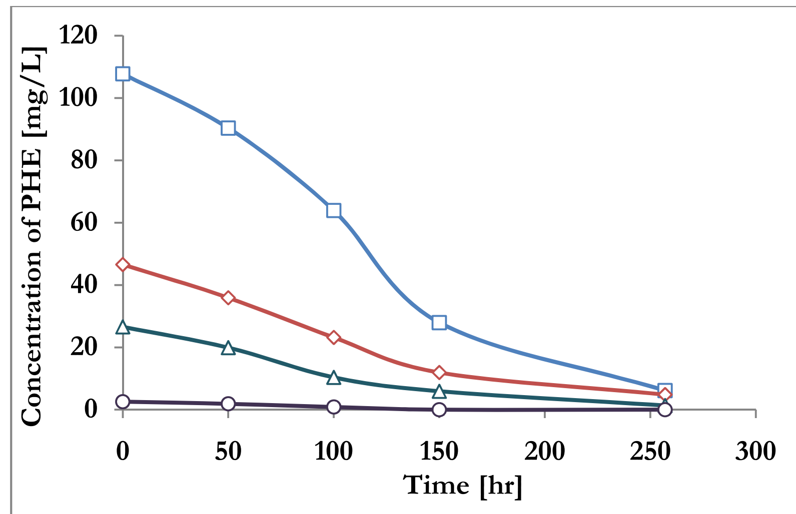


Figure 5.74 *Variation of PHE concentration in presence of different concentration of Triton X100 during the experiments:* □ *$[PHE]_0$ +Triton X100 0.5%;* ◇ *$[PHE]_0$ +Triton X100 0.2%;* △ *$[PHE]_0$ +Triton X100 0.1%,* ○ *$[PHE]_0$ +Triton X100 0.05%.*

The figures 5.75 show the variation of bacterial growth in terms of optical density in presence of different concentrations of Triton X100 and $[PHE]_0$ during the experiments.

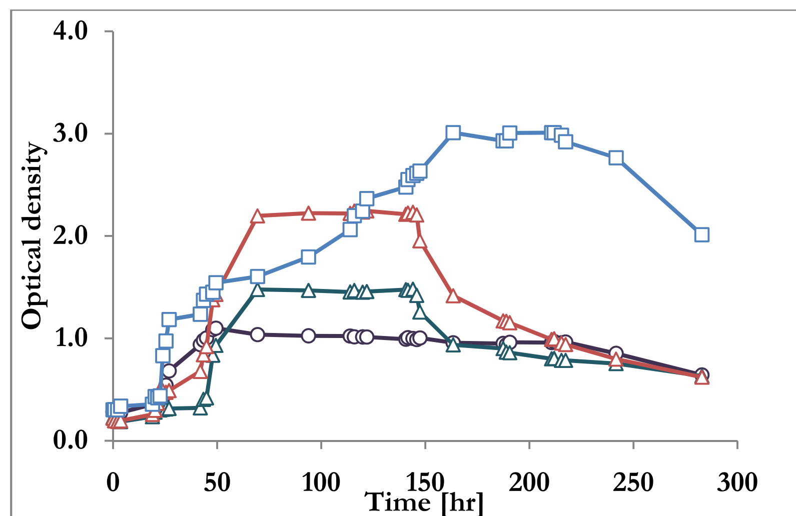


Figure 5.75 *Bacterial growth curves in presence of different concentration of PHE_0 and Triton X100 during the experiments:* □ *$[PHE]_0$ +Triton X100 0.5%;* ◇ *$[PHE]_0$ +Triton X100 0.2%;* △ *$[PHE]_0$ +Triton X100 0.1%,* ○ *$[PHE]_0$ +Triton X100 0.05%.*

Below given figures from 5.76 to 5.79 report the graphs of all experiments at different concentrations of PHE and Triton X100, in semi-logarithmic scale with their interpolation lines and equations (the slope of these lines is precisely the growth rate).

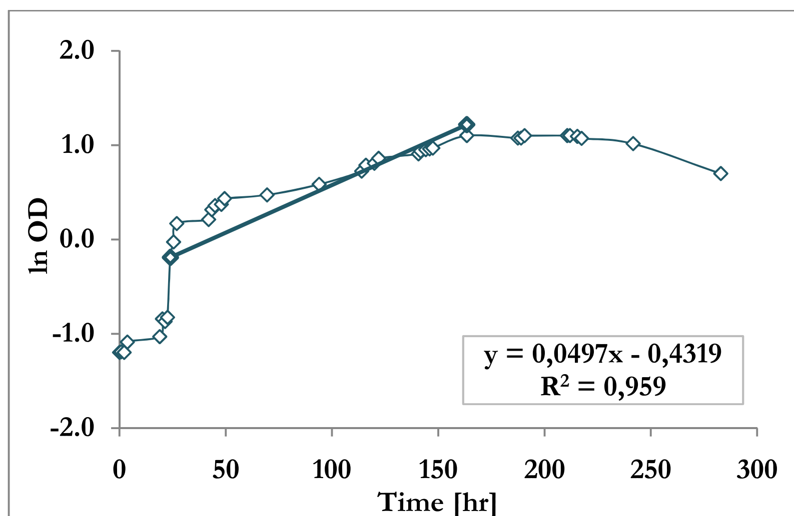


Figure 5.76 *Growth curve of microorganisms on [Phe]$_0$ and Triton X100 0.5% in semi logarithmic scale and the calculation of growth rate.*

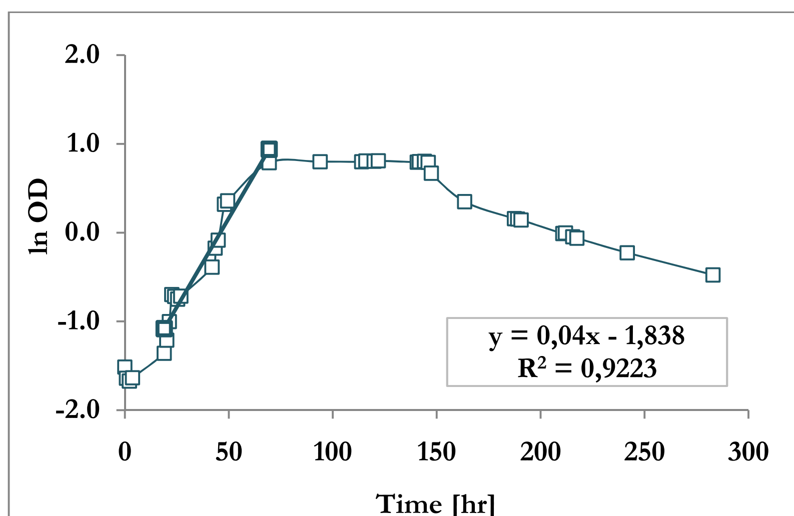


Figure 5.77 *Growth curve of bacteria on [Phe]$_0$ and Triton X100 0.2% in semi logarithmic scale and the calculation of growth rate.*

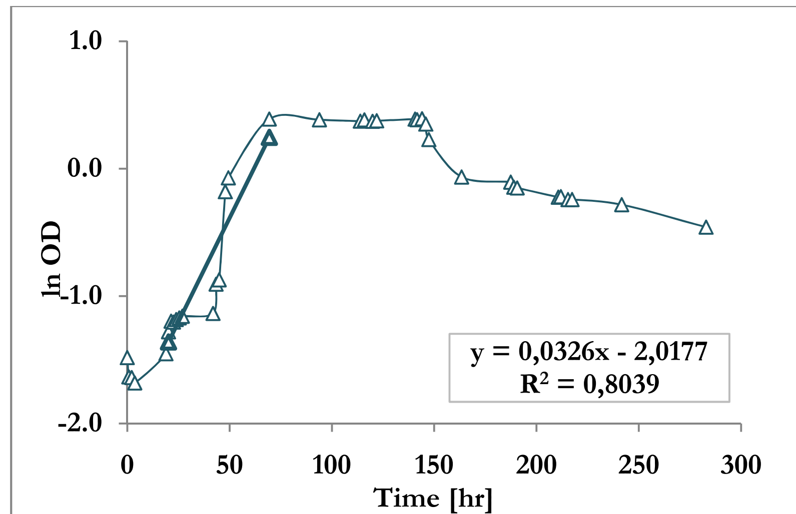


Figure 5.78 *Growth curve of bacteria on* $[Phe]_0$ *and Triton X100 0.1% in semi logarithmic scale and the calculation of growth rate.*

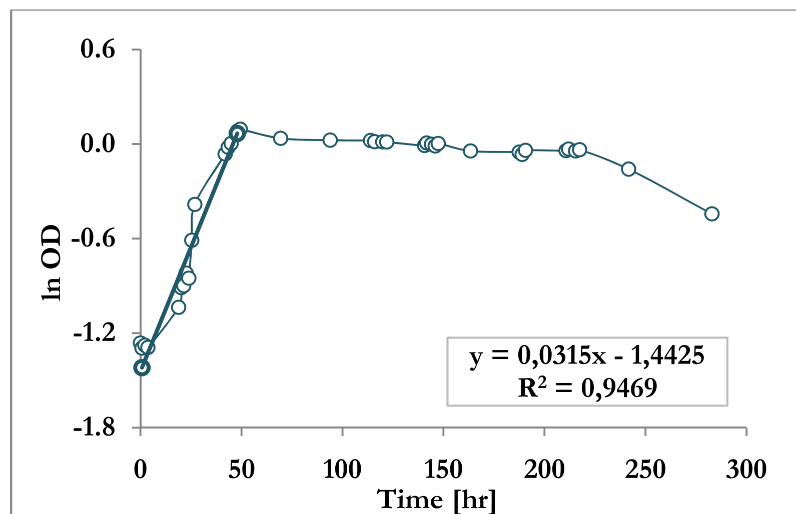


Figure 5.79 *Growth curve of bacteria on* $[Phe]_0$ *and Triton X100 0.05% in semi logarithmic scale and the calculation of growth rate.*

In table 5.18 are reported the previous kinetic results and show the values of the rate growth depending on the concentration of PHE and Triton X100.

[Triton X100] %	$[PHE]_0$ mg/L	Growth velocity 1/hr
0.05	2.57	0.0315
0.1	26.58	0.0326
0.2	46.58	0.04
0.5	107.77	0.0497

Table 5.18 *Growth rate of microorganisms at different concentrations of PHE and Triton X100.*

The figures 5.80 and 5.81 show how the growth velocity of microorganisms increase with the increasing concentration of PHE and Triton X100 and the percentage removal of PHE was observed during the experiment.

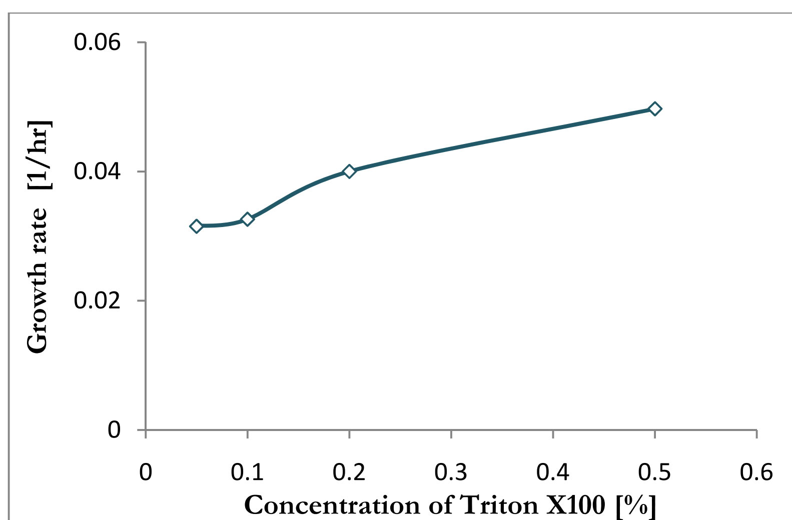


Figure 5.80 *Growth rate of microorganisms at different concentrations of PHE and Triton X100.*

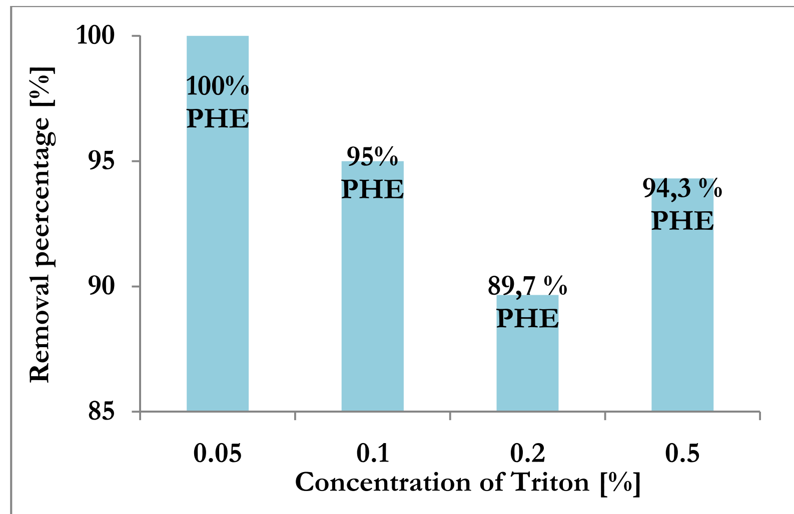


Figure 5.81 *Percentage removal of PHE at the end of experiments.*

5.4.3.2.4 Phenanthrene PHE, Triton X100 and bacterial cultures acclimatised on Tryptic soy broth, TSB.

$[PHE]_0$ mg/L	$[PHE]_{50}$ mg/L	$[PHE]_{100}$ mg/L	$[PHE]_{150}$ mg/L	$[PHE]_{215}$ mg/L
107.77	79.80	53.90	13.5	3.87
46.58	23.16	13.55	6.31	2.17
26.58	19.80	9.89	4.14	0.34
2.57	0.26	0	0	0

Table 5.19 *HPLC results refers of PHE in terms of mg/L during the experiments.*

The figure 5.82 shows the previous data of the table 5.19 and represent the consumption of PHE in terms of concentration only for tryptic soy broth used as an organic substrate in the acclimatization of microorganisms previous to degradation experiments.

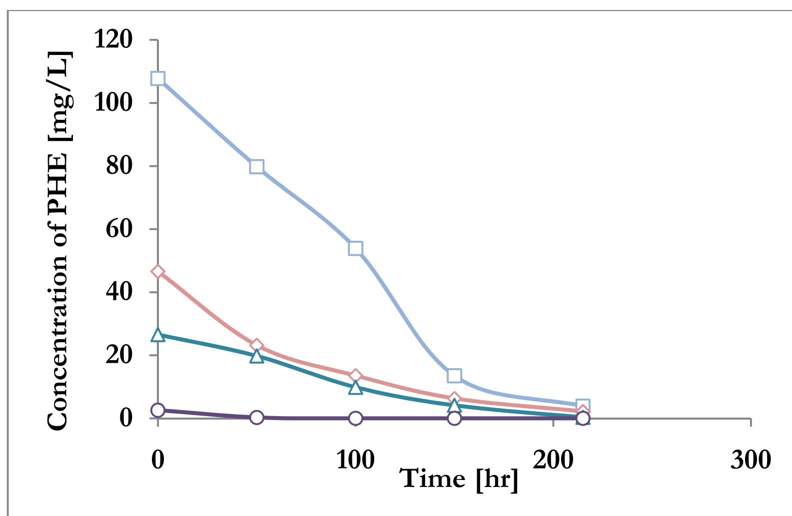


Figure 5.82 *Variation of PHE concentration in presence of different concentration of Triton X100 during the experiments: □ $[PHE]_0$ +Triton X100 0.5%; ◇ $[PHE]_0$ +Triton X100 0.2%; △ $[PHE]_0$ +Triton X100 0.1%; ○ $[PHE]_0$ +Triton X100 0.05%.*

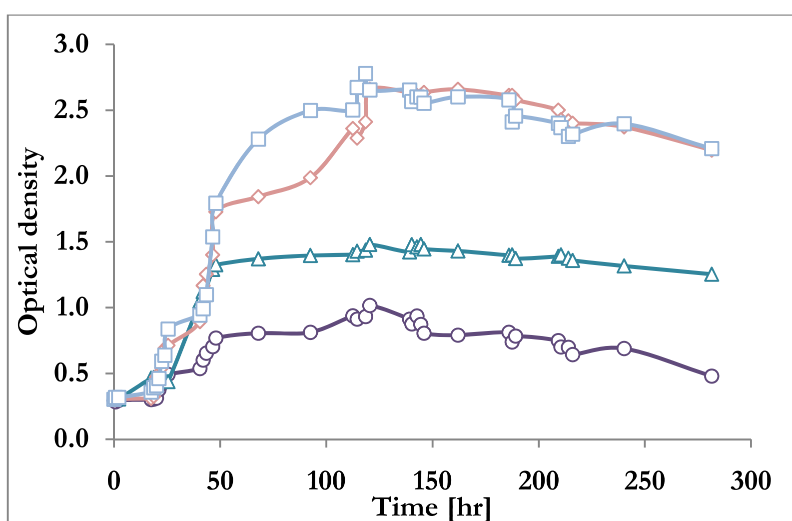


Figure 5.83 *Bacterial growth curves in presence of different concentration of PHE_0 and Triton X100 during the experiments: □ $[PHE]_0$ +Triton X100 0.5%; ◇ $[PHE]_0$ +Triton X100 0.2%; △ $[PHE]_0$ +Triton X100 0.1%; ○ $[PHE]_0$ +Triton X100 0.05%.*

Below the figures from 5.84 to 5.87 report the graphs of all experiments at different concentrations of PHE and Triton X100, in semi logarithmic scale with their interpolation lines and equations (the slope of these lines is precisely

the growth rate). The experimental points used to calculate the growth rate are shown.

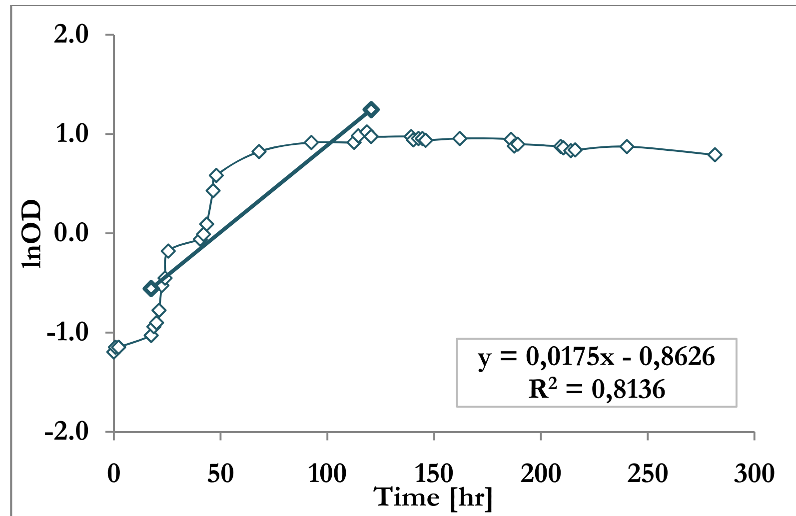


Figure 5.84 *Growth curve of bacteria on [Phe]$_0$ and Triton X100 0.5% in semi logarithmic scale and the calculation of growth rate.*

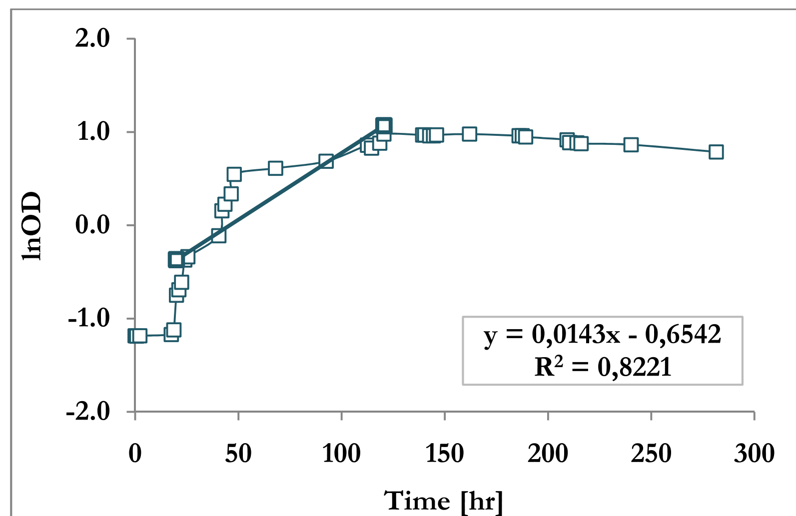


Figure 5.85 *Growth curve of bacteria on [Phe]$_0$ and Triton X100 0.2% in semi logarithmic scale and the calculation of growth rate.*

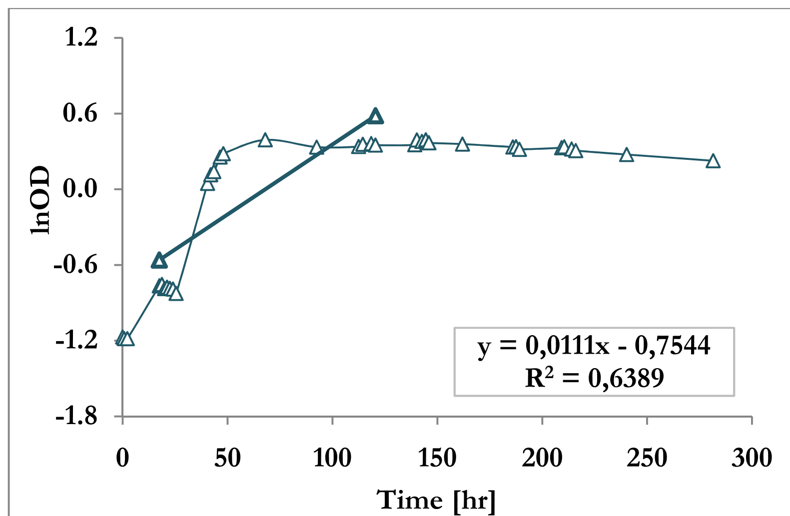


Figure 5.86 *Growth curve of bacteria on $[Phe]_0$ and Triton X100 0.1% in semi logarithmic scale and the calculation of growth rate.*

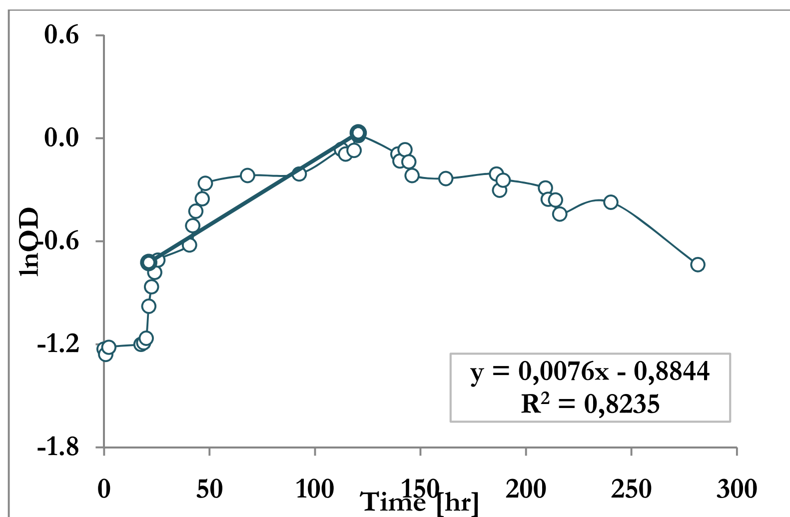


Figure 5.87 *Growth curve of bacteria on $[Phe]_0$ and Triton X100 0.05% in semi logarithmic scale and the calculation of growth rate.*

In table 5.20 are reported the previous kinetic results and show the values of the rate growth depending on the concentration of PHE and Triton X100.

[Triton X100] %	$[PHE]_0$ mg/L	Growth velocity 1/hr
0.05	2.57	0.0076
0.1	26.58	0.0111
0.2	46.58	0.0143
0.5	107.77	0.0175

Table 5.20 *Growth rate of microorganisms at different concentrations of PHE and Triton X100.*

Below the figure 5.88 and 5.89 show how the growth velocity of microorganisms increase with the increasing concentration of PHE and Triton X100 and the percentage removal of PHE was observed during the experiment.

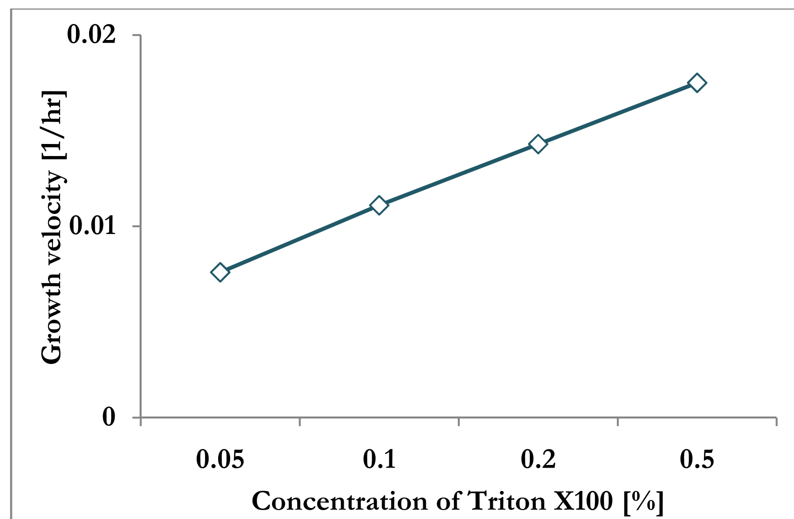


Figure 5.88 *Growth rate of microorganisms at different concentrations of PHE and Triton X100.*

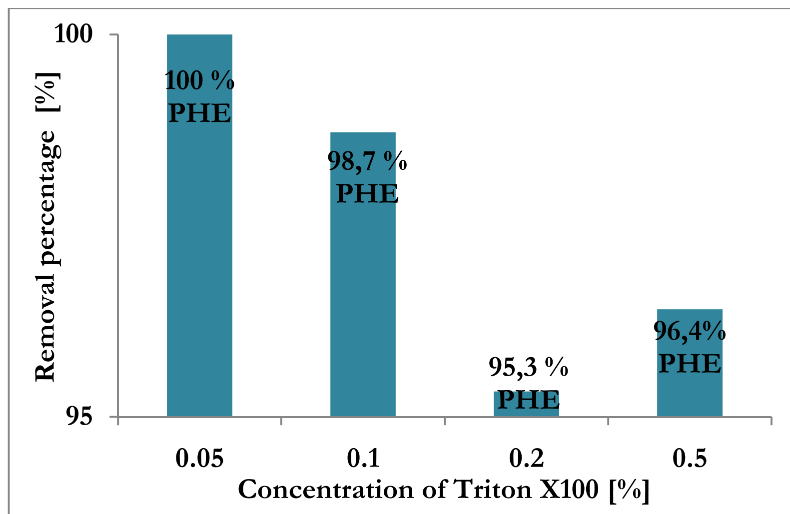


Figure 5.89 *Percentage removal of PHE at the end of experiments.*

Analysis of the data reported in tables and figures describe that:

- the acclimation exhibits a significant effect on the microbial growth and on the substrate consumption:
- the culture acclimatised to GLU showed length of the lag phase, more longer than that of the cultures acclimatised to TSB.
- the bacterial mixed cultures acclimatised on TSB were more efficient in the degradation of PHE than the bacterial cultures acclimatised on GLU for both systems.

Part II

The second part deals with the degradation of *Metal Working Fluids*, MWFs in real aqueous wastes employing biological and chemical treatments

5.5 Degradation of MWFs wastewater employed a biological treatment

5.5.1 Experimental set-up

The biodegradation tests carried out in 150 mL bottle flasks and fermenter 2000 mL where added the stock solutions of MWFs recalcitrant 795B and original fluid that were provided by Microbial Solution, Oxford, UK.

The mixed bacterial pure cultures were *Agrobacterium radiobacter*, *abbrev. form :AG RAO*, *Comamonas testosterone, abbrev. form : COM TEST, Methylobacterium mesophilicum, abbrev. form : METH MES, Microbacterium esteraromaticum, abbrev. form : M EST, Microbacterium saperdae*, *abbrev. form: MIC* and their were cultivated as description in chapter 4.

The pre-cultivated culture was centrifuged at a speed of 4000 rpm for 20 minutes to spin down most of the cell suspended in the media. The cell was then washed (washing was repeated for 3 times) using a phosphate buffer solution for all of the experiments and re-centrifuged. The cell was further re-suspended in the 20 mL and /or 500 mL added to the bottles and /or fermenter containing recalcitrant 795B 75% v/v and original fluid 12.5% v/v MWFs wastewater. The optical density of bacteria consortium in the MSM was 0.3 using a spectrophotometer Schimadzu at 600nm. To achieve faster degradation, was added air bubble in the fermenter experiment using a air pump.

The experiments were conducted in the same conditions, temperature 25ºC, 180 rpm the difference was only the air bubble (with a flow rate air 0.80 L/min) introduced into fermenter.

Sterilized cells were used for the sterile control for the purpose of abiotic activity only for bottle flask. The 250 mL bottles flask were then kept on a shaker for thorough mixing at 180 rpm at 25°C .Samples were drawn at regular intervals for COD and TOC analyses.

The experiment was conducted to stimulate the MWFs wastewater degradation by bacteria *consortium* and the initial and final COD respective TOC level are specified in the following tables, table 5.21 and table 5.22.

Fermenter with original fluid 12.5% [v/v]		
	COD [mg/L]	TOC [mg/L]
t_0 [hr]	38200	11747
t_f [hr]	17751	5917

Tabel 5.21 *COD and TOC values of original fluid 12.5% MWFs in fermenter experiments at zero time and end time of experiment.*

Fermenter with recalcitrant 795 B 75% [v/v]		
	COD [mg/L]	TOC [mg/L]
t_0 [hr]	8234	2812
t_f [hr]	4490	1494

Tabel 5.22 *COD and TOC values of recalcitrant 795B 75% MWFs used in fermenter experiments at zero time and end time of experiment.*

5.5.2 Summary of results

The figures 5.90 and 5.91 refer to removal of COD and TOC level of original fluid and recalcitrant 595B MWFs wastewater in presence of bacterial mixed cultures during the experiment into bench top fermenter.

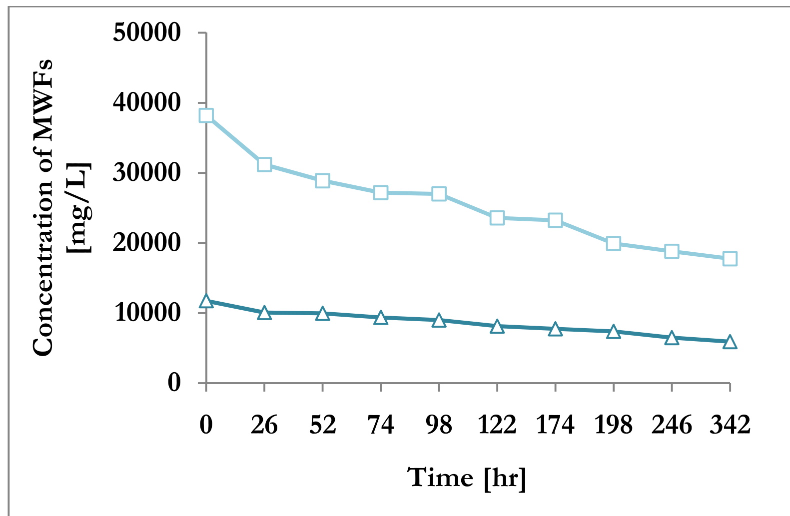


Figure 5.90 *Variation of chemical oxygen demand and total organic carbon in original MWFs during the experiments:* □*COD [mg/L];*△TOC [mg/L].

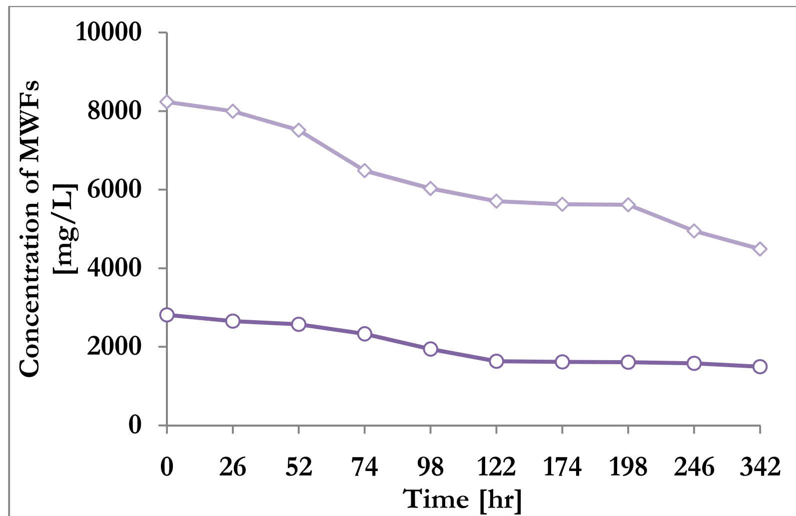


Figure 5.91 *Variation of chemical oxygen demand and total organic carbon in recalcitrant 595B MWFs during the experiments:* ◇*COD [mg/L];*O*TOC [mg/L].*

The curves in the figures 5.92 and 5.93 report the removal of COD and TOC level for both types of MWFs wastewater in presence of bacterial mixed cultures during the experiment into bottle flask and an abiotic control.

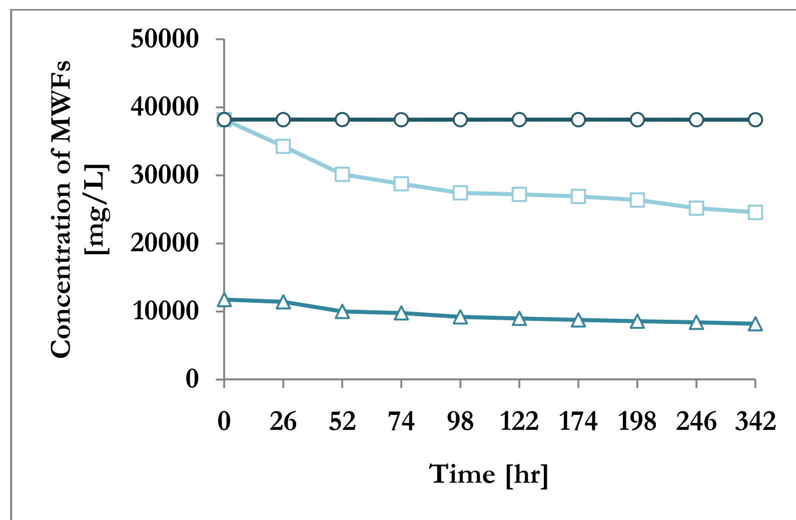


Figure 5.92 *Variation of chemical oxygen demand and total organic carbon in original MWFs during the experiments:* □*COD [mg/L];*△TOC [mg/L];O *Abiotic control.*

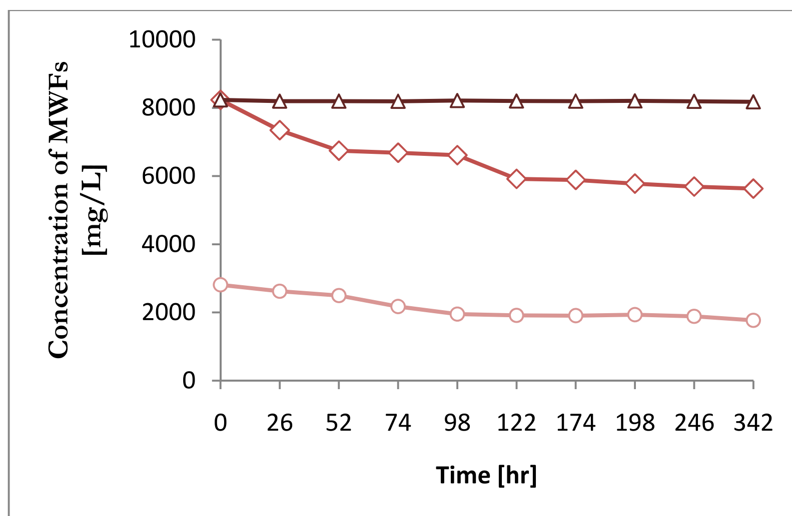


Figure 5.93 *Variation of chemical oxygen demand and total organic carbon in recalcitrant 595B MWFs during the experiments: ◇COD [mg/L];○TOC [mg/L];△Abiotic control.*

The capacity of bacterial mixed cultures to reduce the COD and TOC level was not higher as the figures shown for this reason was necessary to apply a chemical treatment.

5.6 Degradation of MWFs wastewater employed a chemical treatment

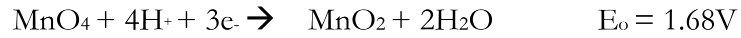
5.6.1 Experimental set-up

The chemical degradation tests are carried out in 100 mL bottle flasks where are added the stock solutions of MWFs recalcitrant 795B and original fluid.

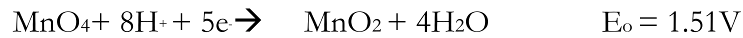
The oxidising agents used were potassium permanganate $KMnO_4$ and hydrogen peroxide H_2O_2 at different concentration and prepared separately.

$KMnO_4$ highly reactive under conditions found in the water industry. It will oxidize a wide variety of inorganic and organic substances. Potassium permanganate (Mn^{7+}) is reduced to manganese dioxide, MnO_2 (Mn^{4+}) which precipitates out of solution . It is a stronger oxidant and the chemical reaction can be describe as follow:

- under acidic conditions:



$$MnO_4 + 4H^+ + 3e^- \rightarrow MnO_2 + 2H_2O \qquad E_o = 1.68V$$



$$MnO_4 + 8H^+ + 5e^- \rightarrow MnO_2 + 4H_2O \qquad E_o = 1.51V$$

- under alkaline conditions:

$$MnO_4 + 2H_2O + 3e^- \rightarrow MnO_2 + 4OH^- \qquad E_o = 0.60V$$

Reaction rates for the oxidation of MWFs are relatively fast and depend on temperature, pH, and dosage.

It is not desirable to maintain a residual of $KMnO_4$ of its tendency to give w a pink colour at waterwastes.

$KMnO_4$ dry crystalline was added to arrived at final concentration about 20 mM for recalcitrant 795B and 50mM for original MWFs and then stirred for 3 hours. The samples was taken every 0.5 hour, filtered in order to remove the precipitate MnO_2 formed. At the liquid phase was added H_2O_2 100mM in order to remove the pink colour given by residual of $KMnO_4$ and then the samples were analyzed by COD and TOC analytical methods. The figures 5.94 and 5.95 reports the image of experiments.

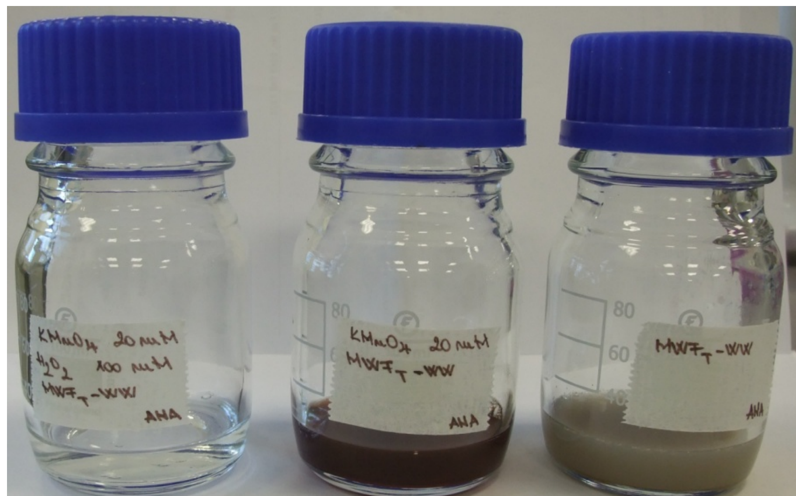
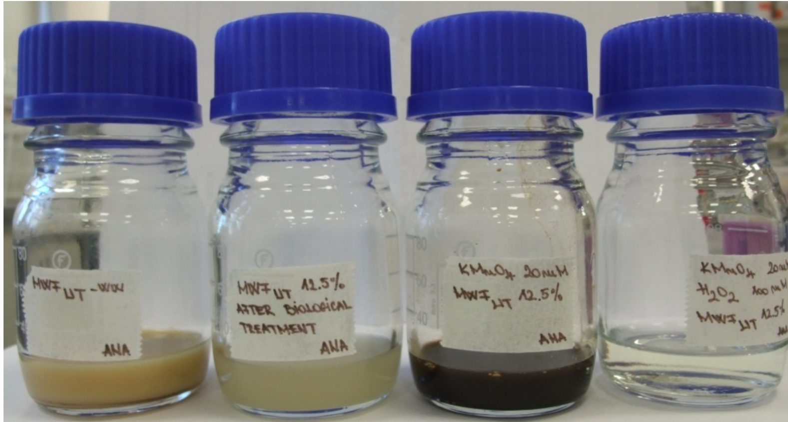


Figure 5.94 *Chemical treatment image for recalcitrant 795B MWFs.*



Figures 5.95 *Chemical treatment image for original fluid MWFs.*

The figure 5.96 report the image of filter paper after filtration with MnO_2 precipitate formed during the chemical treatment. It was analysed and was found lower COD in [mg/L].

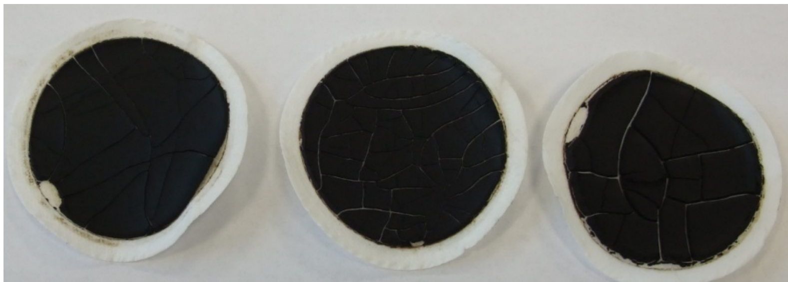


Figure 5.96 *Image of MnO_2 precipitate formed during the chemical treatment.*

The removal in terms of COD and TOC are reported in the following figures for both types of MWFs, figure 5.97 and 5.98.

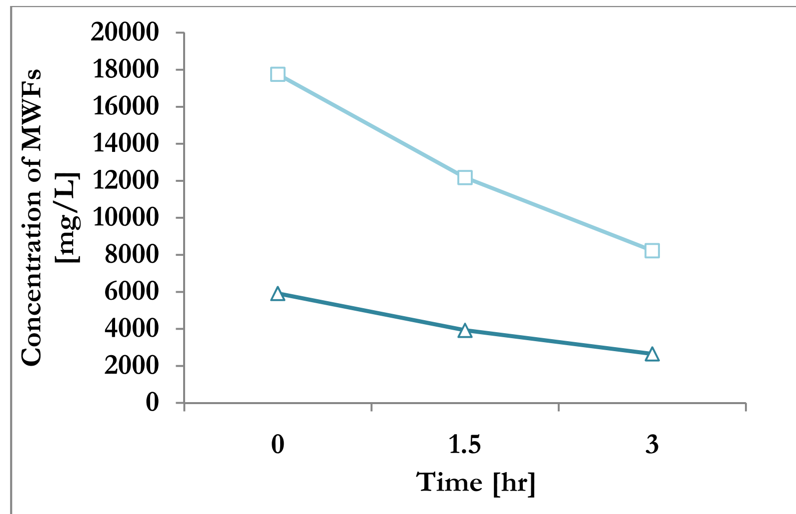


Figure 5.97 *Variation of chemical oxygen demand and total organic carbon in original MWFs during the experiments:* ◻*COD [mg/L];*△TOC [mg/L].

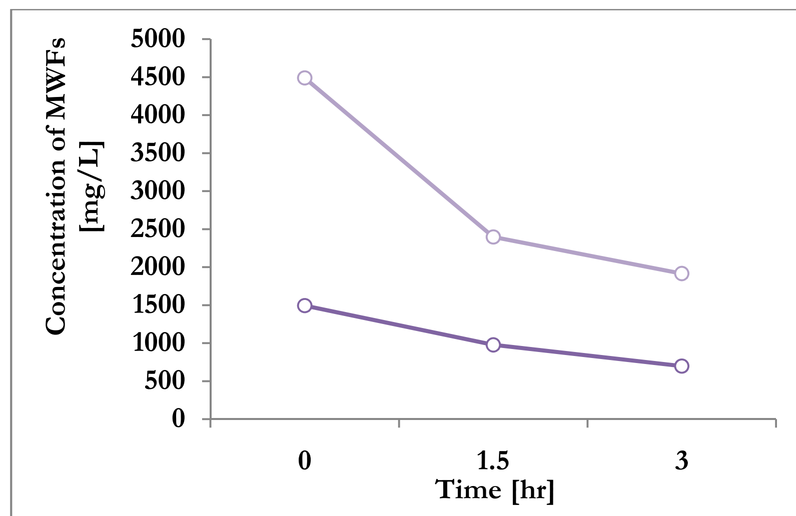


Figure 5.98 *Variation of chemical oxygen demand and total organic carbon in recalcitrant 595B MWFs during the chemical treatment:* ◇*COD [mg/L];* ○*TOC [mg/L].*

The capacity of the oxidising agents to reduce the COD and TOC level was higher as the figures shown but it produce a supplement solid waste, MnO_2 which have to be remediated because the total volume of solid waste depends of the initial volume of MWFs and concentration of $KMnO_4$, and represent increasing in the final economical costs of treatment.

The order of treatments was chosen in this way because the $KMnO_4$ and H_2O_2 dosage produced the inactivation of bacterial mixed pure cultures used. Usually they are used as disinfectants in wastewater treatment.

Chapter 6

Conclusions

This chapter describe the final conclusions.

The aims of this work were to stimulate the degradation of refractory organic compounds in two different situations.

Two aspects of degradation process are investigated: Part I deals with the degradation of phenanthrene, PHE, in synthetic wastewater employing a biological treatment; Part II deals with the degradation of metal working fluids wastewater, MWFs, employing a combination of biological and chemical treatment.

In particular, in the Part I we wanted to clarify the following aspects:

- The response of bacterial mixed cultures Bulab 5733 & Bulab 5738 to the addition of the mobility agents used and their biocompatibility and bioavailability against the bacteria;
- The effectiveness of Bulab 5733 & Bulab 5738 acclimatised on different organic substrate in the degradation of refractory organic compounds;
- The influence of different parameters such: initial concentration of PHE and the type of mobility agent on the efficiency of the degradation process.

Three case studies are analyzed:

- the first case is referred to solubilisation of PHE using different mobility agents: organic solvents, Methanol, Acetone and n-Hexane and surfactants Tween 80 and Triton X100;
- the second case is referred to biocompatibility and bioavailability of mobility agents against the bacterial mixed cultures Bulab 5733 & Bulab 5738;
- the third case is referred to biodegradation of PHE and/or surfactants.

In every case study analysed the main conclusions we have reached are as follows:

- The addition of mobility agents increases the solubility of solid PHE in aqueous solution at higher concentrations in case of organic

solvents and at slightly concentrations of surfactants but more higher than the value of them critical micellar concentration, CMC;

- The solubility of solid PHE in addition in MSM and distilled water increase linearly with the concentration of organic solvents and began immediately;

- The solubility of solid PHE in addition in MSM and distilled water increase approximately linearly with the concentration of surfactants above the CMC value of them and began after 48 hours;

- The solubility of PHE in aqueous solution decreases with increasing concentration of salts in the MSM: the concentration of Tween 80 0.5% the percentage of PHE solubilised goes from 46% in distilled water to 25,7% in MSM and for Triton X100 0.5% the percentage of PHE solubilised goes from 33% in distilled water to 27% in MSM. This phenomenon is also found in the literature and is known as "salting out";

- The mobility agents are not all biocompatible only Methanol, Triton X100 present this property: in fact the growth is not inhibited in the their presence but the lag phase is more longer for Methanol in confront with Triton X100 where it does not exist. n-Hexane are not biocompatible, it exerts a toxic action against microorganisms;

- Tween 80 and Acetone were biocompatible but observed a slightly growth. However, it was found a decrease in growth rate with increasing concentration of Tween 80 and Acetone, which may suggest that both exert, at higher concentrations, a toxic action against microorganisms;

- Methanol and Triton X100 are bioavailable for the microorganisms instead Tween 80, Acetone and n-Hexane are not bioavailable;

- The efficiency of PHE degradation or removal PHE efficiency is influenced by the amount of surfactants used and it was greater for surfactants concentration above the CMC and lower for 0.5%;

- The choice of organic substrate for acclimation of microorganisms has a significant effect on the microbial growth and on the substrate consumption. The results obtained using bacterial cultures acclimated to TSB showed that the removal of PHE were slightly higher than those obtained with the acclimation at GLU.

In case of organic solvents the degradation experiments of PHE were not performed because their higher vapour pressure can lead to a transfer of PHE from the liquid phase to the gas phase and then in the atmosphere.

Regarding the use of Tween 80 as a surfactant can be seen that the PHE disappeared from the liquid phase but really it was found accumulated on the biomass sludge. Differently, the use of Triton X100 as a surfactant did not lead to the same accumulation.

In conclusions, the comparison between the two surfactants seems to be strongly in favor of Triton X100, since it constitutes a source of carbon and energy for microorganisms and it is decomposed.

In the second part, part II, is presented the experimental investigation carried out during research activity developed at Oxford University, Department of Engineering Science, Oxford, UK.

Two types of metalworking fluids wastewater provided by Microbial Solution, Oxford, UK were tested in laboratory employing a biological and chemical treatment in order to stimulate their degradation and reduce the highest COD level which characterized those wastewater and optimise the methods in term of efficiency and economical costs.

The combination of both treatment were respectively tested and their effectiveness on COD removal was confirmed.

Unfortunately, the chemical treatment produced a sludge precipitate MnO_4 and this is a disadvantage because some of hydrophobic compounds can be absorbed into it.

These results represent a disadvantage because the sludge need further treatments for remediation and in this case the economical costs can increase.

Further work is needed for remediation of MWFs wastewater and different methods should also be used in order to avoid the formation of sludge.

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List of abbreviations and symbols

ROCs	Refractory organic compounds
WWT	Wastewater treatment plant
DWW	Domestic wastewater
IWW	Industrial wastewater
AOPs	Advanced oxidation process
PAHs	Polycyclic aromatic hydrocarbons
PHE	Phenathrene
MWFs	Metal working fluids
L	Liter
g	Gram
mg	Milligrams
mL	Milliliters
mg/L	Milligrams per liter
ppm	Parts per million
rpm	rotation per minute
ºC	degree Celsius
OD	Optical density
TOC	Total organic carbon
COD	Chemical oxygen demand
HPLC	High performance liquid chromatography
MSM	Minimal salts medium
$KMnO_4$	Potassium permanganate
H_2O_2	Hydrogen peroxide.

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Chapter 2

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