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INVESTIGATION INTO THE MECHANISM(S) WHICH PERMIT THE HIGH-RATE, DEGRADATION OF PAHS AND RELATED PETROLEUM HYDROCARBONS IN SEQUENCING BATCH REACTORS BY ATTACHED CELLS IN A CONTROLLED MIXED BACTERIAL COMMUNITY.

by

Emad Hussein

Under the direction of George E. Pierce

Abstract:

A stable mixed culture, deposited as ATCC 55644, previously shown to degrade petroleum hydrocarbons at relatively high concentrations was used as the source of inoculum. This culture was grown in Stanier's minimal media, either in the presence of different concentrations of naphthalene, nitrobenzene and toluene (NNT) or naphthalene and toluene (NT) as the sole source of C and/or N. Results showed that the majority of the strains isolated from the mixed culture were able to grow in the presence of NNT or NT. A total of 20 different isolates were isolated from the mixed culture. Individual isolates were grown in Stanier's minimal medium containing a single hydrocarbon as the source of carbon or carbon and nitrogen. Only one strain was found to grow solely in the presence

of nitrobenzene as the source of C and N. Most of the other isolates were able to grow in the presence of naphthalene, toluene, acenaphthene, anthracene, fluoranthene and phenanthrene, n-dodecane, hexadecane, n-pentadecane, n-tetradecane, and n-octadecane. Planktonic and immobilized cells of the controlled mixed culture (ATCC 55644) were grown in separate Sequential Batch Reactors (SBR) using Stanier's media, to which naphthalene, nitrobenzene and toluene were added as the sole source of C and/or N. Biodegradation was determined by measuring the residual hydrocarbon in the SBR and the amount of trapped volatile organic carbon (VOC) and the evolved CO_2. Gas chromatography data showed that immobilized cells were able to degrade NNT faster than the planktonic cells. This observation was confirmed by CO_2 evolution. Over time the loading of hydrocarbon was significantly increased from a starting level of 400 ppm (Naphthalene), 100 ppm (Nitrobenzene), and 500 ppm (toluene), to a final level of 3000 ppm (Naphthalene), 400 ppm (Nitrobenzene), and 1600 ppm (toluene). While increasing nutrient loading, the frequency of re-feeding with hydrocarbons was changed from an initial re-feeding every 60 hrs to a final re-feeding frequency of 18 hrs.

The experiments clearly showed that the attached, mixed microbial community was able to effectively and rapidly degrade high concentrations of hydrocarbons. This demonstrated the practical advantages of employing attached, mixed microbial cultures in a SBR.

INDEX WORDS: Jordan Oil Refinery, Middle East, Jordan, CO_2 trap, synthetic waste, titration, Dehydrogenase enzyme, Naphthalene oxygenase, Evolved CO_2, Volatile organic carbons, Triphenyl-Tetrazolium chloride, Biomass, Granular activated carbon (GAC), Bioreactors, Fermentor, DAP 2, VOC trap

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by

Emad Hussein

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2006

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Emad Hussein
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December 2006

DEDICATION

I dedicate this work to my loving wife Sawsan, my wonderful son Suleiman and to my precious daughters Marah, Farah and Sarah. Without their patience, understanding, support, and most of all love, the completion of this work would not have been possible.

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List Of Abbreviations

ATCC	American Type culture collection
PPM	Part Per Million
NNT	Naphthalene, Nitrobenzene And Toluene
NT	Naphthalene And Toluene
TSA	Tryptic (Trypticase) Soy Agar
TSB	Tryptic (Trypticase) Soy Broth
PAHs	Polycyclic Aromatic Hydrocarbons
CO_2	Carbon Dioxide
GAC	Granular Activated Carbon
VOC	Volatile Organic Carbon
SBR	Sequential Batch Reactor
PAC	Powdered Activated Carbon
PAF	*Pseudomonas* Agar F
PAP	*Pseudomonas* Agar P
EMB	Eosin Methylene Blue
TTC	2,3,5-Triphenyltetrazolium Chloride
TPF	Triphenylformazan
CFU	Colony Forming Unit
NG	No Growth
SEM	Scanning Electron Microscopy

Introduction

Bioremediation is a process in which microbial action destroys environmental pollutants. The term bioremediation is often used to describe a variety of quite different microbial processes occurring in natural ecosystems, (such as mineralization or co-metabolism) (Alexander, 1980). Certain microorganisms can function with or without oxygen to degrade toxic organic compounds in the environment (Sims and Bass, 1984). These microorganisms derive energy and may increase in biomass from these processes (Lee and Ward, 1985). Bioremediation of hydrocarbon contaminated sites is a good example of the application of principles of bioremediation.

Hydrocarbons from both biogenic and abiogenic sources are widespread in the environment (Alexander, 1994). Significant amounts of hydrocarbons are formed by biological processes. The major industrial source of hydrocarbons is petroleum and its associated natural gases, formed geochemically from biomass under conditions of high pressure and temperature. Petroleum products (hydrocarbons) are a rich source of energy. Some naturally occurring bacteria are able to take advantage of that energy and use hydrocarbons as carbon or carbon and energy source, which results in the degradation of the complex molecules, potentially leaving only harmless carbon dioxide and water (Alexander, 1994).

The cleaning up of environments contaminated with hydrocarbon waste is of great interest, as hydrocarbons are considered the major environmental pollutant (Lazar *et al*., 1995). Lazar *et al*. (1995) showed that microbial treatment of hydrocarbon polluted sites

is an effective alternative to traditional methods in cleaning up polluted environments. At present, the bioremediation of polluted sites is considered an effective biotechnology with a range of advantages compared to traditional technologies, as bioremediation is based on the ability of microorganisms to degrade the hydrocarbons to non-toxic products such as H_2O, CO_2 and biomass (Leahy and Colwell, 1990; Hiebert *et al*., 1993; Dave *et al*., 1994).

While hydrocarbon-degrading microorganisms are ubiquitous, hydrocarbon-degraders normally constitute less than 1% of the total microbial community. When oil pollutants are present these hydrocarbon-degrading populations increase, typically to 10% of the community (Alexander, 1994). Rates of natural degradation typically have been found to be low and are limited by environmental factors, such as, the contaminant or nutrient (N and P) bioavailability, physical conditions (e.g. temperature, salinity, pH) or microbial competition (Atlas, 1991). The goal of bioremediation is to increase the rates of degradation. This process causes minimal ecological effects because it is natural to the problem of oil pollutants.

There are two basic approaches used for bioremediation of hydrocarbons. The first approach depends on aeration and/or fertilization to maximize the rates of hydrocarbon degradation, which is based on the metabolic capabilities of the indigenous microbial populations. The rationale for this approach is that indigenous microbial populations may not have all the necessary nutrients and/or aeration. The second approach is accomplished by the addition of exogenous microbial populations (seeding). The seed cultures are selected for their hydrocarbon-degradation activities (Prince, 1992). The rationale for this

approach is that indigenous microbial populations might not be capable of degrading the wide range of potential substrates present in the environment.

Bioremediation, accomplished by the application of fertilizer, stimulates the metabolism of the indigenous oil-degrading microorganisms. Prince (1992) showed that the stimulation caused by the application of fertilizers was reflected by an increase in the hydrocarbon biodegradation rate, and increased numbers of oil-degrading microorganisms in the fertilized environment. Fertilization was successfully applied for the treatment of the 1989 Alaskan oil spill in Prince William Sound, Alaska. The spill portions of the (200,000 barrels of crude oil from the oil tanker *Exxon Valdez)*, were treated by bioremediation to remove petroleum pollutants. The *Exxon Valdez* spill formed the basis for a major study on bioremediation through fertilizer application and was the largest application of this emerging technology (Atlas and Atlas, 1991).

"The use of microorganisms to treat waste or waste contaminated material is well documented. At the February, 1990 symposium which preceded the EPA-Industry Meeting on Environmental Applications of Biotechnology, the EPA noted that biotechnology has been successfully utilized to treat soils and sludges from superfund sites which include contaminants from multiple and varied sources. Economic and environmental considerations indicate that bioprocessing technologies offer a significant potential for the remediation and treatment of waste and waste contaminated materials." (Pierce and Smith, 1997).

Alexander (1994) examined the occurrence of bacterial communities in oily sludges from petroleum industry and their role in bioremediation of such polluted environments. The existence of pure populations of microorganisms is more or less a laboratory phenomenon. In nature most bacteria do not exist as pure cultures, and significant proportions of all microorganisms coexist together. It is well known that bacteria interact in the environment and each bacterial species has its own role in the ecosystem and mixed communities (Alexander, 1994). In nature, microorganisms may interact positively or negatively with each other in a variety of ways. In our study we will concentrate on the positive side of the interaction. There are many examples of microbial processes that need bacterial interactions. For example, anaerobic digestion of organic matter requires association of syntrophic H_2-producing acetogens and H_2-consuming methanogens (Conrad *et al*.,1985; MacLeod *et al*., 1990). Also, during degradation of many xenobiotic compounds, such as chlorinated herbicides (Lappin *et al*., 1985; Wolfaardt *et al*., 1994), nitrate esters (Ramos *et al*., 1996), naphthalene derivatives (Rozgaj and Glancer-oljan, 1992), and alkylbenzene sulfonates (Jiménez *et al*., 1991), the combined action of several species present in bacterial communities enhances or is required for complete mineralization of these compounds. The biodegradation of PAHs by mixed cultures has been the subject of numerous investigations, often based on the natural microflora of contaminated soils (Mueller *et al*., 1989; Shiaris, 1989). These cultures frequently have significant degradative capabilities because the single isolates can complement one another due to their physiological properties, with some members of the culture providing important degradative enzymes whereas others supply surfactants or growth factors.

Studies have shown that to enhance the rate of biodegradation of toxic compounds (including hydrocarbons) in the environment, different strategies can be considered, for example, chemotaxis and immobilization (Irvine *et al*., 1984; Irvine and Ketchum, 1988; Atkinson and Mavituna, 1991; Ozaki *et al*., 1991; Jerabkova *et al*., 1997; Witt *et al*., 1999; Marx and Aitken, 2000). Although chemotaxis is a phenomenon that has been known for some time, it is a complex process in which bacterial cells detect temporal changes in the concentrations of specific chemicals, respond behaviorally to these changes, and then adapt to the new concentration of the chemical stimuli. Some toxic organic compounds are chemoattractants for different bacterial species, which could lead to improved degradation (Witt *et al*., 1999; Marx and Aitken, 2000).

Since the main purpose of this study is to achieve and maintain a high rate of hydrocarbon biodegradation using a controlled mixed bacterial community, then it is necessary to address all the factors that enhance that rate (e.g. chemotaxis and immobilization). Marx and Aitken (2000) have shown that for biodegradation in natural environment, microorganisms that have degradation capability and also show chemotaxis towards a compound would be more efficient in bioremediation than non-chemotactic microorganisms. Because they will have more affinity toward that compound and this increases the chance for the bacteria to come in contact with the compound in order to degrade it.

Immobilization may be defined as the physical isolation of a given microorganism from the reaction medium. It may be achieved by physical attachment, chemical attachment, entrapment within a gel, or entrapment within a membrane.

Studies have shown that immobilization of hydrocarbon degrading organisms has many advantages, for example it increases significantly the biodegradation rate, because they were grouped together on the immobilization matrix, so they can withstand higher concentration of the contaminant and degrade it faster (Irvine *et al*., 1984; Irvine and Ketchum, 1988; Atkinson and Mavituna, 1991; Ozaki *et al*., 1991; Jerabkova *et al*., 1997). Immobilized biomass may be dried and stored at room temperature for re-use within several years (Atkinson and Mavituna, 1991), and it is more resistant to environmental changes and attack by other microorganisms (Irvine *et al*., 1984), proteolytic enzymes (Irvine and Ketchum, 1988), and toxic chemicals (Jerabkova *et al*., 1997). Also, when free cells in a bioprocess are subjected to high flow rates, the biomass has a tendency to wash out, whereas immobilized cells attached to a matrix do not wash out easily (Ozaki *et al*., 1991). In addition to their use in pharmaceutical and food biotransformation (Eikmeier and Rehm, 1987), immobilized cell processes have been used in the degradation of toxic compounds (i.e., Phenol) during wastewater treatment (Ehrhardt and Rehm, 1985; Ghozick and Irvine, 1991; Jerabkova *et al*., 1997).

Immobilized and entrapped bacterial processes have been established for many years (Atkinson and Mavituna, 1991). These processes are believed to provide additional advantages with respect to improving the microorganism's tolerance to their environment.

Improved stability to oxygen deprivation and pH shocking in an immobilized continuous culture reactor versus free swimming bacteria has been shown by Dickman *et al*. (1990). Also it has been found that immobilized cells of *Alcaligenes* species degrade 4-chlorophenol at faster rates than do free-swimming cells when fed 4-chlorophenol at low concentrations (Westmeier and Rehm, 1985). Crump *et al*. (1998) reported that the degree of bacterial diversity in estuarine environments is expected to be high due to a combination of the mixing of seawater and freshwater and the resuspension of sediments and particles from many sources. However, only a fraction of these bacteria may be active as consumers of organic matter. Work in the Columbia River estuary showed that the fraction of bacteria attached (immobilized) to particles accounted for approximately 90% of the heterotrophic bacterial activity in the water column and that these bacteria were 10 to 100 times more active than free-living bacteria (Crump and Baross, 1996).

Different matrixes have been used for immobilization of microorganisms, for example, granular activated carbon (GAC) or powdered activated carbon (PAC). Suidan *et al*. (1986) studied the degradation of phenol using a packed bed containing microorganisms attached to a GAC. Phenol degradation by *Candida* species and *Pseudomonas* species immobilized on activated carbon was investigated. Due to its great adsorptive surface, activated carbon is suited as a supporting material for microorganisms and also provides a high adsorption capacity for phenol. Immobilization by adsorption avoids any nonphysiological treatment of the microorganisms. One gram of activated C adsorbed in 10 h approximately 4×10^9 *pseudomonas* species cells and 3×10^8 *Candida* species cells. While the free cells did not tolerate >1.5 g phenol/L, the adsorbed microorganisms

survived temporarily high phenol concentration of <15 g/L and degraded approximately 90% of the adsorbed phenol. The activated C operated like a "sponge": the adsorbed phenol diffused out of the C and could be metabolized by the microorganisms (Ehrhardt and Rehm, 1985; Suidan *et al*., 1986).

Ghozick and Irvine (1991) studied integration of GAC into a sequencing batch reactor (SBR) for treatment of VOC contaminated waters using a process known as the granular activated carbon sequencing batch reactor (GAC-SBR). The SBR is a periodically-operated unsteady state suspended growth multistage treatment system with an operating cycle that consists of five discrete stages (i.e. Fill, React, Settle, Draw and IDLE) which take place in a time sequence (Irvine and Ketchum, 1988). Kold and Wilderer (1995) developed two types of activated carbon membrane biofilm reactors for degradation of volatile organic pollutants. They combined membrane aeration technology and activated carbon adsorption with biological treatment in order to biologically degrade VOCs in industrial wastewater. In further studies, Kold and Wilderer (1997) tested a GAC-SBR to treat industrial wastewater. They found that a combination of activated carbon and biodegradation technology produced additional advantages.

In hydrocarbon treatment, reactors with immobilized microbial cells may offer several advantages over processes with suspended biomass. These advantages include: (a) retention of a high concentration of microorganisms in the reactor, (b) protection of cells from toxic substances, (c) prevention of suspended particles from being washed out, (d) immobilized bacterial bioreactors are ideal for small manufacturers and commercial

laboratories, which generally have neither the space nor the existing conventional free cell-treatment plants (Ozaki *et al*., 1991). As a result, the immobilized cell reactors are favored over free cells because they provide economical and high efficiency treatment with somewhat compact reactors.

In this study, we have used a stable mixed culture, previously deposited as ATCC 55644 (DAP 2), which is known to degrade petroleum hydrocarbons at relatively high concentrations. The microorganisms which comprise the mixed culture have been successfully used to treat aromatics/PAHs wastes associated with the manufacture and synthesis of dyes from light petroleum oils (Pierce and Smith, 1997). In this project, we have used this culture in Sequential Batch Reactor (SBR) for the treatment of petroleum waste. Also, we investigated the conditions that allow the different organisms in the mixed culture to maintain their high degradation rate and reach very high population levels.

Rationale

The majority of the published literature on biodegradation uses pure cultures for the degradation of single pure chemicals. Moreover, the majority of the work that deals with the degradation of the pure chemicals has been done using very low concentrations of those chemicals, which is not the case in the real-world situations at environmental contaminated sites.

In real situations bacteria don't generally exist as pure culture and in any hydrocarbon contaminated site in the environment, there will be more than one substrate needing to be

removed. This study will mimic realistic conditions using a mixed culture and mixed substrate.

Another reason to use both a mixed culture and complex or mixed substrate is that, most members of the mixed culture have a greater potential to degrade a variety of substrates. So, it has to be subjected to these substrates in order to trigger its full potential to degrade it. The mixed culture and mixed substrate will result in a different physiology and biochemistry than in pure culture and pure substrate. In each of these degrading bacteria we have inducible systems that under the right conditions will be triggered to degrade the target substrate. These induced systems might be modulated by chemotaxis and quorum sensing. So, clearly there is a need to study these induced systems using mixed population and mixed substrate.

Petroleum hydrocarbons including polycyclic aromatic hydrocarbons (PAHs) are common environmental pollutants that are of concern because they are suspected carcinogens. Therefore, there is a need to develop methods that are reliable, efficient, and economical to biodegrade these complex compounds beginning at relatively high concentrations leaving very low residual levels. So, the hypothesis of this dissertation is that SBR and immobilized cells can be used to enhance petroleum hydrocarbon biodegradation.

Using a mixed culture that is known to degrade a wide range of hydrocarbons, we will address the following issues:

a- Optimization of immobilization bacterial cells and hydrocarbons using GAC.

b- Investigate the conditions that allow our mixed culture to grow to very high densities and maintain its degradation characteristics (e.g. chemotaxis, immobilization, etc).

Materials and methods

1- Culture and maintenance conditions (Growth media)

ATCC 55644 is a mixed bacterial culture, (originally designated as DAP 2), isolated from soil and sediments near Bridgewater, NJ, and the mixed culture (cited in U.S. Patents 5,688,685) has numerous applications in the aerobic degradation of aromatic and aliphatic compounds in waste materials and was deposited by Cytec Industries with the American Type culture collection (ATCC).

The mixed culture present in ATCC 55644 was revived and subcultured using Tryptic (Trypticase) Soy Agar (TSA) and Tryptic (Trypticase) Soy broth (TSB) media as recommended by the ATCC. Plates and tubes were incubated at 30º C for 24h, then the mixed culture was routinely grown in Stanier's minimal media supplemented with Naphthalene, Toluene or/and Nitrobenzene as the sole source of C or C/N and incubated in a 3L reaction vessel, which provided source of inocula for further experiments. Experimental apparatus used for inoculum maintenance is shown in Figure 1.

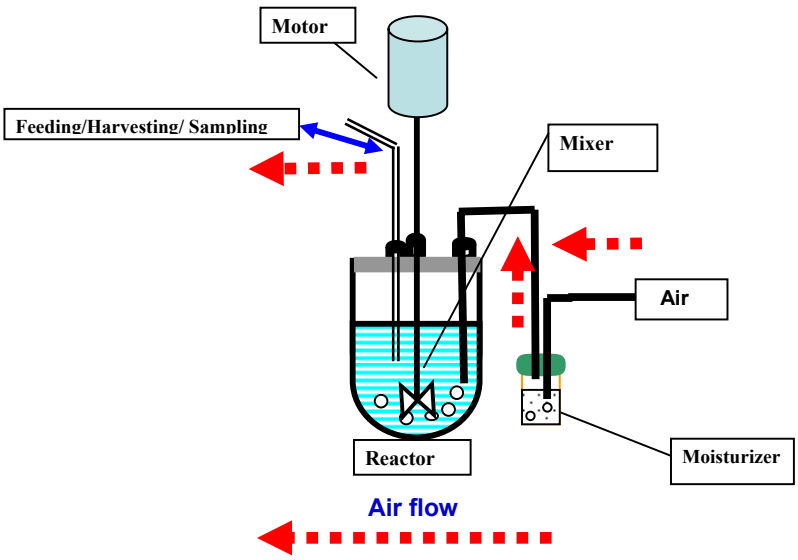

Figure 1. Experimental apparatus using 3 liter vessel (inoculum maintenance)
Reaction vessel (LG8079A) 3000 ml, cylindrical, indented with O-ring flange; reaction vessel lid (LG8073), four Necks 24/40, with O-ring flange; clamp (LG7316), Heavy-duty laboratory stirrer, PTFE stirring shaft, PTFE turbine agitator, holders, joints, and bearings (ACE Glass).

2- Chemicals

A range of different hydrocarbon compounds was chosen to test for the degradation capacity of the mixed culture. Hydrocarbons used (was based on real waste composition) and their respective sources are shown in table 1.

3- Isolation and purification of different bacterial isolates from the mixed culture

A total of 20 different isolates were isolated from the mixed culture DAP 2. The purified isolates were identified with the aid of Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). Isolated bacterial strains were grown on four different media, *Pseudomonas* agar F (PAF), *Pseudomonas* agar P (PAP), eosin methylene blue (EMB) and TSA and incubated at different incubation temperatures (4º C, 25º C, 30º C and 42º C). These cultures were characterized depending on their morphology, Gram staining, spore staining, motility, oxidase, catalase, oxidation/fermentation, gas production, ammonia formation, nitrate and nitrite reduction, indole production test, methyl-red and Voges-Proskauer test, citrate and mannitol utilization test, hydrolysis of gelatin, starch, urea and lipid.

Table 1. Chemical list

Chemical	Source	Purity
Naphthalene	Sigma-Aldrich	99%
Nitrobenzene	Aldrich	99%
Acenaphthene	Eastman	97%
Fluoranthene	Eastman	98%
Octadecane	Eastman	97%
Anthracene	MCB	96%
N-Dodecane	Fisher	98%
Toluene	Burdick and Jackson	99.9%
2-Methylnaphthalene	Riedel-de haen	98.6%
Ethylbenzene	Fluka	99.5%
N-Hexadecane	Sigma	97%
N-Pentadecane	Sigma	99%
Phenanthrene	Sigma	96%
N-Tetradecane	Sigma	98%
Sodium Benzoate	Sigma	99%
P-Xylene	Sigma	99%

4- Preparation of Hydrocarbon media

4.1- Broth and agar media

Stanier's minimal medium stock solutions were prepared separately and then supplemented with varying concentrations of naphthalene, nitrobenzene and toluene (Stanier *et al*., 1966).

The hydrocarbon containing Stanier's media were prepared both with and without ammonium sulfate. In addition, solid media were made using 1% and 2% agar to check for twitching microorganisms. After autoclaving, the different concentrations of the NNT mixed solution were added aseptically. For media without ammonium sulfate, nitrobenzene was considered the sole source of nitrogen present. Also, different concentrations of the NT mixed solution were added under aseptic conditions to the Stanier's media, containing ammonium sulfate after being autoclaved .
These different media, which contain 1 or 2% agar, were poured aseptically and cooled at room temperature; the broth media were cooled before inoculation.

4.2- Biphasic media

This media consists of two parts, solid (agar) and liquid (broth). The solid part of the biphasic media was prepared as TN media (Appendix I). 190 ml of TN agar media were prepared in a 500 ml flask and autoclaved separately. Different concentrations of hydrocarbons (NNT/NT) were added to the cooled TN media. The liquid part of the biphasic media (60 ml) was either Stanier's or TSB. The same hydrocarbon (NNT/NT) concentrations used in the preparation of the TN agar media were supplied aseptically and

was added to the cooled broth. The corresponding hydrocarbon containing broth media was poured into the corresponding (NNT/NT) solidified TN media.

5- Growth of bacterial isolates on solid media containing hydrocarbons as the only carbon and/or nitrogen source

Colonies were picked from the stored TSA plates, using a sterile toothpick, and were inoculated (stab-technique) into each of the different media. Inoculated plates were wrapped with parafilm (there were no sign of interaction between the chemicals and parafilm) to prevent evaporation of the NNT and NT and incubated at 30 ºC for several days.

6- Growth of bacterial isolates in broth media containing hydrocarbons as the only carbon and/or nitrogen source

Broth media containing the different concentrations of hydrocarbons (NNT or NT) were inoculated using the original mixed culture. All of the inoculated flasks (two sets of each) were incubated at both room temperature and at 30 ºC in a gyratory shaker (160 rpm) for 4 weeks. The cultures were maintained at their respective temperatures until turbidity was noted. Sub-samples were withdrawn weekly and used to inoculate specific hydrocarbon media plates in order to check for the growth of the hydrocarbon degraders. At the end of 4 weeks, the broth was centrifuged at 10,000 rpm/4 ºC. The cells were suspended in a minimal volume of broth to achieve a 20x cell concentrate and were stored at –20 ºC to be used in the inoculation of the three liter fermentor vessel.

7- Growth of bacterial isolates in a biphasic media containing hydrocarbons as the only carbon and/or nitrogen source

The prepared biphasic media containing different concentrations of hydrocarbons (NNT or NT) were inoculated using the hydrocarbon degrading cells previously stored at -20 ºC. All the inoculated flasks (two sets of each) were incubated either at room temperature or at 30 ºC for several days, with shaking (160 rpm). The cultures were kept at that temperature until turbidity was formed. Weekly samples from these flasks were withdrawn and used to inoculate hydrocarbon media plates in order to check for the growth of the hydrocarbon degraders.

8- Setup of the 3L fermentation vessel and growth of the mixed Bacterial culture

8.1 Stanier's broth media with sodium benzoate:

Two liters of Stanier's broth supplemented with sterile sodium benzoate (500 ppm) was added to the 3L fermentation vessel, Naphthalene (150 ppm) was added aseptically to the cooled medium. Next the inoculum (500 ml) was added aseptically. An air pump was used to pass moist, sterile air continuously through the vessel.

8.2 Growth of the mixed culture bacteria in the 3 L fermentor

Cells previously stored at -20 ºC were used as the inoculum, which was added through the feeding neck of the fermentor under sterile conditions. Upon the increase in turbidity after 24-72 h, both the air and the mixing were stopped for 10-15 min, to allow the cells to settle. Then 1200 ml of the clear medium was removed and stored in sterile flask to be centrifuged in order to retrieve any remaining cells. The retrieved cells were added back to the fermentor. Fresh, sterile 1200 ml Stanier's media containing sodium benzoate and naphthalene was added to keep the cells under continuous feed. Agitation and aeration

were resumed. Samples (2 ml) were collected every 48 h. A 0.1 ml sample was spread on Stanier's agar media supplemented with 300 ppm NNT/NT and 500 ppm NT and 1000 ppm NNT. Once dry, the plates were placed in the incubator at 30°C for 5-7 days.

9- Viable Bacterial Count

The samples were serially diluted to 10^{-6} using Stanier's minimal medium as the diluent. Using a fresh pipette tip and starting with the highest dilution, 0.1 ml of dilutions were plated onto Stanier's media with sodium benzoate or naphthalene or NNT or NT. The bacterial dilution was spread over the whole surface of the plate using a spreader. Once dry, the plates were placed in the incubator at 30 °C for 3-5 days. Evaluation and plate counts were conducted for all plates.

10- Isolation and identification of individual hydrocarbon degraders

10.1 Media preparation and preliminary isolation

Stanier's minimal medium containing 500 ppm of Naphthalene or Nitrobenzene or Toluene or NT or NNT or sodium benzoate or NT and sodium benzoate or NNT and sodium benzoate were prepared with or without ammonium sulfate (ammonium sulfate was only added to media that did not contain nitrobenzene). Both ammonium sulfate and sodium benzoate were dissolved in the Stanier's media prior to autoclaving. Hydrocarbons were added after autoclaving and after cooling.

A 3 ml sample was collected from the fermentor. Serial dilution was done using Stanier's broth media. Fractions (0.1 ml) with the highest dilution were plated (in duplicates) on the freshly prepared hydrocarbon media and let stand until they were dry. Plates were incubated at 30 °C for seven to ten days after being individually wrapped with parafilm.

After 10 days all different colonies (see results section) grown in each medium were picked and transferred into a fresh medium containing the same hydrocarbon that previously supported growth, all plates were incubated at 30 °C for 7-10 days. All growing cells were enriched using TN and Stanier's media.

10.2 Enrichment of the isolates using TN and Stanier's media

10.2.1 Enrichment using TN media

TN media containing selected hydrocarbons were prepared as mentioned in the appendix. Cells obtained in the previous section were inoculated onto TN plates supplemented with hydrocarbons, and these plates were then incubated at 35 °C for 24-48 h. Isolated colonies were then transferred to vials containing TN media supplemented with individual hydrocarbon, and then vials were incubated at 35 °C for 24-48h.

10.2.2 Enrichment using Stanier's minimal medium

Stanier's minimal medium was prepared and aliquoted into test tubes (8 ml/tube), and then autoclaved. After cooling, individual hydrocarbon was added to each set of tubes. Cells growing in TN media with hydrocarbons were transferred to the corresponding Stanier's broth media with the same hydrocarbon and incubated at 30°C for 10-20 days until tubes become turbid. The content of each tube was centrifuged at 4000g/4°C and washed twice with phosphate buffer saline (PBS) to wash excess hydrocarbons. The washed cells were used as an inoculum in the next step.

10.3 Preparation of hydrocarbon-Stanier's media

Stanier's medium was prepared containing one of the following hydrocarbons (500 ppm): acenaphthene, anthracene, fluoranthene, naphthalene, n-dodecane, n-hexadecane, n-pentadecane, n-tetradecane, octadecane, phenanthrene, sodium benzoate, toluene, and

xylene. Another set of Stanier's medium was prepared containing a mixture of the following hydrocarbons (500 ppm each): I-acenaphthene, anthracene, fluoranthene, and phenanthrene II- naphthalene, sodium benzoate, toluene, and xylene III- n-dodecane, n-hexadecane, n-pentadecane, n-tetradecane, octadecane.

The washed cells were inoculated (stabbing) in every plate containing either the individual hydrocarbon or the mixture, in order to see if the bacterial isolates were able to grow on which one of the different hydrocarbons.

11- Inoculum preparation, planktonic and immobilized cells.

The mixed culture grown in two 3L vessel was maintained throughout the experiment in minimal media supplemented with hydrocarbons. Granular activated carbon, 300g (GAC, water treatment grade F-300), was added to one 3L vessel. GAC was used to adsorb hydrocarbons and to serve as a substratum for the entrainment and attachment of microorganisms. The bacterial cells in the second 3L vessel will remain planktonic. Both immobilized and planktonic cells were maintained under the same cultural conditions to be used as a source of inoculum in the biodegradation experiments comparing the biodegradation of hydrocarbon between immobilized vs. planktonic cells.

12- Biodegradation experiment

12.1 Experimental setup

Apparatus used for this research consisted of a 5L reactor (Fig.2), Ballston Unit that removes CO_2 from house air, a humidifier, VOC trap and two CO_2 traps. The reactor is equipped with ports for: DO, sampling, feeding/ harvesting, aeration and VOC/CO_2 venting port. It is tightly closed so VOC/CO_2 can not escape without being captured and

analyzed. Since carbon dioxide was removed from the incoming air by the Ballston Unit, captured CO_2 can be attributed entirely to biodegradation. A humidifier containing sterile distilled water was used to raise the water content of the incoming air to nearly 100% relative humidity at room temperature. This process was to prevent the content in the reactor from drying. Experimental apparatus was placed in a laminar flow-hood at room temperature to minimize the exposure to the volatile organics during sampling. In house air or compressed air/O_2 introduced at a suitable rate monitored by a mass flow meter to the reactor.

Biodegradation of hydrocarbon was determined by measuring the residual hydrocarbon in the fermentor and the VOC trapped using GC according to a standard protocol. Evolved CO_2 was analyzed by titration (Standard Methods for the Examination of Water and Wastewater, 1975). The same setup was run as a control but without the mixed culture in order to account for the level of volatilization and loss of hydrocarbons due to adsorption to the GAC (the adsorbed fraction that can't be recovered or extracted to be analyzed), reaction vessel, and tubes.

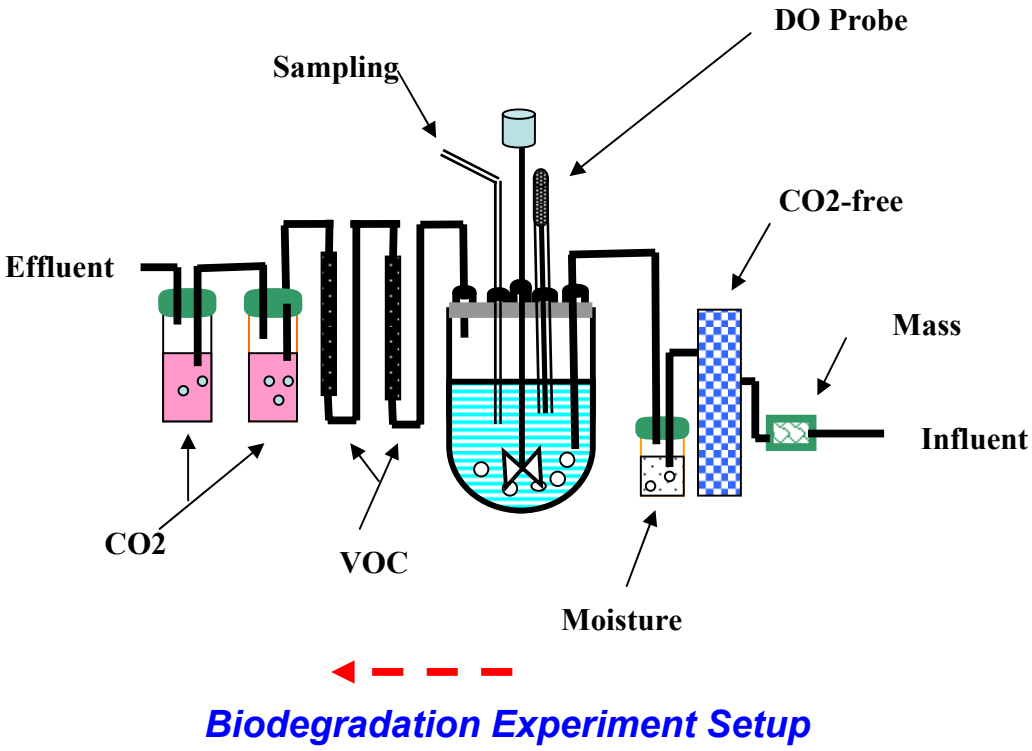

Figure 2. Experimental apparatus using 5 liter vessel

12.2 Degradation of different concentrations of NNT

Planktonic and immobilized cells (ATCC 55644) were grown in separate Sequential Batch Reactors (SBR) in the presence of Stanier's media. Naphthalene (400 ppm-3000 ppm), nitrobenzene (100-400 ppm) and toluene (500 ppm-1600 ppm) were added as the sole source of C and/or N. Samples were collected over a 18hr period from both vessels and plated on Stanier's media containing different concentrations of naphthalene, nitrobenzene and toluene (NNT) or naphthalene and toluene (NT). Biodegradation of hydrocarbon was determined by measuring the residual hydrocarbon in the SBR and the amount of trapped volatile organic carbon (VOC) and evolved CO_2.

12.3 Degradation of synthetic waste

The composition of this waste is shown in Table 2. Planktonic and immobilized cells (ATCC 55644) were grown in a separate 5L Sequential Batch Reactors (SBR) in Stanier's media. Structured synthetic waste were added as the sole source of C and/or N. Biodegradation of synthetic waste constituents was determined by measuring the residuals in the SBR and the amount of trapped volatile organic carbon (VOC) using GC and the evolved CO_2 by titration.

Table 2. Industrial and synthetic waste composition

Chemical	Actual Waste ppm	Structured Waste in SBR
Chloroform	200	100
Toluene	1360	600
Chlorobenzene	350	50
Ethyl benzene	450	60
O-Xylene	400	60
Aniline	100	100
Nitrobenzene	2800	100
Naphthalene	14000	3000-5000
Me-naphthalene	4400	100

A. Based on actual waste sludges and sediments associated with the waste impoundments at Bound Brook NJ former manufacturing site.

13- Process control and monitoring

13.1 Monitor Volatilization

Volatile organic compounds (VOCs) from the reactor were collected using VOC trap. These traps were prepared (15cm) using stainless steel tubing, washed using GC grade toluene and dried for 10 min using N_2. Granular Activated Carbon (GAC) was packed inside these stainless steel tubes then closed from both ends using glass-wool to prevent the escape of GAC but still allow the VOC to pass through. The VOCs were solvent extracted with methanol/methylene chloride) (10:9) and analyzed using GC (CYTEC standard protocol) to determine volatilization rate at each sampling interval (Appendix A).

13.2 Monitor CO_2 efflux

Evolved CO_2 was trapped in a solution of 4 N NaOH or KOH. CO_2 traps were replaced and analyzed by titration (Standard Methods for the Examination of Water and Wastewater, 1975) to determine the concentration of evolved CO_2 (Appendix B).

13.3 Enumeration of cells

Samples were collected from the fermentor for analysis purposes (residual hydrocarbon and biomass). Viable cells were counted using serial dilution technique (Stanier's media as the diluent). Dilutions (100ul) were spread on Stanier's hydrocarbon agar media. Once dry, the plates were placed in the incubator at 30°C for 3-5 days. The semi-automatic counter was used to count the colonies.

14- Hydrocarbon analysis

14.1 Residual hydrocarbon (liquid phase, sorbed to the glass wall, sorbed to GAC or VOC)

Samples for GC analysis were prepared by extraction according to CYTEC standard protocol. The ratio of sample to solvent was suitable. The extract was analyzed by gas chromatography (Hewlett Packard Model) using SPB-1 column (60m x 032mm; SUPELCO, Philadelphia, Pa., USA) equipped with a flame ionization detector. The initial temperature and temperature progress rate was selected based on the retention time of the spiked compounds. The injection port and detector temperatures were 225/300º C respectively. Hydrogen gas and air flow rates for the flame ionization detector were set (24 ml/min). Nitrogen gas was used as a carrier. A stock standard solution for each individual hydrocarbon present in the waste was prepared (Appendix G).

14.2 Enzyme assays

14.2.1 Dehydrogenase activity

Dehydrogenase activity, an indicator of microbial activity, was measured spectrophotometrically using characteristics of TTC (2, 3, 5-triphenyltetrazolium chloride) reduction to TPF (triphenylformazan). This method was direct, fast and required a small sample size (Appendix C). The color intensity was determined (at 485 nm) using a spectrophotometer with methanol as a blank. Measurement of dehydrogenase activity will indicate whether the organisms in the mixed culture are metabolically active or not. This activity may contribute to the biodegradation process.

14.2.2 Naphthalene oxygenase

Naphthalene oxygenase activity was assayed using a whole-cell, spectrophotometric assay (Shamsuzzaman and Barnsley, 1974). The disappearance of naphthalene was monitored as the decrease in absorbance at 276nm as a function of time (Appendix D).

14.3 Total protein analysis of the mixed community and individual members

Bacterial growth was measured by both the determination of colony-forming units and/or by the determination of total protein contents. Cells were harvested by centrifugation and broken by sonication, and then total protein contents could be determined using either the Bradford dye-binding assay (Bradford, 1976) or the Lowry assay (Lowry et al., 1951) using bovine serum albumin as the standard.

Results

1- Bacterial growth

A number of different colony types were observed. Twenty colonies were picked based upon morphological differences (Table 3).

2- Identification of the isolated bacterial strains

Table 4 shows the results of the taxonomic tests for all the isolates. All isolates were found to be Gram-negative, catalase positive, and oxidase negative bacilli except for isolates B and C, which were oxidase positive. All other biochemical characteristics are shown on table 4. All tests were performed according to the methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994).

3- Growth of initial bacterial isolates on media containing hydrocarbons as the only carbon and/or nitrogen source

3.1- Growth in the presence of NT hydrocarbon mixed solution

Tables 5 and 6 show the level of growth of each bacterial isolate under each NT concentration. The levels of growth are shown in numbers ranging from 1.0 -5.0, and all numbers shown are average of triplicate plates.

Table 3. Morphological characterization of cultures obtained from the mixed culture DAP 2.

Culture	Morphological Characteristics
A	Circular, white, large, creamy and concave.
B	Circular, brown red (pale orange), medium and concave.
C	Circular, light brown center, medium size and concave
D	Circular, small, pale white and concave
E	Circular, small, yellow, center and concave.
F	Circular, large, white circles and flat.
G	Circular, pinpoint, on top of cells, yellow center and flat.
H	Circular, brown center and concave.
I	Circular, brown red (pale orange), medium and concave.
J	Circular, brown center and concave.
K	Circular, large, white circles and flat.
L	Circular, white, large, creamy and concave.
M	Circular, brown red (pale orange), medium and concave.
N	Circular, small, yellow, center and concave.
O	Circular, large, white circles and flat.
P	Circular, white, large, creamy and concave.
Q	Circular, brown red (pale orange), medium and concave.
R	Circular, brown center and concave.
S	Circular, large, white circles and flat.
T	Circular, small, yellow, center and concave.

Table 4. Differential biochemical characteristics of all bacterial isolates and growth of individual isolates in the presence of single hydrocarbon compound

Test \ Isolate	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
Gram Stain	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose utilization	+	+	+	+	+	+	+	+	+		+	+	+	+	+	+	+	+	+	+
Lactose utilization	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+
Fructose utilization	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+
Mannitol utilization	-	-	+	-	-	-	-	-	-	-	-	+	-	+	-	-	-	+	-	+
Citrate	NR	NR	-	NR	NR	NR	-	NR	NR	NR	NR	-	NR	-	NR	NR	NR	-	NR	-
Urease	+	+	-	+	+	+	-	+	+		+	-	+	+	+	+	+	-	-	-
H2S production	-	-	+	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	+
Gelatin hydrolysis	+	-	+	-	+	-	-	+	-	+	+	+	+	+	-	+	+	+	-	-
Lysine Deacaboxylase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Ornithine Deacarboxylase	-	-	+	-	-	-	+	-	-	-	-	+	-	-	-	-	-	+		+
Argenine Dehydrolase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
O/F	+/-	+/-	+/+	+/-	+/-	+/-	+/+	+/-	+/-	+/-	+/-	+/+	+/-	+/+	+/-	+/-	+/-	+/+	+/-	+/+
Growth at RT																				
TSA	+/no pig	+/no pig	+/no pig	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAF	+/no pig	+/no pig	+/no pig	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAP	+/no pig	+/no pig	+/no pig	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 30º C																				
TSA	+/no pig	+/piga	+/pig[a]	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAF	+/no pig	+/pig[b]	+/no pig	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PAP	+/no pig	+/pig[c]	+/no pig	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 42º C																				
TSA	+/no pig	+/no pig	+/no pig	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	-	+
PAF	+/no pig	+/no pig	+/no pig	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	-	+
PAP	+/no pig	+/no pig	+/no pig	-	+	-	+	+	-	+	+	+	+	+	-	+	+	+	-	+
Acenaphthene	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+
Anthracene	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+
Fluoranthene	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+
Phenanthrene	+	-	+	-	-	-	+	-	+	-	-	+	-	-	-	-	-	-	-	+
N – Dodecane	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-	+	+
N – Pentadecane	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-	+	+
N – Tetradecane	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-	+	+
N – Octadecane	+	+	+	-	+	-	+	-	+	+	-	-	-	-	+	-	+	-	+	+
Naphthalene	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrobenzene	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Toluene	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	-	+	-

- Incubation Temperature for all biochemical tests is 30º C
- Incubation Time 24h. – Two weeks.
- TSA: Tryptic (Trypticase) Soy Agar
- PAF: *Pseudomonas* Agar F for enhancing fluorescein production.
- PAP: *Pseudomonas* Agar P for enhancing pyocyanin production.
- [a] light-green pigment in daylight
- [b] Blue-green pigment in daylight
- [c] Yellow-green pigment in daylight
- NR: no result

Table 5. Growth in the presence of different concentration of NT hydrocarbon mixed solution in Stanier's media containing 2% agar

Isolate	100 ppm NT	300 ppm NT	500 ppm NT	1000 ppm NT
A	2.0	2.0	1.0	2.0
B	2.0	1.0	1.5	2.5
C	3.5	1.0	3.5	4.0
D	3.0	4.0	3.0	2.0
E	5.0	2.0	3.0	4.0
F	5.0	4.0	3.0	3.0
G	5.0	3.0	1.0	5.0
H	2.5	1.0	1.0	1.0

- 1.0 Single, small size colony.
- 2.0 Single, medium size colony.
- 3.0 Single, large size colony.
- 4.0 Two to three, small size colony.
- 5.0 Whole mass of growth in the form of a lane of growth (circle) surrounding the growing colony.

Table 6. Growth in the presence of different concentration of NT hydrocarbon mixed solution in Stanier's media containing 1% agar

Isolate	100 ppm NT	300 ppm NT	500 ppm NT	1000 ppm NT
A	2.0	1.0	1.0	2.0
B	2.0	1.0	1.0	1.0
C	3.0	1.0	1.0	1.5
D	3.0	3.0	2.0	0.5
E	3.0	2.0	2.0	1.0
F	1.0	2.0	1.0	0.5
G	3.0	1.0	2.5	1.5
H	1.0	1.5	1.5	2.0

- 1.0 Single, small size colony.
- 2.0 Single, medium size colony.
- 3.0 Single, large size colony.
- 4.0 Two to three, small size colony.
- 5.0 Whole mass of growth in the form of a lane of growth (circle) surrounding the growing colony.

3.2- Growth in the presence of NNT hydrocarbon mixed solution

Tables 7 and 8 show the level of growth of each bacterial isolate under each NNT concentration. The levels of growth are shown in numbers ranging from 1.0 -5.0, and all numbers shown are averages from triplicate plates.

Table 7. Growth in the presence of different concentration of NNT hydrocarbon mixed solution in Stanier's media containing 2% agar

Isolate	200 ppm NNT	500 ppm NNT
A	3.0	0.0
B	3.0	1.0
C	2.0	1.0
D	2.0	1.5
E	1.0	0.5
F	2.0	1.0
G	2.5	1.0
H	1.5	0.5

- 1.0 Single, small size colony.
- 2.0 Single, medium size colony.
- 3.0 Single, large size colony.
- 4.0 Two to three, small size colony.
- 5.0 Whole mass of growth in the form of a lane of growth (circle) surrounding the growing colony.

Table 8. Growth in the presence of different concentration of NNT hydrocarbon mixed solution in Stanier's media containing 1% agar

Isolate	200 ppm NNT	500 ppm NNT
A	2.0	1.0
B	2.0	1.0
C	2.5	2.0
D	2.0	1.5
E	1.0	2.0
F	1.5	1.0
G	2.5	1.5
H	2.0	1.5

- 1.0 Single, small size colony.
- 2.0 Single, medium size colony.
- 3.0 Single, large size colony.
- 4.0 Two to three, small size colony.
- 5.0 Whole mass of growth in the form of a lane of growth (circle) surrounding the growing colony.

4- Growth of mixed culture isolates on broth media containing hydrocarbons as the only carbon and/or nitrogen source (Growth in the presence of NNT and NT hydrocarbon mixed solution)

Stanier's minimal medium supplemented with different concentrations of NNT and NT solution was used to grow the mixed bacterial culture. Weekly samples were collected and plated in Stanier's media plates containing different concentrations of NNT and NT. Tables 9 and 10 show the growth of bacterial isolate under each NNT and NT concentration respectively. The levels of growth are shown in numbers ranging from 1.0 - 4.0, and all numbers shown are average of duplicate plates.

Table 9. Growth in the presence of different concentration of NNT hydrocarbon mixed solution in Stanier's media

Sample	300 ppm NNT	1000 ppm NNT
Week 1	1.0	1.0
Week 2	1.0	1.0
Week 3	2.0	1.0
Week 4	2.0	2.0

- 1.0 Single, large size colony.
- 2.0 Two to three, small size colony.

Table 10. Growth in the presence of different concentration of NT hydrocarbon mixed solution in Stanier's media.

Sample	300 ppm NT	1000 ppm NT
Week 1	1.0	1.0
Week 2	1.0	1.0
Week 3	2.0	2.0
Week 4	3.0	2.0

- 1.0 Single, large size colony.
- 2.0 Two to three, small size colony.

5- Growth of mixed culture in a biphasic media containing hydrocarbons as the only carbon and/or nitrogen source (Growth in the presence of NNT and NT hydrocarbon mixed solution)

Stanier's minimal broth and TN agar media containing different concentrations of NNT and NT solutions respectively were used to grow the mixed bacterial culture. Weekly samples have been collected and plated in Stanier's media plates containing different concentrations of NNT.

Tables 11 and 12 show the growth of bacterial isolate under each NNT and NT concentration respectively. The levels of growth are shown in numbers ranging from 1.0 - 4.0, and all numbers shown are average of duplicate plates.

Table 11. Growth in the presence of different concentration of NNT hydrocarbon mixed solution in Stanier's agar media

Sample	300 ppm NNT	1000 ppm NNT
Week 1	1.0	1.0
Week 2	2.0	1.0
Week 3	2.0	2.0
Week 4	3.0	2.0

- 1.0 Single, large size colony.
- 2.0 Two to three, small size colony.

Table 12. Growth in the presence of different concentration of NT hydrocarbon mixed solution in Stanier's media.

Sample	300 ppm NT	1000 ppm NT
Week 1	1.0	1.0
Week 2	1.0	1.0
Week 3	2.0	2.0
Week 4	3.0	2.0

- 1.0 Single, large size colony.
- 2.0 Two to three, small size colony.

6- Growth of bacteria collected from the 3 liter vessel using Stanier's media containing NNT/NT

6.1- Growth of bacteria collected from the fermentation vessel containing 500 ppm sodium benzoate, in Stanier's media containing NNT and NT respectively

Stanier's minimal broth containing 500 ppm Sodium Benzoate was used to grow the mixed bacterial culture. Samples have been collected and plated in Stanier's media plates containing different concentrations of NNT and NT respectively. Tables 13 and 14 show the growth of bacterial isolate under each NNT and NT respectively concentration. The levels of growth are shown in numbers ranging from 1.0 -4.0, and all numbers shown are average of duplicate plates.

Table 13. Growth of mixed culture samples collected from the fermentation vessel in Stanier's media containing NNT

Sample	300 ppm NNT	1000 ppm NNT
Sample 1	2.0	1.0
Sample2	3.0	2.0
Sample3	4.0	2.0
Sample4	4.0	3.0
Sample5	4.0	2.0
Sample6	4.0	3.0

- 1.0 Single, large size colony.
- 2.0 Two to three, small size colony.

Table 14. Growth of mixed culture samples collected from the fermentation vessel in Stanier's media containing NT.

Sample	300 ppm NT	1000 ppm NT
Sample 1	3.0	2.0
Sample2	3.0	2.0
Sample3	4.0	3.0
Sample4	4.0	4.0
Sample5	4.0	4.0
Sample6	4.0	4.0

- 1.0 Single, large size colony.
- 2.0 Two to three, small size colony.

6.2- Viable Bacterial Count

Table 15, shows the viable bacterial count of the collected samples grown in the 3 liter fermentor in the presence of 500 ppm Sodium Benzoate with/without 150 ppm Naphthalene. The total CFUs in the presence of: Sodium Benzoate was $8.7x10^8$ CFUs; Naphthalene was $9.7x10^6$ CFUs; NNT was $4.1x10^5$ CFUs; NT was $5.7x10^5$ CFUs.

7- Isolation and identification of individual hydrocarbon degraders

7.1- Microorganism isolated using Nitrobenzene as sole C and N

The following microorganism was isolated from the fermentor on a minimal medium containing only nitrobenzene as the sole source of carbon, nitrogen, and energy.

Strain A is a *Pseudomonas* species Gram-negative motile rod occasionally seen in pairs, and when grown on nutrient agar the colonies appear white to creamy. Strain A is further characterized as shown in Table 4.

Table 15. Viable count of bacteria grown on media containing different hydrocarbons

Chemical Compound\Dilution	**10^{-1}** (CFUs)	**10^{-2}** (CFUs)	**10^{-3}** (CFUs)	**10^{-4}** (CFUs)	**10^{-5}** (CFUs)	**10^{-6}** (CFUs)	**10^{-7}** (CFUs)
Sodium Benzoate	TNTC	TNTC	TNTC	TNTC	TNTC	87	0
Naphthalene*	TNTC	TNTC	TNTC	97	0	0	0
NT*	TNTC	180	57	0	0	0	0
NNT*	TNTC	155	41	0	0	0	0

- These numbers indicates the colony forming units (CFUs) in each dilution.
- Results are averages from triplicate experiments.
- * Without sodium benzoate.

7.2- Microorganisms isolated using naphthalene as sole C

The following microorganisms were isolated from the fermenter on minimal medium containing only naphthalene as the sole source of carbon and energy. Isolates A, B, D, E, F, H, I, J, K, M, O, P, Q and S belong to *Pseudomonas* species They are Gram-negative motile rod where the rods are seen singly, in pairs or in long chains. These bacterial isolates are further characterized as shown in Table 4. Isolates C, G, L, N, R, and T belong to *Aeromonas* species. They are Gram-negative motile rod. The colonies appear white on TSA. All these isolates are positive for twitching motility on twitching plates. These bacterial isolates are further characterized as shown in Table 4.

7.3- Microorganisms isolated using other hydrocarbons

The following microorganisms were isolated from the fermentor on minimal medium containing only toluene as the sole source of carbon and energy. Table 4, shows that all isolated strains were able to grow in the presence of toluene except isolates J, P, R, and T. Only strain A was able to grow in the presence of nitrobenzene. All isolated strains were able to grow in the presence of Naphthalene, and only isolates A, C, G, I, L, T were able to grow in the presence of Acenaphthene, Anthracene, Fluoranthene and Phenanthrene. On the other hand, most of the isolates were able to grow in the presence of n-dodecane, n-pentadecane, n-tetradecane, and n-octadecane except isolates D, F, H, K, L, M, N, P, and R (Table 4).

7.4- Microorganisms isolated from the two 5 liter vessels

Table 16 shows nine new isolates from the 5 liter fermentor, isolated, identified and grown in the presence of different hydrocarbons.

Table 16. Differential Biochemical Characteristics and growth of individual isolates in the presence of hydrocarbon compound

Test \ Isolate	HPRA-1	HPRA-2	HPRA-3	HPRA-4	HPRB-1	HPRB-2	HPRB-3	HPRB-4	HPRB-5
Gram Stain	-R	-R	-R	-R	-R	-R	-R	-R	-R
Catalase	+	+	+	+	+	+	+		
Oxidase	-	+	+	+	+	+	+	+	+
Glucose utilization	+	+	+	+	+	+	+	-	-
Lactose utilization	–	–	+	–	–	+	–	+	+
Mannitol utilization	+	+	+	–	+	+	+	–	–
Fructose utilization	+	+	+	–	+	+	+	+	+
Mannose	+	+	+	–	+	-	+	+	+
Indole	-	-	-	–	-	-	-	+	+
Citrate	+	+	+	–	+	+	+	+	+
Urease	+	+	+	–	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+
Gelatin hydrolysis	+	+	-	-	+	-	+	+	+
Lysine Deacaboxylase	+	+	-	–	+	-	+	+	+
Ornithine Deacarboxylase	+	–	-	–	–	-	–	+	+
Argenine Deacarboxylase	+	–	-	–	–	-	–	+	+
Growth at 4º C	+	+	+	+	+	+	+	+	+
Growth at 25º C	+	+	+	+	+	+	+	+	+
Growth at 30º C	+	+	+	+	+	+	+	+	+
Growth at 42º C	+	+	-	-	+	-	+	+	+
Toluene* (200 ppm)	3	2	3	NG	3	NG	2	2	3
Naphthalene* (200 ppm)	4	3	3	2	3	3	2	3	4
Nitrobenzene (200 ppm)	2	NG	2	NG	3	NG	NG	3	3
NT* (200 ppm)	4	3	2	3	4	3	3	3	4
NNT (200 ppm)	2	2	2	1	3	1	1	3	3
NNT* (200 ppm)	4	3	4	3	4	3	3	4	4

- * With ammonium sulfate.
- 1-4 represent the size of growth.
- NG: no growth.
- HPRA-1, HPRB-4 and HPRB-5 (*Aeromonas*).
- HPRA-2, HPRA-3, HPRA-4, HPRB-1, HPRB-2 and HPRB-3 (*Pseudomonas species*).

8- SEM

Figure 3 is an SEM image of bacterial cells immobilized on GAC particles. Different types of cells (in large numbers) can be clearly observed on the surface of GAC (Samples have been collected from 5 liter vessel after being fed with 1000 ppm naphthalene, 400 ppm nitrobenzene, 1000 ppm toluene, for 24 h over two months period).

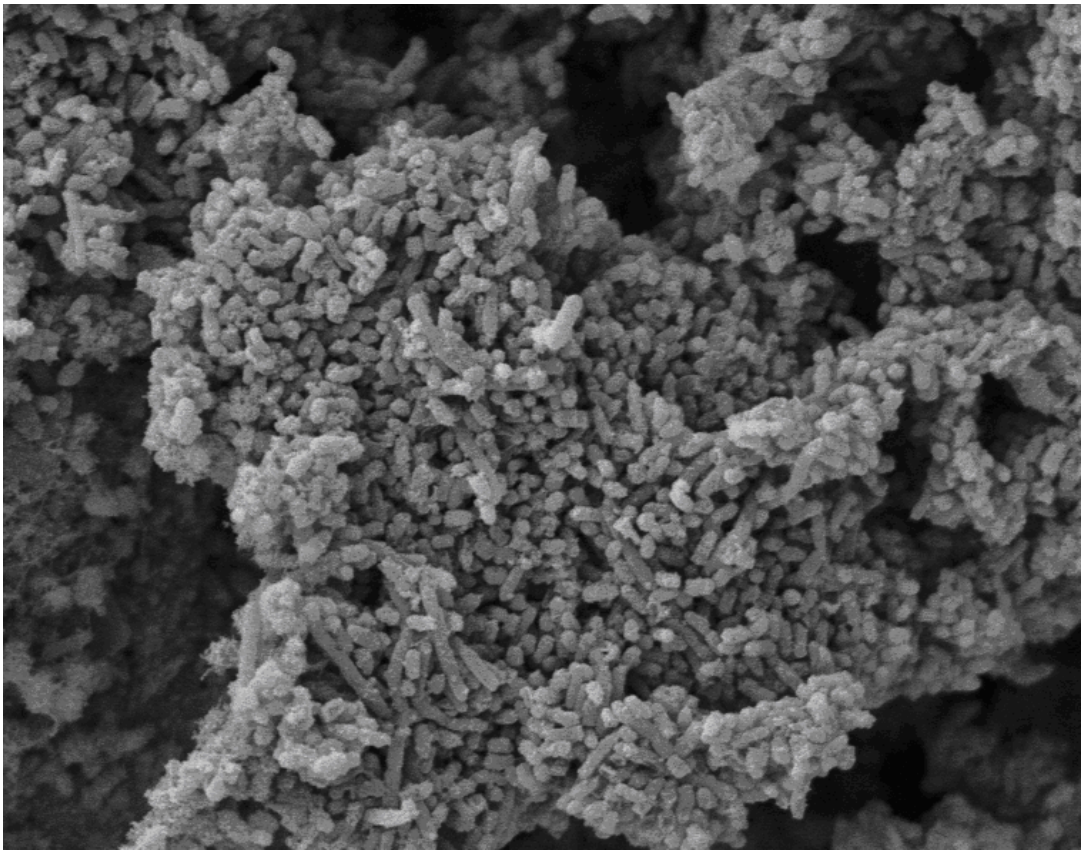

Figure 3. SEM of immobilized cells on granular activated carbon, sampled from 5 liter vessel, after being fed with 1000 ppm naphthalene, 400 ppm nitrobenzene, 1000 ppm toluene. Magnification: 5000 X.

9- Analysis of biodegradation

9.1 Hydrocarbon determination

9.1.1- Residual hydrocarbon determination

Figures 4 and 5 show the degradation of NNT in the presence of immobilized and planktonic cells respectively over 60 h period. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm. Immobilized cells were able to degrade toluene and naphthalene to a level below 30 ppm within 48 h, while it took the planktonic cells 60 h to degrade toluene and naphthalene to a level below 30 ppm.

On the other hand, nitrobenzene was shown to be degraded to a level below 400 ppm at 8 h in the presence of immobilized cells, while it took the planktonic cells more than 24 h to degrade it to below 400 ppm. Finally, nitrobenzene was degraded to a level below 100 ppm in the presence of immobilized cells within 48 h, while it took the planktonic cells 60 h to degrade it to a level below 100 ppm.

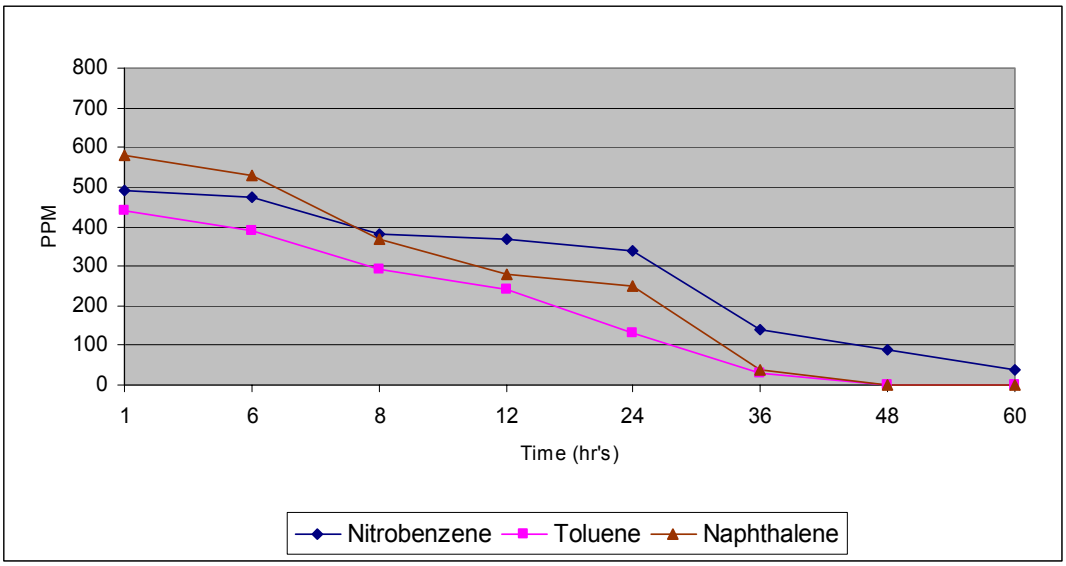

Figure 4. Degradation of NNT in the presence of immobilized cells over 60 h period. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

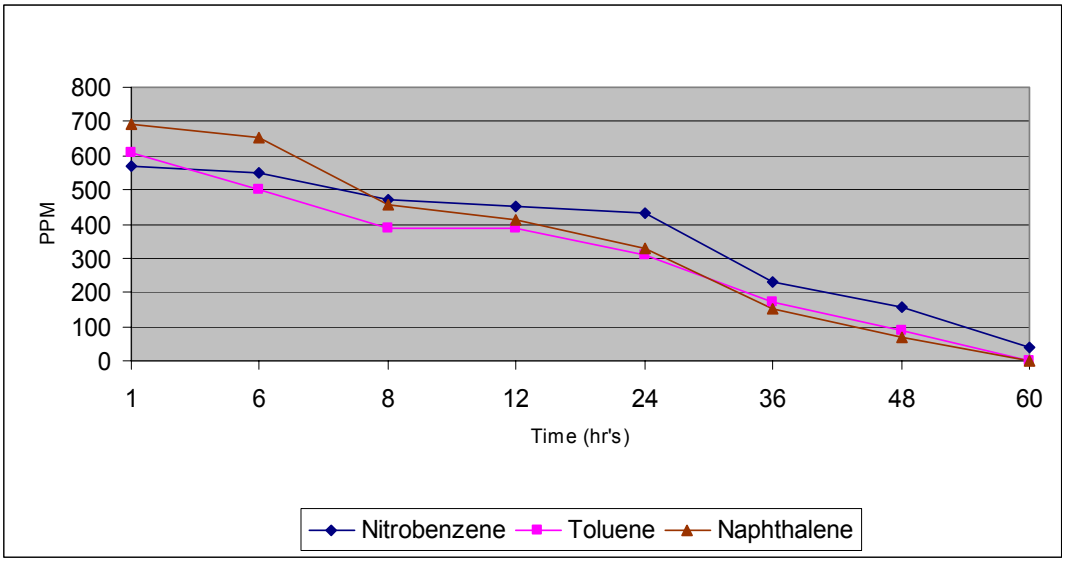

Figure 5. Degradation of NNT in the presence of planktonic cells over a 60 h period. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

9.1.2. Volatilization

Figures 6 and 7 show the level of volatile organic carbon (VOC) produced after the addition of NNT in the presence of immobilized and planktonic cells respectively over 60 h period. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm. The level of VOC detected from toluene and naphthalene was higher in the presence of immobilized cells compared to planktonic cells within the first 12 h.

On the other hand, the level of VOC detected from nitrobenzene was higher in the presence of planktonic cells compared to immobilized cells within the first 24 h. Finally, no VOC was detected after 48 h.

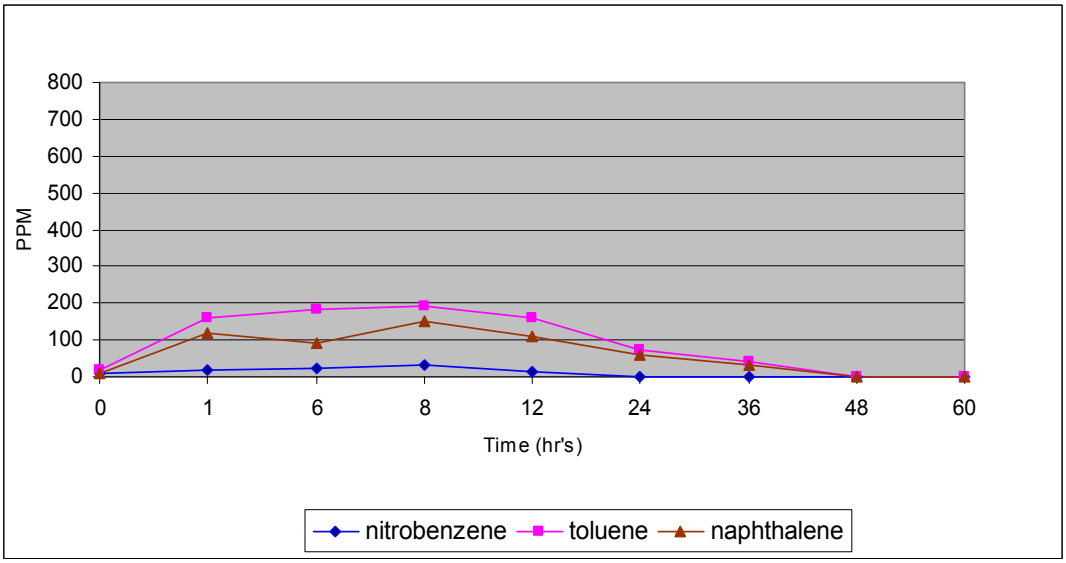

Figure 6. GC analysis of VOC trap after the addition of NNT to immobilized cells over a 60 h period. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

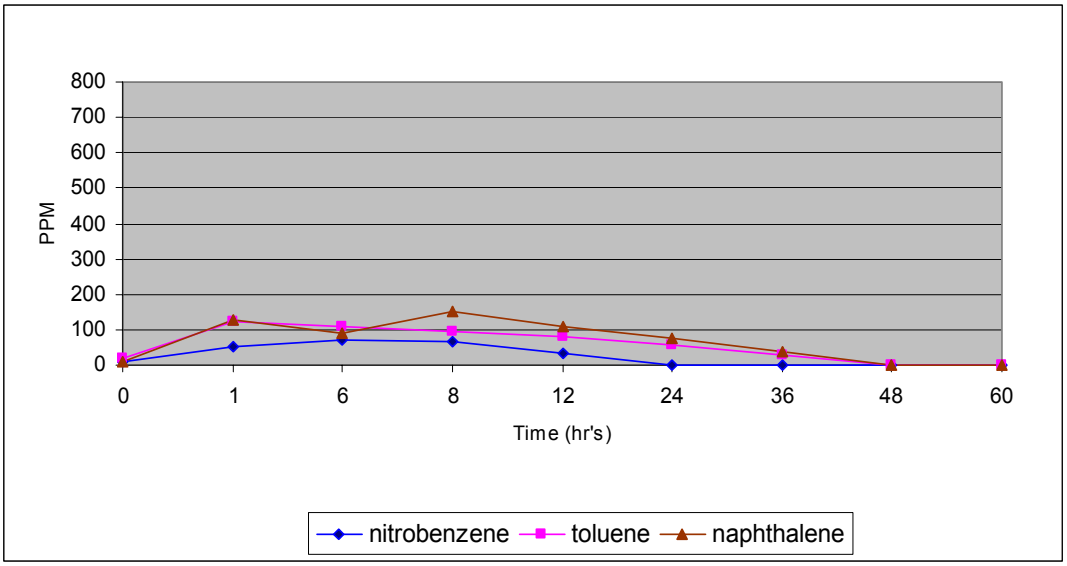

Figure 7. GC analysis of VOC trap after the addition of NNT to planktonic cells over a 60 h period. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

9.1.3- Degradation of individual hydrocarbons

Figure 8, shows the degradation of nitrobenzene (600 ppm) in the presence of immobilized and planktonic cells. The degradation of nitrobenzene was higher in the presence of immobilized cells compared to planktonic cells. Immobilized cells were able to degrade nitrobenzene to a level below 400 ppm within 8 h, while it took the planktonic cells more than 24 h to degrade nitrobenzene to a level below 400 ppm. Finally, nitrobenzene was degraded to a level below 100 ppm in the presence of immobilized cells within 48 h, while it took the planktonic cells 60 h to degrade it to a level below 100 ppm.

Figure 9, shows the degradation of naphthalene (700 ppm) in the presence of immobilized and planktonic cells. The degradation of naphthalene was higher in the presence of immobilized cells compared to planktonic cells. Immobilized cells were able to degrade naphthalene to a level below 400 ppm within 8 h, while it took the planktonic cells more than 24 h to degrade naphthalene to a level below 400 ppm. Finally, naphthalene was degraded to a level below 30 ppm in the presence of immobilized cells within 48 h, while it took the planktonic cells 60 h to degrade it to a level below 30 ppm.

Figure 10, shows the degradation of toluene (700 ppm) in the presence of immobilized and planktonic cells. The degradation of toluene was higher in the presence of immobilized cells compared to planktonic cells. Immobilized cells were able to degrade toluene to a level below 300 ppm within 8 h, while it took the planktonic cells more than 24 h to degrade naphthalene to a level below 300 ppm. Finally, toluene was degraded to a

level below 30 ppm in the presence of immobilized cells within 36 h, while it took the planktonic cells more than 48 h to degrade it to a level below 30 ppm.

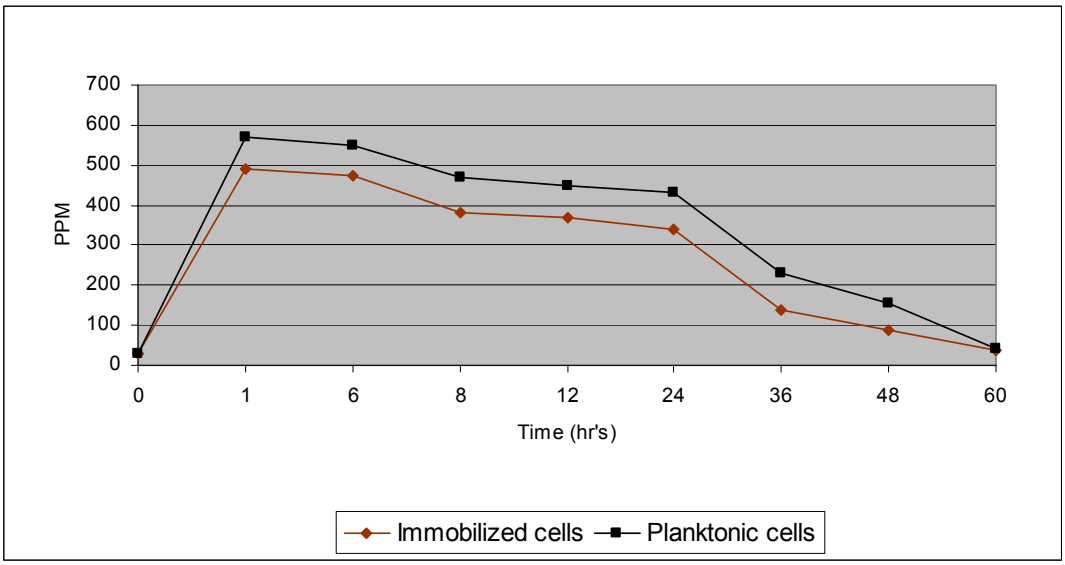

Figure 8. Degradation of Nitrobenzene (600 ppm) in the presence of immobilized and planktonic cells over a 60 h period.

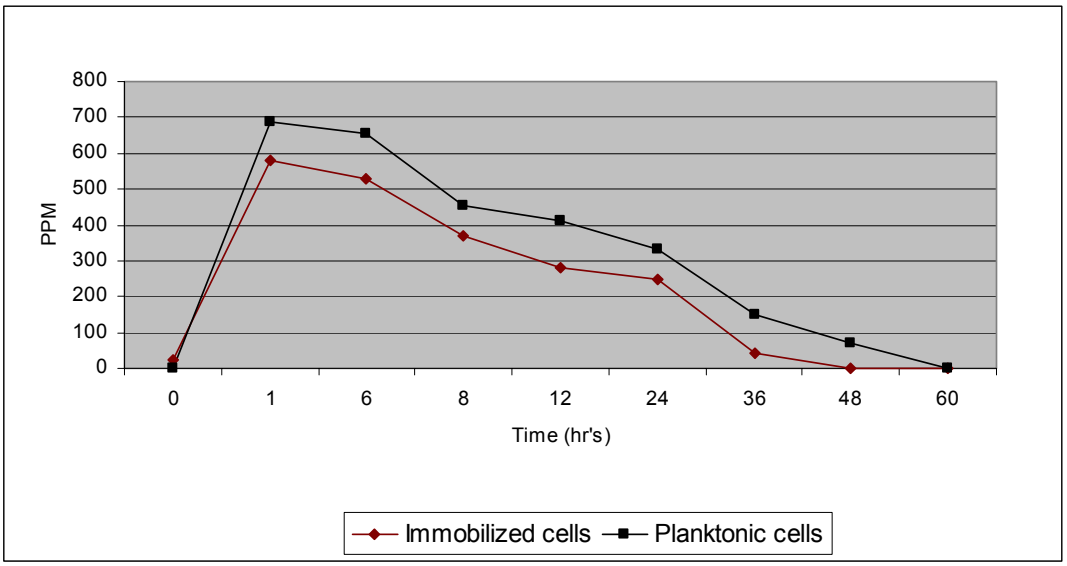

Figure 9. Degradation of Naphthalene (700 ppm) in the presence of immobilized and planktonic cells over a 60 h period.

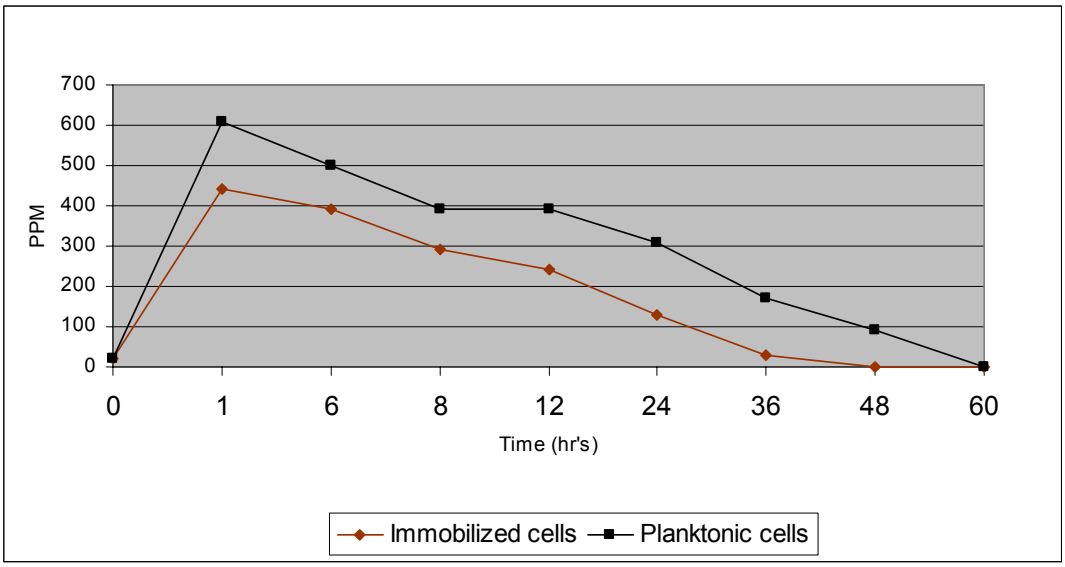

Figure 10. Degradation of Toluene (700 ppm) in the presence of immobilized and planktonic cells over a 60 h period.

9.1.4- Degradation of hydrocarbons over four 20 h cycles

Figures 11 and 12 show the degradation of NNT in the presence of immobilized and planktonic cells respectively over four cycles (each cycle 20 h). These cycles were run under the same conditions as previously described in figures 4 and 5, except the time for each run and the feed concentration. The initial feed was Naphthalene 600 ppm, Nitrobenzene 300 ppm and Toluene 500 ppm. These cycles shown in figures 11 and 12 were the average of multiple SBR cycles that were run under the same conditions, to show the consistency of the system over time.

Immobilized cells were able to degrade toluene and naphthalene to a level below 50 ppm within 20 h, while it took the planktonic cells 20 h to degrade toluene and naphthalene to a level above 140 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 120 ppm within 20 h in the presence of immobilized cells, while it took the planktonic cells 20 h to degrade it to a level below 200 ppm.

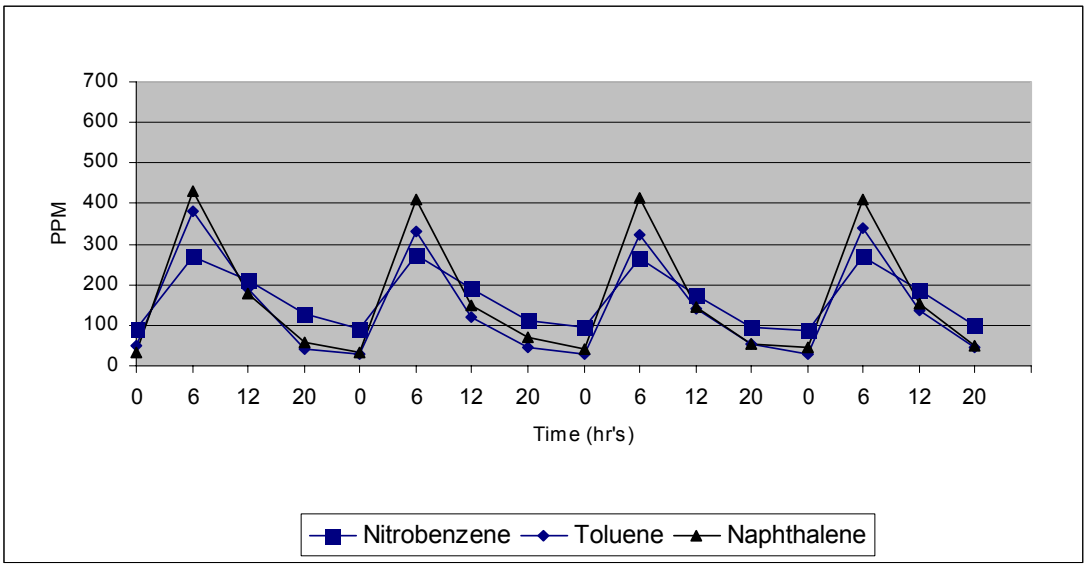

Figure 11. Degradation of NNT in the presence of immobilized cells over a 4 cycle (20 h) re-feeding (Naphthalene 600 ppm, Nitrobenzene 300 ppm and Toluene 500 ppm).

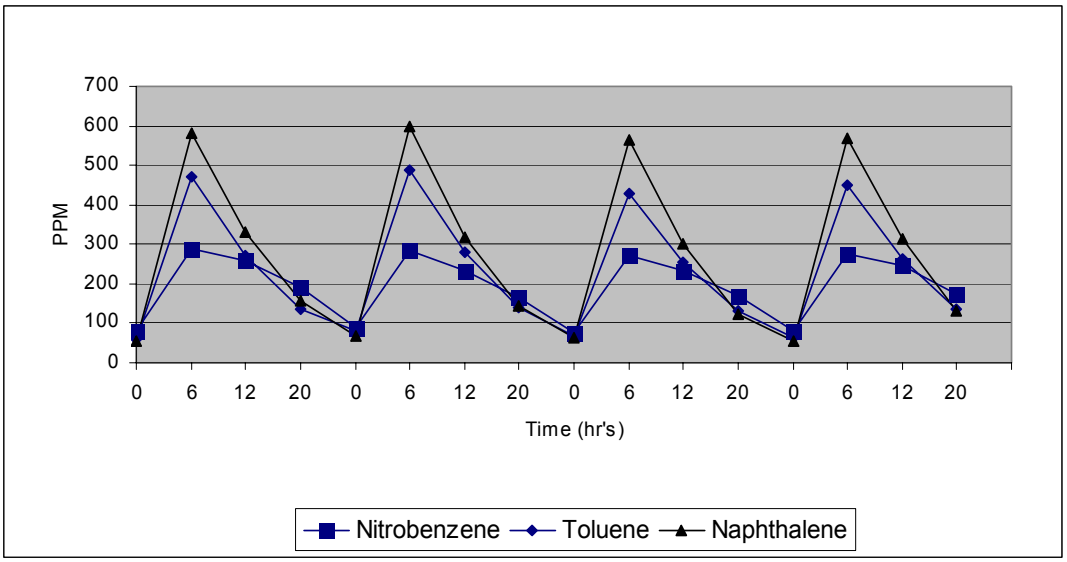

Figure 12. Degradation of NNT in the presence of planktonic cells over a 4 cycle (20h) re-feeding (Naphthalene 600 ppm, Nitrobenzene 300 ppm and Toluene 500 ppm).

9.1.5- Level of NNT-degrading microorganisms

Figures 13 and 14, show the count of NNT-degrading bacteria of the collected samples grown in the presence of naphthalene 600 ppm, nitrobenzene 300 ppm, and toluene 500 ppm.

The level NNT-degrading organisms in the presence of granular activated carbon were above $6.4x10^5$ CFUs after 20 h over different cycles. The level planktonic NNT-degrading organisms were below $6.0x10^5$ CFUs after 20 h over different cycles. These cycles were run under the same conditions as described in figures 11 and 12.

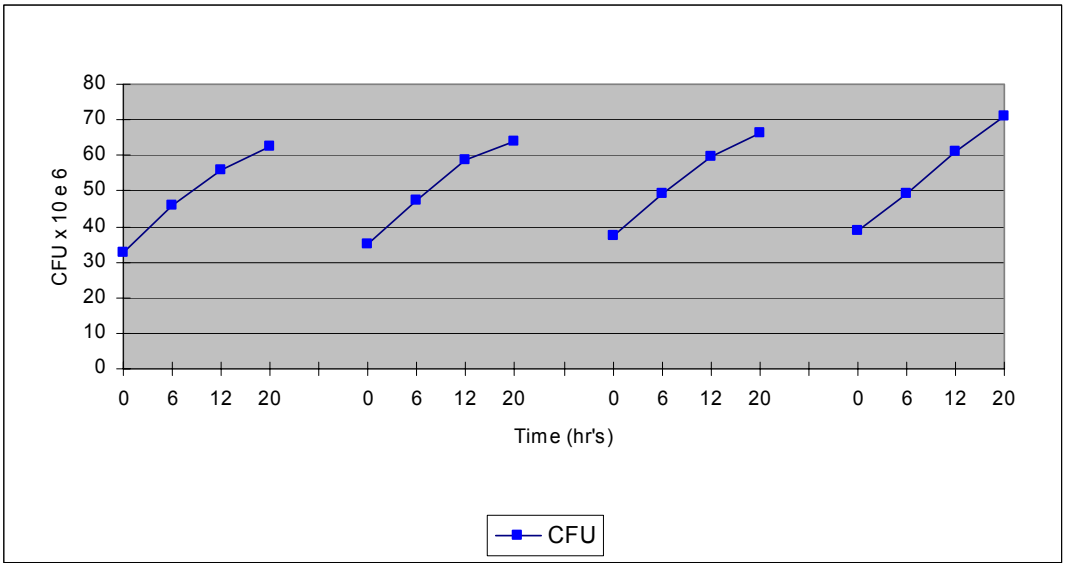

Figure 13. Level of immobilized NNT-degrading microorganisms in the liquid samples from reactor.

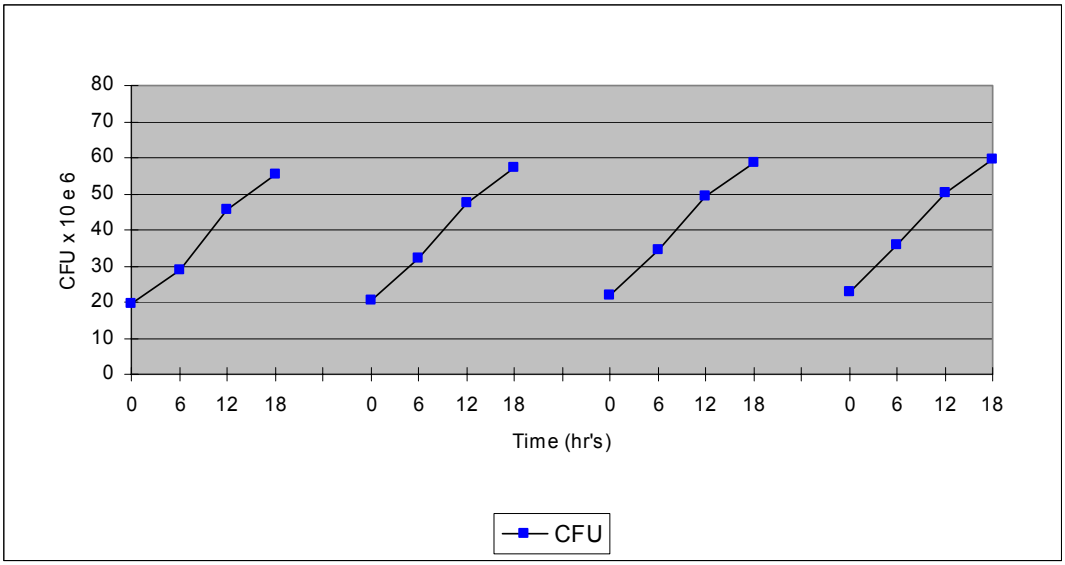

Figure 14. Level of planktonic NNT-degrading microorganisms in the liquid samples from reactor.

9.1.6- Degradation of different concentrations of NNT

Figure 15 and 16, show the degradation of NNT in the presence of immobilized and planktonic cells respectively over 4 (20 h) cycles. These cycles were run under the same conditions as previously described in figures 11 and 12, except the time for each run and the feed concentration. The initial feed was Naphthalene 1000 ppm, Nitrobenzene 400 ppm and Toluene 1000 ppm. Immobilized cells were able to degrade toluene and naphthalene to a level below 100 ppm within 20 h, while it took the planktonic cells 20 h to degrade toluene and naphthalene to a level above 190 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 100 ppm within 20 h in the presence of immobilized cells, while it took the planktonic cells 20 h to degrade it to below 190 ppm.

Figure 17 and 18, show the degradation of NNT in the presence of immobilized and planktonic cells respectively over 18 h period. These cycles were run under the same conditions as previously described in figures 15 and 16, except the time for each run and the feed concentration. The feed was Naphthalene 2000 ppm, Nitrobenzene 400 ppm and Toluene 1600 ppm. These first two curves shown in figures 17 and 18 were the first 2 cycles after changing the cycles to 18 hr, and the last 2 curves were from later cycles that were run under the same conditions, to show the consistency of the system over time. Immobilized cells were able to degrade toluene and naphthalene to a level below 270 ppm within 18 h, while it took the planktonic cells 18 h to degrade toluene and naphthalene to a level of 500 ppm. On the other hand, nitrobenzene was shown to be degraded to a level

below 110 ppm within 18 h in the presence of immobilized cells, while it took the planktonic cells 18 h to degrade it to below 230 ppm.

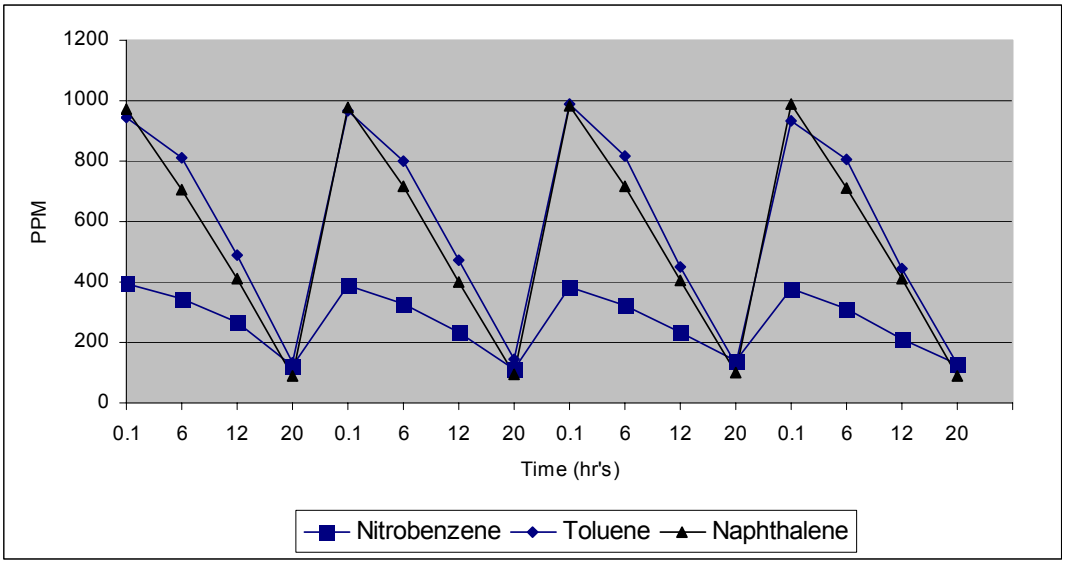

Figure 15. Degradation of NNT in the presence of immobilized cells over a 4 cycle (20h) re-feeding (Naphthalene 1000 ppm, Nitrobenzene 400 ppm and Toluene 1000 ppm).

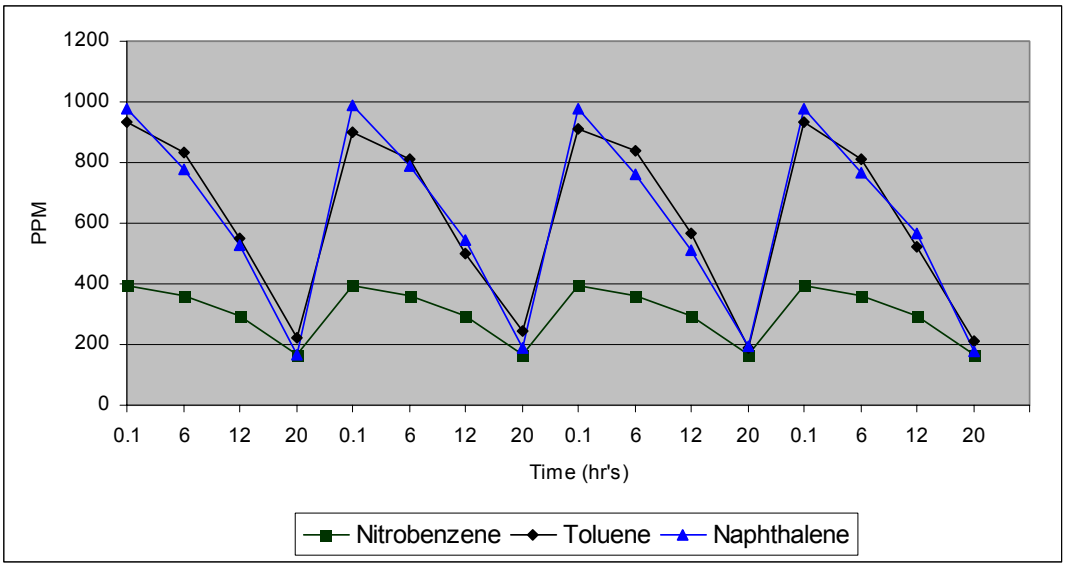

Figure 16. Degradation of NNT in the presence of planktonic cells over a 4 cycle (20h) re-feeding (Naphthalene 1000 ppm, Nitrobenzene 400 ppm and Toluene 1000 ppm).

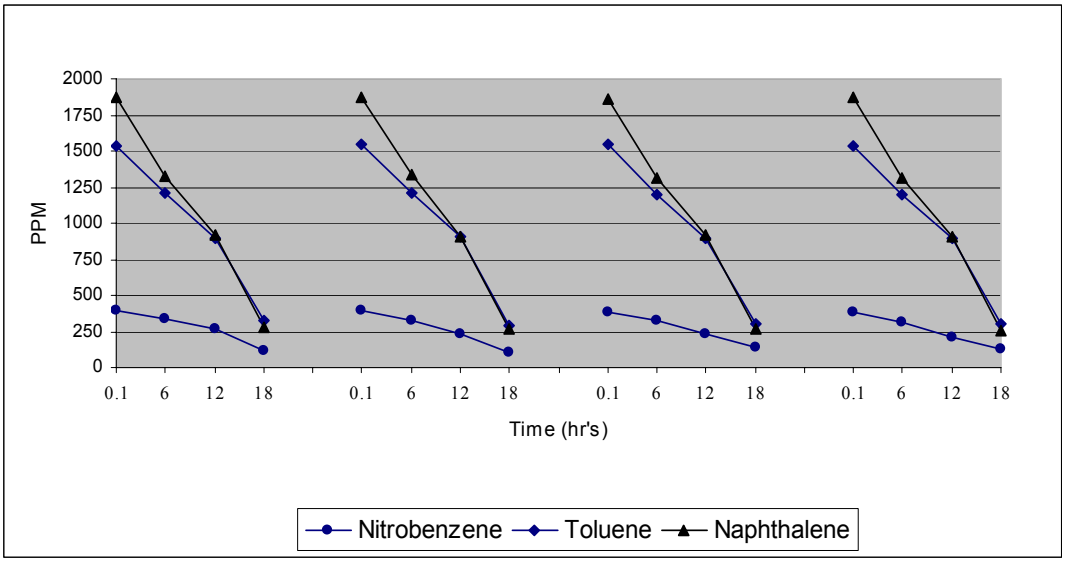

Figure 17. Degradation of NNT in the presence of immobilized cells in Stanier's media supplemented with ammonium sulfate over 18 h period for 4 days. The feed concentration was Naphthalene 2000 ppm, Nitrobenzene 400 ppm and Toluene 1600 ppm.

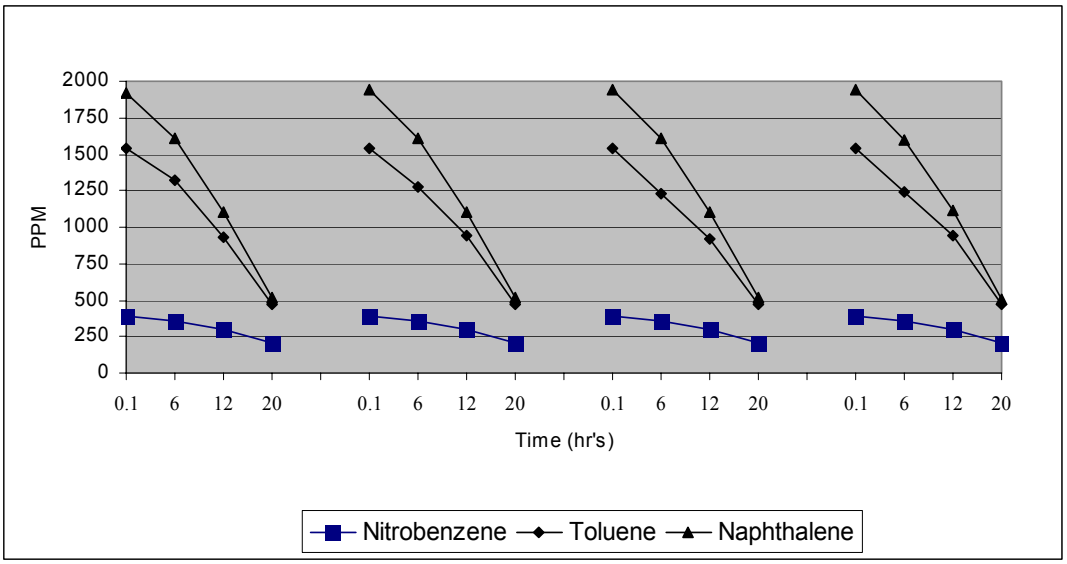

Figure 18. Degradation of NNT in the presence of planktonic cells in Stanier's media supplemented with ammonium sulfate over 18 h period for 4 days. The feed concentration was Naphthalene 2000 ppm, Nitrobenzene 400 ppm and Toluene 1600 ppm.

Figure 19 and 20, show a summary of the degradation of NNT in the presence of immobilized and planktonic cells respectively over five different stages within 18 h period each. These cycles were run under the same conditions as previously described in previous figures 17 and 18 except feed concentration. The feed for each stage was different from the others.

Stage 1and 2, the feed was Naphthalene 700, 1000 ppm, Nitrobenzene 400 ppm and Toluene 1000 ppm. Immobilized cells were able to degrade toluene and naphthalene to a level below 200 ppm within 18 h, while it took the planktonic cells 18 h to degrade toluene and naphthalene to a level of 250 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 210 ppm within 18 h in the presence of immobilized cells, while it took the planktonic cells 18 h to degrade it to below 280 ppm.

Stage 3, the feed was Naphthalene 1500 ppm, Nitrobenzene 400 ppm and Toluene 1500 ppm. Immobilized cells were able to degrade toluene and naphthalene to a level below 230 ppm within 18 h, while it took the planktonic cells 18 h to degrade toluene and naphthalene to a level below 410 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 200 ppm within 18 h in the presence of immobilized cells, while it took the planktonic cells 18 h to degrade it to below 230 ppm.

Stage 4, the feed was Naphthalene 2000 ppm, Nitrobenzene 400 ppm and Toluene 1500 ppm. Immobilized cells were able to degrade toluene and naphthalene to a level below 230 ppm within 18 h, while it took the planktonic cells 18 h to degrade toluene and

naphthalene to a level below 430 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 180 ppm within 18 h in the presence of immobilized cells, while it took the planktonic cells 18 h to degrade it to 200 ppm.

Stage 5, the feed was Naphthalene 3000 ppm, Nitrobenzene 400 ppm and Toluene 1500 ppm. Immobilized cells were able to degrade toluene to 370 ppm and naphthalene to 560 ppm within 18 h, while it took the planktonic cells 18 h to degrade toluene to 420 ppm and naphthalene to 970 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 180 ppm within 18 h in the presence of immobilized cells, while it took the planktonic cells 18 h to degrade it to 200 ppm.

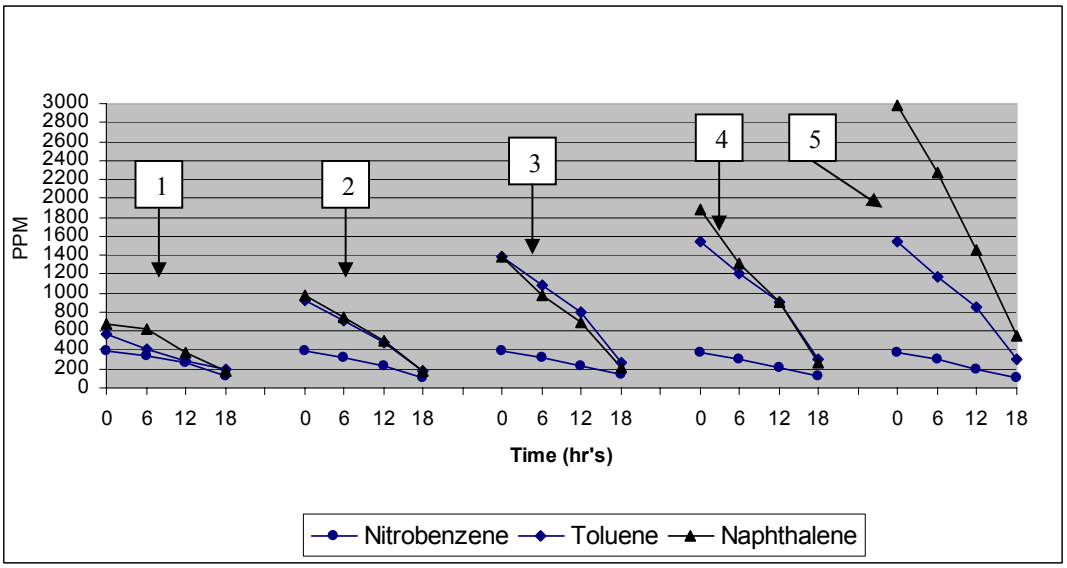

Figure 19. Degradation of different concentrations of NNT at different stages in the presence of immobilized cells.

Stage	Naphthalene	Nitrobenzene	Toluene
Stage 1	700ppm	400ppm	1000ppm
Stage 2	1000ppm	400ppm	1000ppm
Stage 3	1500ppm	400ppm	1500ppm
Stage 4	2000ppm	400ppm	1500ppm
Stage 5	3000ppm	400ppm	1500ppm

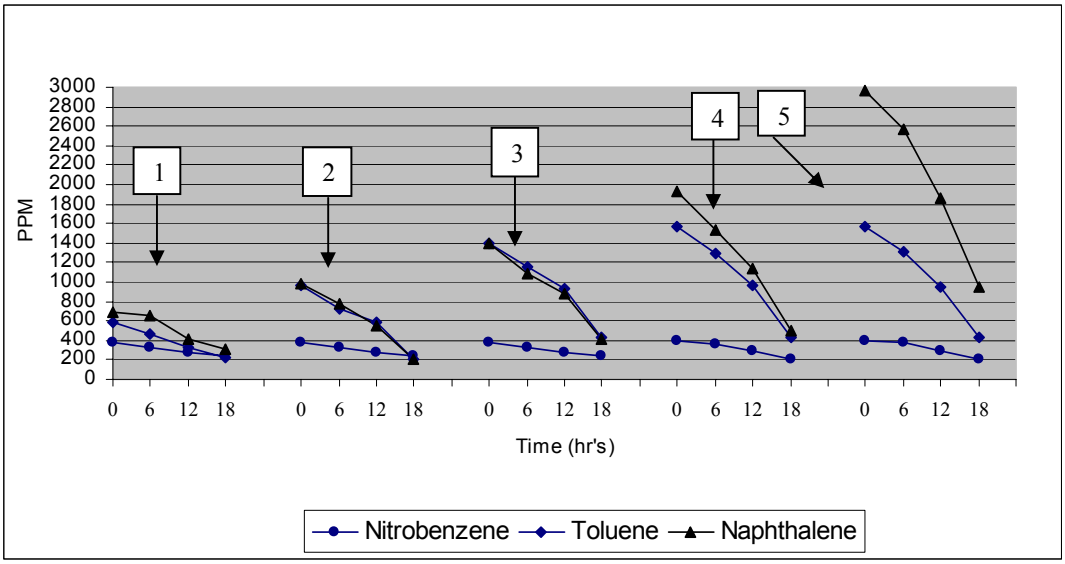

Figure 20. Degradation of different concentrations of NNT at different stages in the presence of planktonic cells.

Stage	Naphthalene	Nitrobenzene	Toluene
Stage 1	700ppm	400ppm	1000ppm
Stage 2	1000ppm	400ppm	1000ppm
Stage 3	1500ppm	400ppm	1500ppm
Stage 4	2000ppm	400ppm	1500ppm
Stage 5	3000ppm	400ppm	1500ppm

9.2- Degradation of synthetic waste

Figure 21, 22, 23, and 24, show the degradation of synthetic waste in the presence of immobilized and planktonic cells respectively 18 h period. The feed was chloroform, aniline, me-naphthalene and nitrobenzene 100 ppm, phenanthrene, ethyl benzene and o-xylene 60 ppm, chlorobenzene 50 ppm, naphthalene 1500 ppm, toluene 600 ppm. Immobilized cells were able to degrade toluene and naphthalene to a level below 200 ppm within 18 h, while it took the planktonic cells 18 h to degrade toluene to 200 ppm and naphthalene to 390 ppm. On the other hand, nitrobenzene was shown to be degraded to a level below 30 ppm within 18 h in the presence of immobilized cells, while it took the planktonic cells 18 h to degrade it to below 60 ppm.

Chloroform, aniline, ethylbenzene, chlorobenzene, and O-xylene, were shown to be degraded to a level below 25 ppm in the presence of immobilized cells, while in the presence of planktonic cells it has been shown to be degraded to a level above 35 ppm. Finally, me-naphthalene was shown to be degraded to a level below 40 ppm in the presence of immobilized cells, while in the presence of planktonic cells it was degraded to 50 ppm.

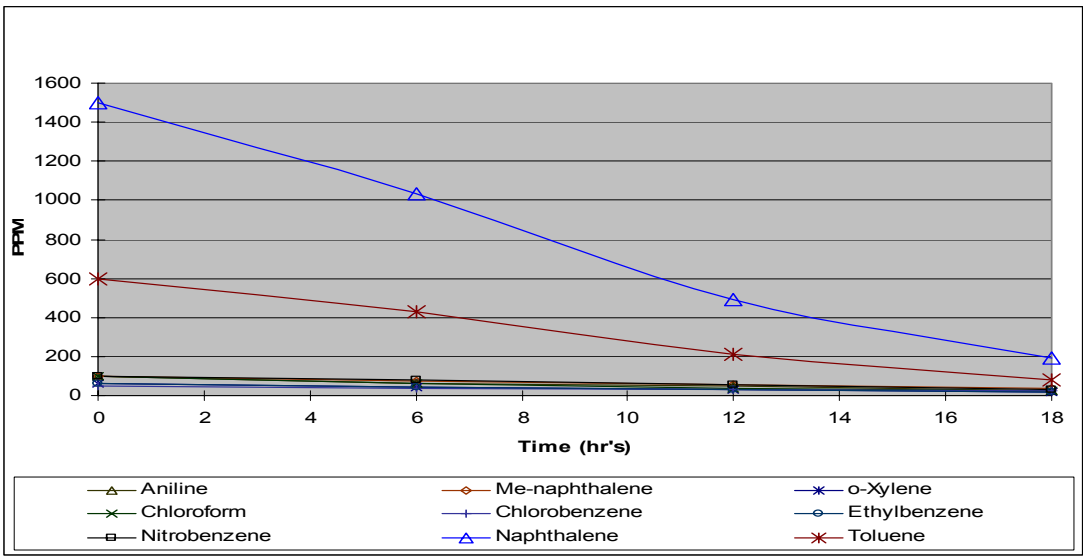

Figure 21. Degradation of synthetic waste in the presence of immobilized cells. Chloroform, Aniline, Me-naphthalene and Nitrobenzene 100 ppm, Ethyl benzene and O-Xylene 60 ppm, Chlorobenzene 50 ppm, Naphthalene 1500 ppm, Toluene 600 ppm.

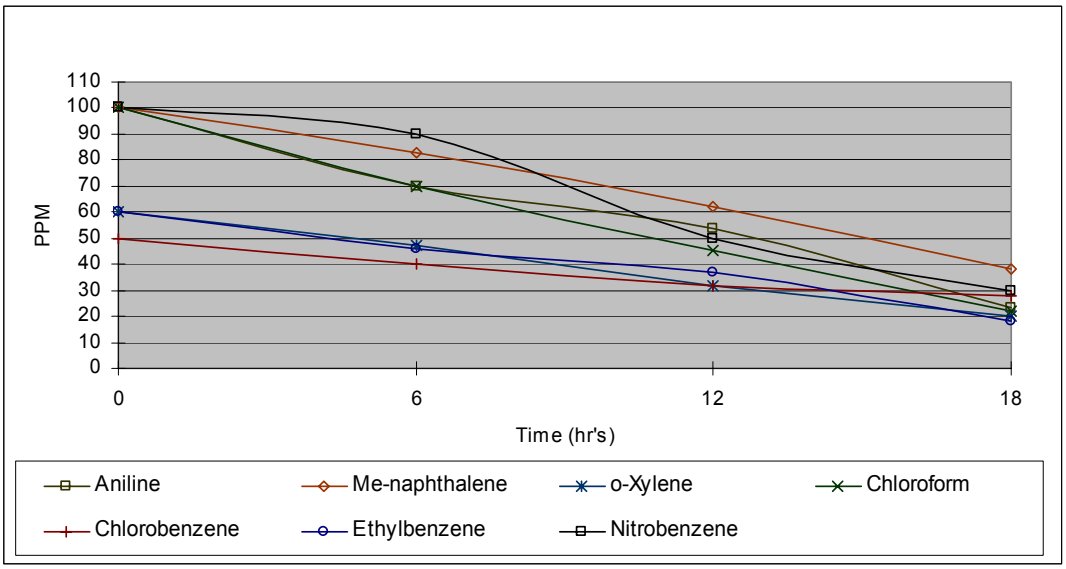

Figure 22. Degradation of synthetic waste in the presence of immobilized cells without Naphthalene and Toluene.
Chloroform, Aniline, Me-naphthalene and Nitrobenzene 100 ppm, Ethyl benzene and O-Xylene 60 ppm, Chlorobenzene 50 ppm, Naphthalene 1500 ppm, Toluene 600 ppm.

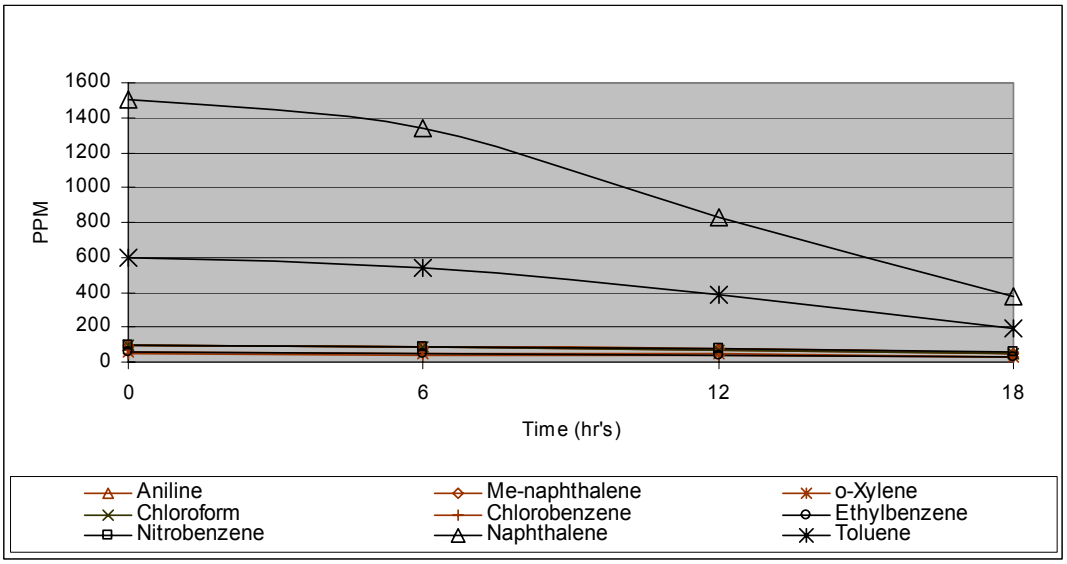

Figure 23. Degradation of synthetic waste in the presence of planktonic cells. Chloroform, Aniline, Me-naphthalene and Nitrobenzene 100 ppm, Ethyl benzene and O-Xylene 60 ppm, Chlorobenzene 50 ppm, Naphthalene 1500 ppm, Toluene 600 ppm.

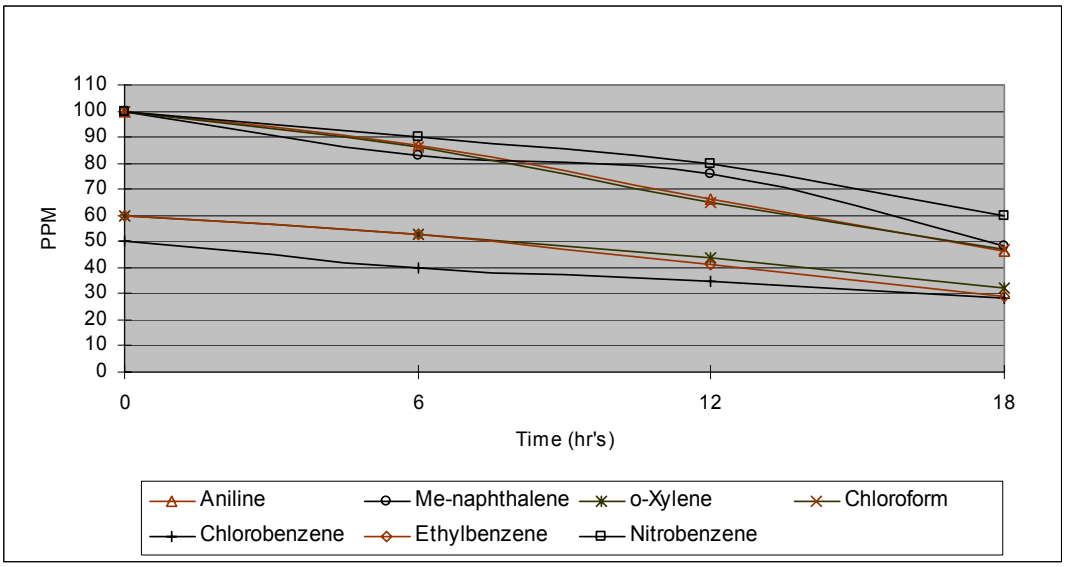

Figure 24. Degradation of synthetic waste in the presence of planktonic cells without Naphthalene and Toluene.
Chloroform, Aniline, Me-naphthalene and Nitrobenzene 100 ppm, Ethyl benzene and O-Xylene 60 ppm, Chlorobenzene 50 ppm, Naphthalene 1500 ppm, Toluene 600 ppm.

9.3- Determination of evolved CO_2 and volatile organic carbons (VOCs)

Figure 25 and 26, show the level of cumulative carbon dioxide after the addition of NNT in the presence of immobilized and planktonic cells respectively. Immobilized cells were shown to produce more carbon dioxide compared to planktonic cells. On the other hand, figures 27 and 28, show the level of cumulative and theoretical carbon dioxide after the addition of NNT in the presence of immobilized and planktonic cells. Immobilized cells were shown to produce more than 75% carbon dioxide compared to planktonic cells which produce lower than 50%, compared to the theoretical.

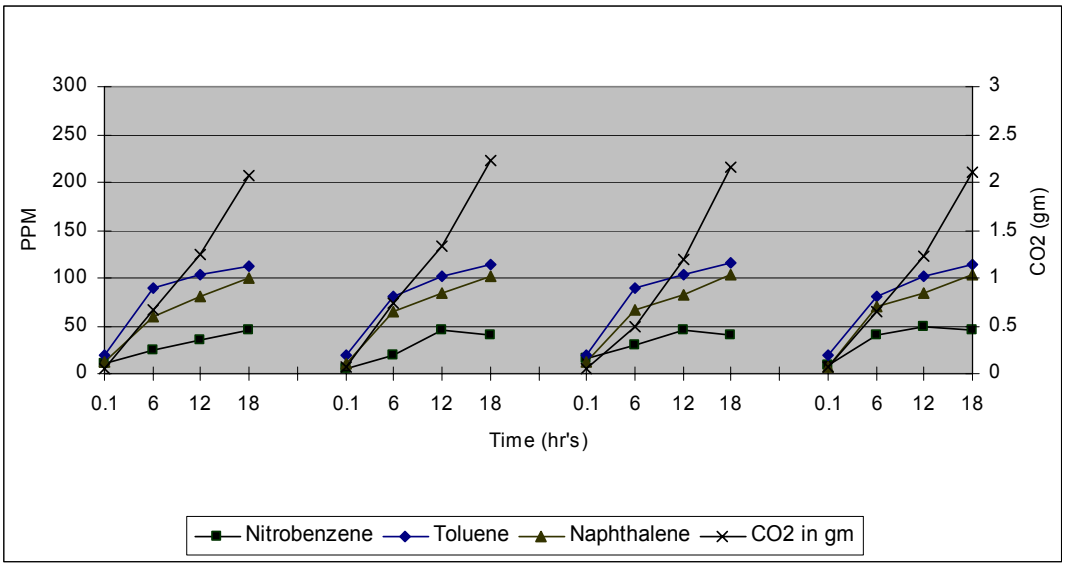

Figure 25. Level of cumulative evolved CO_2 and trapped VOC after the addition of NNT to immobilized cells over 4 day period.

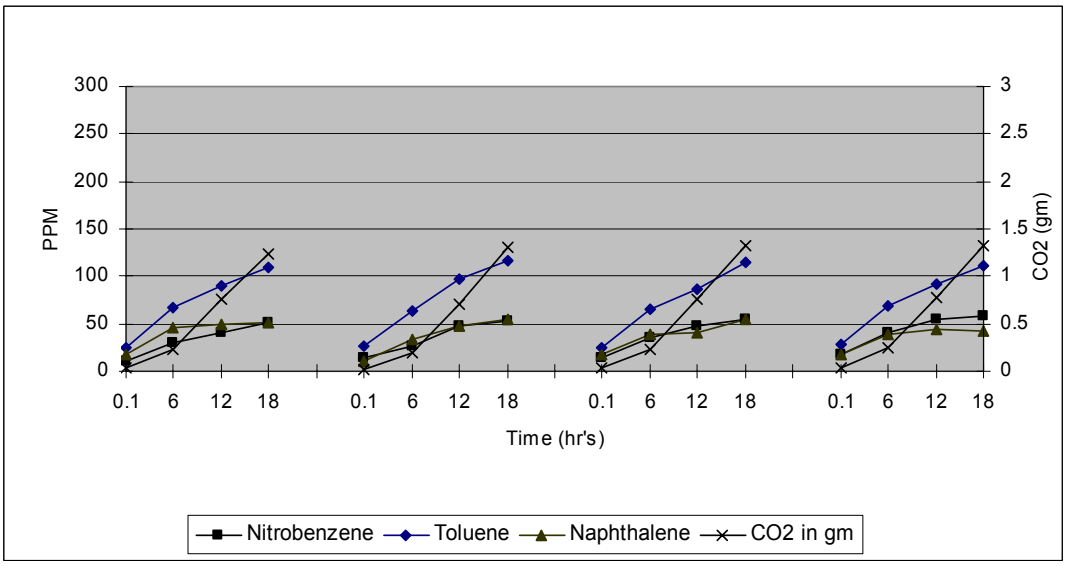

Figure 26. Level of cumulative evolved CO_2 and trapped VOC after the addition of NNT to planktonic cells over 4 day period.

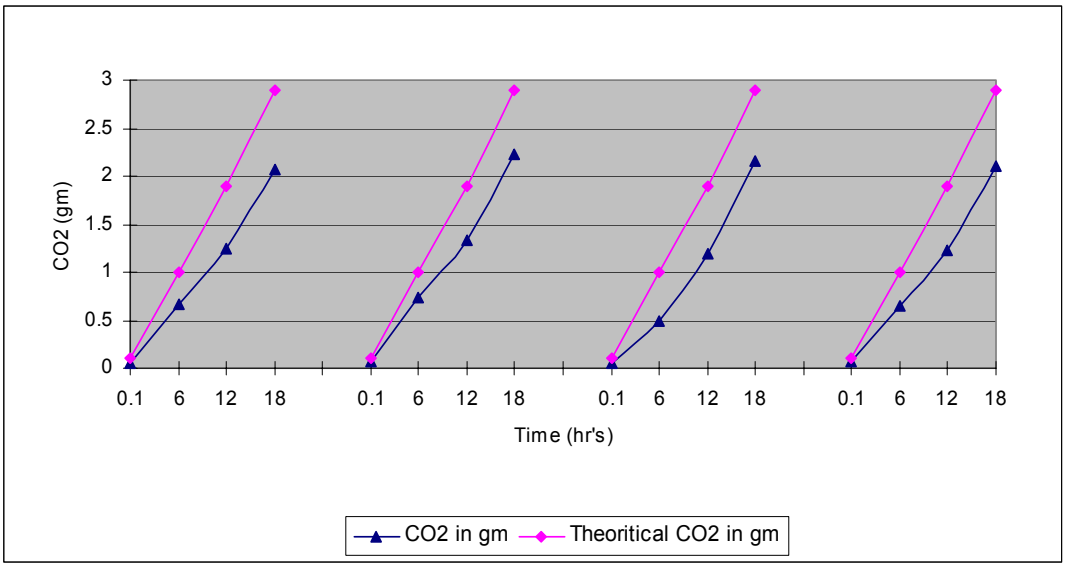

Figure 27. Level of cumulative CO_2 and theoretical CO_2 evolved after the addition of NNT to immobilized cells over 4 day period.

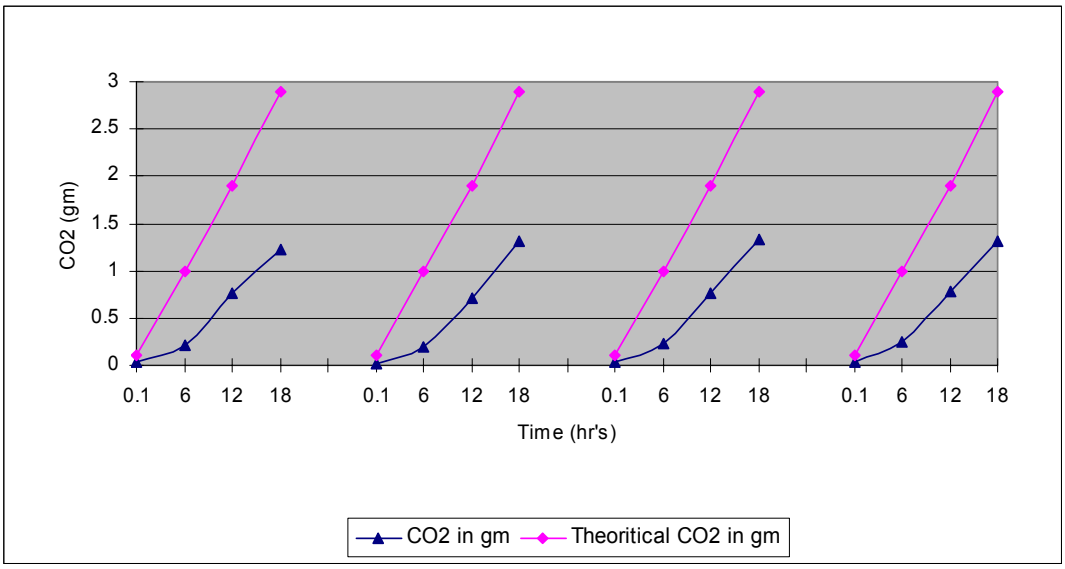

Figure 28. Level of cumulative CO_2 and theoretical CO_2 evolved after the addition of NNT to planktonic cells over 4 day period.

10- Monitoring the activity and viability of the cultures

10.1- Triphenyl-Tetrazolium chloride (TTC) dehydrogenase activity assay

Figure 29 and 30, show the growth of immobilized and planktonic cells respectively and the activity of dehydrogenase enzyme after the addition of NNT. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm. The enzyme activity in the presence of immobilized cells was shown to reach 12 mmole/min mg after 24 h, compared to 9.8 mmole/min mg after 24 h, for planktonic cells.

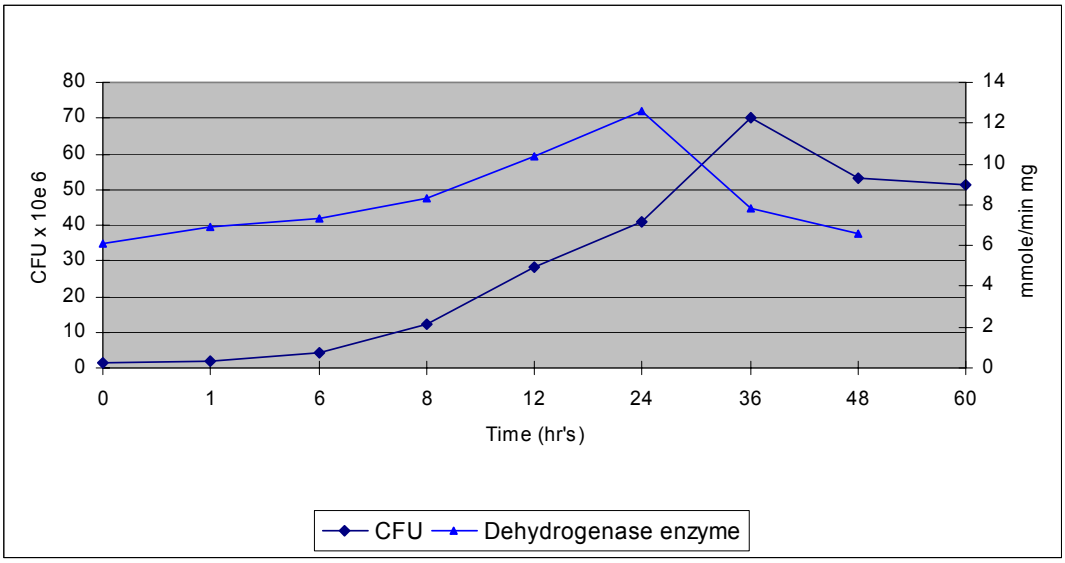

Figure 29. Growth of immobilized cells and activity of dehydrogenase enzyme after the addition of NNT. Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

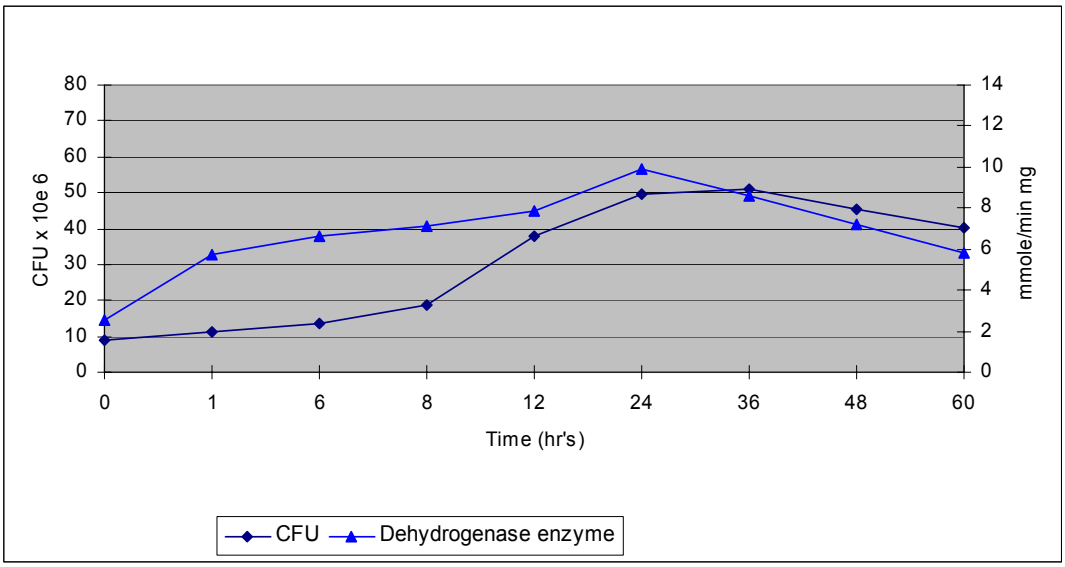

Figure 30. Growth of planktonic cells and the activity of dehydrogenase enzyme after the addition of NNT. Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

10.2- Naphthalene Oxygenase activity assay.

Figure 31 and 32, show the growth of immobilized and planktonic cells respectively and the activity of naphthalene oxygenase enzyme after the addition of NNT. The initial feed was Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm. The enzyme activity in the presence of immobilized cells was shown to reach 0.44 umole/min mg after 24 h compared to 0.4 umole/min mg after 24 h, for planktonic cells.

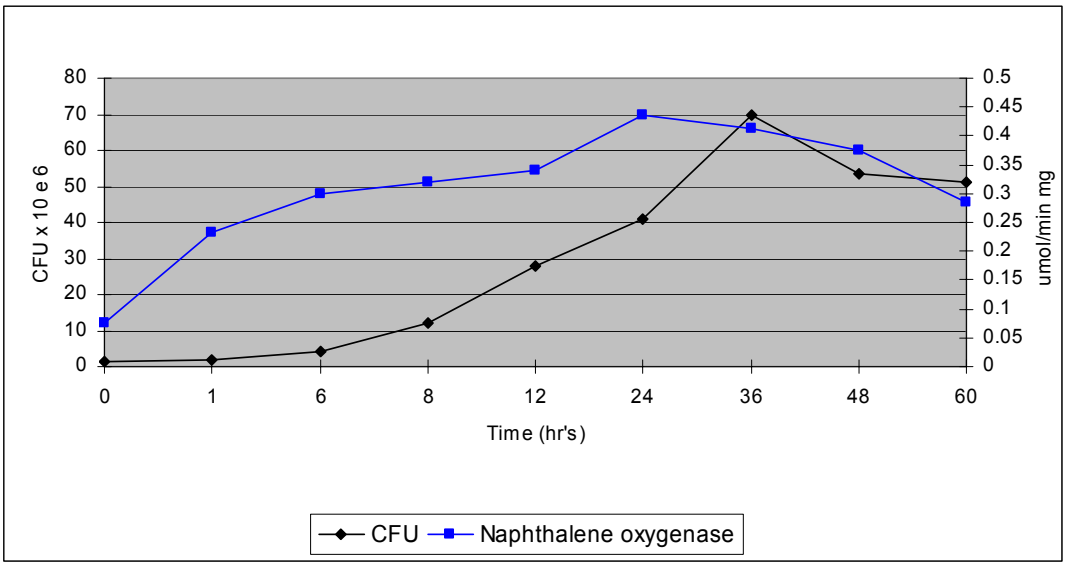

Figure 31. Growth of immobilized cells and naphthalene oxygenase enzyme activity after the addition of NNT. Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

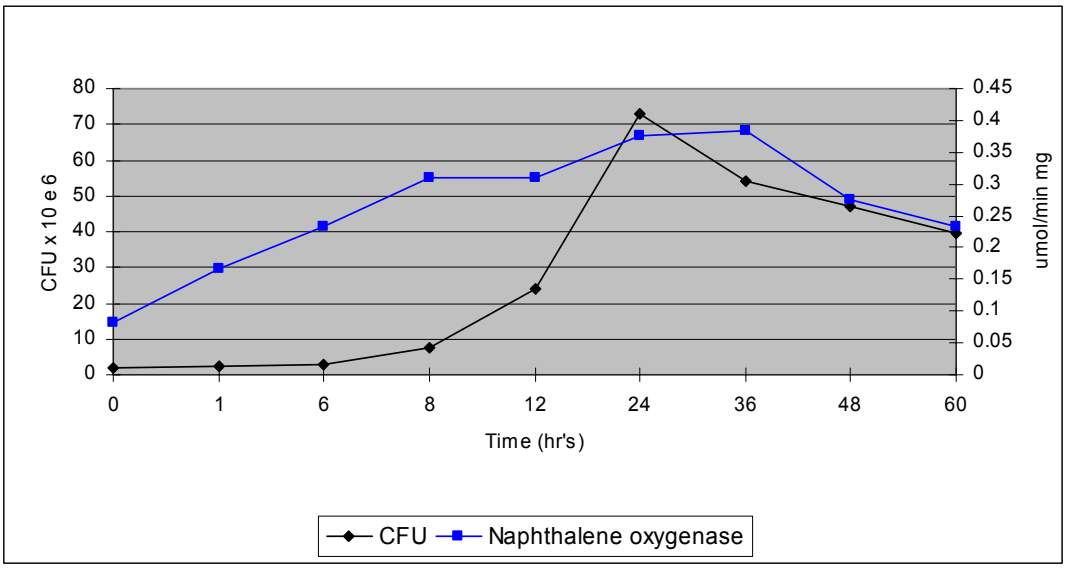

Figure 32. Growth of planktonic cells and naphthalene oxygenase enzyme activity after the addition of NNT. Naphthalene 700 ppm, Nitrobenzene 600 ppm and Toluene 700 ppm.

Discussion

This study provides a comparison between planktonic and immobilized cells of the controlled mixed culture DAP-2 (ATCC 55644) that were grown in separate Sequential Batch Reactors (SBR) using Stanier's media, to which naphthalene, nitrobenzene and toluene were added as the sole source of C and/or N.

Of the twenty cultures that were isolated primarily from the 3 liter fermentors, only isolate A was able to grow on nitrobenzene. Isolates A, B, D, E, F, H, I, and K were able to grow in the presence of naphthalene. All isolated organisms were able to grow in the presence of toluene except isolates J, P, R, and T. Only isolates A, C, G, I, L, and T were able to grow in the presence of acenaphthene, anthracene, fluoranthene and phenanthrene. On the other hand, most of the isolated organisms were able to grow in the presence of n-dodecane, hexadecane, n-pentadecane, n-tetradecane, and n-octadecane except isolates D, F, H, K, L, M, N, P, and R.

Isolates A, B, D, E, F, H, I, J, K, M, O, P, Q and S are *Pseudomonas* species, while isolates C, G, L, N, R, and T are *Aeromonas* species. All these isolates were positive for twitching motility on twitching plates. Further characterization of these is shown in Table 4.

Isolates HPRA-1, 2, 3 and 4 were isolated from the planktonic SBR, while HPRB-1, 2, 3, 4 and 5 were isolated from the immobilized-cell SBR (Table 16). Most of these new isolates, in contrast to the original cultures, were able to grow in the presence of

nitrobenzene, except HPRA-2, and 4, and HPRB 2 and 3. All of the nine new isolated organisms were able to grow in the presence of toluene, or naphthalene, or NT and NNT (with/without ammonium sulfate added). Isolates HPRA-2, 3, and 4, and HPRB-1, 2, and 3 were identified as *Pseudomonas* species. Isolates HPRA -1, HPRB-4 and 5 were identified as *Aeromonas* species. Further characterization of these is shown in Table 16.

The mixed culture was grown in Stanier's minimal media, either in the presence of different concentrations of naphthalene, nitrobenzene and toluene (NNT) or naphthalene and toluene (NT) as the sole source of C and/or N.

Figures 13 and 14 clearly show that the level of NNT-degrading bacteria increased with re-feeding in both the immobilized and planktonic SBR but the greatest increase in NNT-degrading bacteria was seen in the immobilized SBR. The observations made based upon the levels of NNT-degrading bacteria was confirmed when the extent of degradation was monitored in the two SBRs by measuring the residual hydrocarbon in the SBR and the amount of trapped volatile organic carbon (VOC), and the evolved CO_2.

Figures 19 and 20 show the degradation level of different NNT concentrations in the presence of immobilized and planktonic cells at different treatment stages. In the presence of immobilized cells (Fig.19), 90% of both Naphthalene (700-3000 ppm) and Toluene (600-1500 ppm) were degraded within 18 h, while 60% of Nitrobenzene (400 ppm) was degraded within the same time frame. On the other hand, in the presence of planktonic cells (Fig.20), only 65% of both Naphthalene (700-3000 ppm) and Toluene

(600-1600 ppm) were degraded within 18 h, while less than 50% of Nitrobenzene (400 ppm) was degraded within the same time frame.

Figures 25 and 26, show the level of cumulative evolved CO_2 in the presence of immobilized cells and planktonic cells. The results clearly show that the level of cumulative evolved CO_2 for immobilized cells was higher than planktonic cells; reaching 2.1 gm and 1.3 gm respectively. This difference further provides support that the level of NNT degradation in the presence of immobilized cells was significantly higher than of planktonic form.

Figures 27 and 28, provide a comparison of the level of observed cumulative and theoretical carbon dioxide evolved in the presence of immobilized and planktonic cells. Immobilized cells were shown to produce more than 75% of the theoretical carbon dioxide as compared to planktonic cells which produce less than 50%, of theoretical carbon dioxide evolved. This difference further provides support that the level of NNT mineralization in the presence of immobilized cells was significantly higher than of planktonic form.

The superiority of the SBR mode, employing immobilized/attached microorganisms versus planktonic cells was clearly established. Most importantly the immobilized-cell SBR system showed: 1- increased cell mass/number of specific degraders, 2- faster degradation rates, 3- ability to handle higher-levels of mixed organics, and 4- achieved lower residual levels of contaminants than the planktonic reactor.

Gas chromatographic data showed that immobilized cells were able to degrade NNT faster than the planktonic cells (Tables 17 and 18). This observation was confirmed by measuring CO_2 evolution.

Over time the loading of hydrocarbon was significantly increased from a starting level of 400 ppm (Naphthalene), 100 ppm (Nitrobenzene), and 500 ppm (Toluene), to a final level of 3000 ppm (Naphthalene), 400 ppm (Nitrobenzene), and 1600 ppm (Toluene). While increasing nutrient loading, the frequency of re-feeding with hydrocarbons also was increased from an initial re-feeding every 60 h to a final re-feeding frequency of 18 h (Tables 17 and 18).

The experiments clearly showed that the attached, mixed microbial community was able to effectively and rapidly degrade high concentrations of hydrocarbons. This demonstrating the practical advantages of employing attached, mixed microbial cultures in a SBR.

Most of the published literature, on biodegradation, is focused on the degradation of one or two pure chemicals at very low concentrations by pure cultures or a mixed culture of two or three organisms but not on the degradation of complex mixtures of organic pollutants by mixed cultures. The extent of hydrocarbon biodegradation demonstrated in this study was higher than that obtained in other studies. Limbert and Betts (1994), in their study showed, three bacterial isolates were required to treat a mixture of compounds consisted of benzene, o-xylene, nitrobenzene, naphthalene, and other chemicals at

extremely low concentrations of 15-60 ppm. Their isolates were able to degrade up to 60% of these chemicals, in 18 h. Grant *et al*. (2002) have shown how the concentration of a target chemical can influence the growth of bacteria. In their experiments, higher concentrations resulted in lower cell growth due to the increased toxicity of that chemical Arcangeli and Arvin (1992) used very low concentrations of toluene, less than 1 ppm to 6 ppm, in their study. Heitkamp *et al*. (1987) showed that it takes 17 to 31 days to effectively degrade naphthalene, when added to selected soil microcosms at levels of less than 1 ppm. Speitel *et al*. (1989) demonstrated the degradation of phenols (e.g. p-nitrophenol, 2, 4-dinitrophenol, and pentachlorophenol) at very low levels, i.e., 1-100 ppb.

These and similar studies all argue that aerobic degradation of mixtures of toxic organics is limited to dilute aqueous solutions. However, the experiments conducted at Georgia State University clearly demonstrated that a defined population of hydrocarbon degraders could be maintained stably for extended periods while at the same time, effectively degrading mixtures of toxic organics present at very high concentrations which further demonstrate the practicality of the SBR approach.

Thus the performance demonstrated by the attached, mixed culture in a SBR showed that effective microbial remediation is not limited to the treatment of dilute aqueous waste but can accommodate very high strength organic wastes.

Table 17. Improvement of the treatment process over five separate SBR treatment cycles in the presence of immobilized cells

Condition\ Stage	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
Time (h)	up to 60	up to 20	up to 18	up to 18	up to 18
Starting naphthalene conc.	**400 ppm**	**1000 ppm**	**1500 ppm**	**2000 ppm**	**3000 ppm**
Ending naphthalene conc.	30 ppm	155 ppm	200 ppm	220 ppm	470 ppm
Starting toluene conc.	**500 ppm**	**1000 ppm**	**1500 ppm**	**1500 ppm**	**1500 ppm**
Ending toluene conc.	30 ppm	160 ppm	280 ppm	300 ppm	300 ppm
Starting nitrobenzene conc.	**100 ppm**	**400 ppm**	**400 ppm**	**400 ppm**	**400 ppm**
Ending nitrobenzene conc.	70 ppm	160 ppm	160 ppm	140 ppm	130 ppm

Table 18. Improvement of the treatment process over five separate SBR treatment cycles in the presence of planktonic cells

Condition\ Stage	**Stage 1**	**Stage 2**	**Stage 3**	**Stage 4**	**Stage 5**
Feeding time	Up to 18	Up to 20	up to 18	up to 18	up to 18
Starting naphthalene conc.	**400 ppm**	**1000 ppm**	**1500 ppm**	**2000 ppm**	**3000 ppm**
Ending naphthalene conc.	320 ppm	190 ppm	400 ppm	500 ppm	950 ppm
Starting toluene conc.	**500 ppm**	**1000 ppm**	**1500 ppm**	**1500 ppm**	**1500 ppm**
Ending toluene conc.	250 ppm	210 ppm	430 ppm	420 ppm	420 ppm
Starting nitrobenzene conc.	**100 ppm**	**400 ppm**	**400 ppm**	**400 ppm**	**400 ppm**
Ending nitrobenzene conc.	90 ppm	220 ppm	220 ppm	205 ppm	200 ppm

Future direction

The major pollutants and pollution threats in the Middle East region and in Jordan in particular are marine oil spills and discharges, industrial pollutants, uncontained disposal of used motor oil, municipal and ship-based sewage, and solid waste from marine and land based sources (Hashemite Kingdom of Jordan Ministry of Municipal and Rural Affairs and the Environment, IUCN-World Conservation Union. 1991).

Jordan is a country faced with significant volume of oil sludge being produced from the Jordan Oil Refinery and disposed into ponds causing environmental problems. The oil sludge generated from the Jordanian Oil Refinery represents one of the most serious environmental problems in Jordan, and efforts are needed to develop a strategy to solve such problems. The plan is to implement the techniques used in this research to try to help the environment in Jordan and where possible to spread that technology to neighboring countries.

Since, the results achieved in this study, demonstrate that employing this technology with the use of SBR system is effective in significantly reducing the contaminants to a very low level within a short period of time, and since it also helps in reducing the capital costs, a complete economic analysis of this approach will be done. This goal requires the development of an optimal set of operating conditions, which will be determined by the laboratory measurements.

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Appendices

A. Processing of GAC trap for analysis of VOCs

Glass Column
100 ml volumetric flask
Funnel
Cotton

Procedure:

- Push a small piece of cotton into a chromatographic column, so that it closes the effluent end.
- Rinse with methylene chloride, and then make sure the valve is closed.
- Add 10 ml methylene chloride to the GAC trap and mix it well.
- Empty all the GAC with methylene chloride in the trap into the column through the funnel. Be sure to transfer all the GAC, rinsing the trap with methylene chloride if needed. After that, rinse the funnel with methylene chloride.
- Immediately drain off the methylene chloride layer into the volumetric until the methylene chloride reaches the bed level in the column.
- Add 10 mls of methanol to the column and drain off at the rate of 2-3 drops per second into the flask. Never let the liquid level go below the top of the GAC bed.
- Stop the flow when the methanol drains to the top of the bed.
- Swirl the volumetric flask to make sure all water is dissolved. If the solution turns cloudy upon swirling, add 5 mls more methanol and drain to the bed level again at 2-3 drops per second until 100 ml flask is filled to the mark.
- Stop flow.
- Remove flask, cap and mix.
- If no dilution is necessary, pipette with disposable pipette to GC vials.
- Refrigerate until ready for analysis.

(This protocol was developed according to Cytec industries protocol # 5-1/ Dec. 1994.)

B. Analysis of CO_2 evolved

The CO_2 evolved was reacted with excess of NaOH as follows:

$$2NaOH + CO_2 \longrightarrow Na_2CO_3 + H_2O$$

So we'll have in the mixture Na_2CO_3 + NaOH (because NaOH is excess)

This mixture was titrated by HCl in two stages as follows:

1) After the addition of 3 drops of phenolphthalein, titrate by HCl until colorless (record the volume of HCl used:

$$NaOH + HCl \longrightarrow NaCl + H_2O$$

$$Na_2CO_3 + HCl \longrightarrow NaHCO_3 + NaCl$$

2) After the addition of four drops of methyl orange, titrate by HCl until we get an orange color (record the volume of HCl):

$$NaHCO_3 + HCl \longrightarrow H_2O + CO_2 + NaCl$$

The difference in milliliters between the first and second endpoints is used to calculate the CO_2 present in the sample, according to the following formula:

Mass of CO_2 (gm) =

Vol. of Titrant (HCl) in L (1st to 2nd endpoint) × M of standard acid × M. wt of CO_2

C. Triphenyl-Tetrazolium chloride (TTC) dehydrogenase activity assay

- Phosphate buffer (0.1M, pH 7.8).
- 3% aqueous Triphenyl-Tetrazolium chloride (TTC) solution (filter sterilize).
- 500ug/ ml Triphenylformazan (TPF) dissolved in ethanol-acetic acid mix (19:1, v/v).
- Spectrophotometer.

Procedure:

1- Wash cells three times with phosphate buffer.
2- Prepare a cell suspension of 0.2 OD at 600nm, using phosphate buffer. The cell suspension should contain 5 mg/ml protein.
3- Pipette 3ml cell suspension into a screw capped glass test tube.
4- Add 3ml of 3% (w/v) TTC for 24h at 37 C.
5- Pipette 3 ml phosphate buffer into a test tube and add 3 ml of 3% TTC, this will act as the blank.
6- Terminate the TTC reaction with 12 ml of methanol.
7- Extract the product (triphenylformazan) by shaking for 30 min.
8- Centrifuge 10,000 x g for 20min and collect the supernatant.
9- Dilute the TPF standard stock solution (500, 200,100, 50, 25 ug/ml) and use that to prepare the standard curve.
10- Measure the absorbance at 496nm.
11- Activity should be expressed as mmol formazan /min mg protein.

D. Naphthalene oxygenase activity assay

- Phosphate buffer (0.05M, pH 7.0).
- 10mM Naphthalene (1.28 mg/ml) dissolved in ethanol.
- Spectrophotometer.

Procedure:

1- Wash cells three times with sodium phosphate buffer.
2- Prepare a cell suspension of 0.19 OD at 600nm, using sodium phosphate buffer. The cell suspension should contain 5 mg/ml protein.
3- Pipette 3ml cell suspension into a screw capped glass test tube.
4- Add 20ul of naphthalene to the cell suspension at 25 C.
5- Blank the instrument at 276 nm using cell suspension only.
6- Measure the absorbance at 276 nm (follow the decrease in absorbance due to the addition of naphthalene).
7- For calculation:
   1. Naphthalene extinction coefficient at 276 nm is $4.51 mM^{-1} cm^{-1}$.
   2. Results are expressed as umol naphthalene consumed/min/mg protein.
   3. calculation

$$\textbf{Activity } (\text{umol/min} \times \text{mg}_{\text{prot.}}) = \frac{\text{Absorbance}}{4.51 \times Vc \times [\text{Prot.}] \times T}$$

- **Vc** volume of the cell suspension.
- **[Prot.]** Prtein concentration.
- **T** incubation time.

E. Stock standard preparation

I. liquid standards:

1. Use a 1000ul syringe.
2. Add 95 ml of methylene chloride into a 100 ml volumetric flask.
3. Weigh by difference approximately 600ul of each standard into separate 100 ml volumetric flasks containing 95 ml methylene chloride.
4. Inject the standard below the liquid level and reweigh the syringe.
5. Dilute to the mark with methylene chloride and mix well.

II. Solid standards:

1. Use a glass weighing boat.
2. Add 50 ml of methylene chloride into a 100 ml volumetric flask.
3. Use a glass weighing boat and weigh by difference between 0.5000 and 0.6000 grams of Naphthalene and 2-methyl naphthalene into separate 100 ml volumetric flask, containing 50 ml of methylene chloride.

   For 2-chloronaphthalene use 0.2000 grams.

   i. Wash down weighing boat, with methylene chloride.

Important:

- Let the stock standards equilibrate to room temperature before diluting to the mark.
- The stock standards must be kept in the refrigerator at all times when not in use.
- Allow them to come to room temperature before making a working standard.
- Make new stock standards every 3 months.

F. Working standard preparation

1- Bring all stock standards to room temperature (allow an hour for this). After a half hour, open the top of each standard shortly to release pressure.
2- Fill a 100 ml volumetric flask with methylene chloride up to the neck.
3- Inject into volumetric flask (using a micro syringe) the appropriate amount of each stock standard necessary for the standard we are creating (see table 17 below for amounts). Be sure to place the syringe into the liquid while injecting.
4- Fill volumetric flask to line with methylene chloride.
5- Mix and pipette to GC vial.

Table 19. Target compounds

Target compounds	**volume**
Chloroform	100ul
Benzene	100ul
Toluene	100ul
Chlorobebenzene	100ul
Ethylbenzene	100ul
Xylene	100ul
Aniline	100ul
Nitrobenzene	100ul
Naphthalene	100ul
2-metylnaphthalene	100ul
Styrene	100ul
2-chloronaphthalene	200ul

G. Extraction and preparation of samples for GC analysis

1- place 20 ml methanol and a small magnetic bar in a 100 ml volumetric flask
2- shake the capped sample vial from the fermentor
3- Unscrew the cap. Using an eyedropper, remove enough from the sample vial content so that the eyedropper may sit in the sample vial without any of the sample spilling over.
4- Accurately weigh the sample vial, with eyedropper in it, and record.
5- Using the same eyedropper, add approximately 2.5 grams of sample to the 100 ml volumetric flask.
6- Rinse down sides of flask with methanol. Swirl.
7- Weigh the sample vial again, with the eyedropper in it, and record. Subtract to determine the sample weight. Ideal weight is between 2.3 and 2.7 grams.
8- Add approximately 60 ml methylene chloride to the 100 ml volumetric flask. Cap.
9- Stir the contents of the 100 ml volumetric flask on a magnetic stir plate for 10 minutes.
10- Using a second magnetic bar, remove the stir bar from the 100 ml volumetric flask and rinse with methylene chloride. Be careful not to exceed the 100 ml line on the flask.
11- Fill volumetric flask to 100 ml mark with methylene chloride. Cap and shake to mix contents.
12- Using a 5 ml disposable titan syringe, filter sample: pull plunger out of 5 ml syringe and place a 2um filter on the end. Pour about 4 ml of sample into syringe, replace plunger, and filter into 11 ml vial.
13- Dilute sample 5 ml in 10 ml: using a 5 ml volumetric pipet and rubber bulb, add exactly 5 ml of filtered sample to a 10 ml volumetric flask. Fill to line with methylene chloride using an eyedropper. If contents appear cloudy while adding methylene chloride, add methanol drop wise, swirling after each drop, until solution clears. ***[note: never use Drummond pipetor when using methylene chloride].***
14- Shake volumetric to mix contents.
15- Using an eyedropper or pouring, add solution up to the neck of GC vial. Cap. Refrigerate until run time.

(This protocol was developed according to Cytec industries protocol June. 1997.)

H. Stanier's Medium*

A. Stock salt solution:

EDTA	2.5g
$ZnSO_4$, $7H_2O$	10.95g
$FeSO_4$, 7 H_2O	5.0g
$MnSO_4$, 7 H_2O	1.54g
$CuSO_4$, 5 H_2O	392mg
$Co(NO_3)$, 6 H_2O	248mg
$Na_2B_4O_7$, 10 H_2O	177mg
DI H_2O	1000 ml

Add several drops of 2N H_2SO_4 to reduce precipitation. Keep away from light at 4 ºC.

B. Hutner's Mineral Base:

Nitrilotriacetic acid	10.0g (dissolve in DI H_2O neutralized with 7.3g KOH)
$MgSO_4$	14.45g (Monohydrate can be used)
$CaCl_2$, 2 H_2O	3.335g
$(NH_4)_6Mo_7O_{24}$, 4 H_2O	9.25mg
$FeSO_4$, 7 H_2O	99mg
Stock salt solution	50 ml
DI H_2O	to 1000 ml

C. Mineral Base:

Na_2HPO_4+KH_2PO_4 Buffer (1M each; pH 6.8)	40 ml
Hutner's vitamin free mineral base	20 ml
DI H_2O	1000 ml
$(NH_4)_2SO_4$	1.0g/L (N source)
CH_3COONa	10g/L (C source)
Bacto Agar	20g/L (for solid media)

* Stanier RY, Palleroni NJ, Doudoroff M. 1966. The aerobic pseudomonads: a taxonomic study. J. Gen. Microbiol. 43: 159-271.

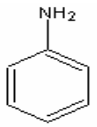
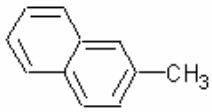
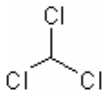
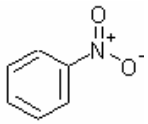
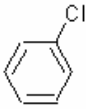
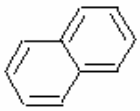
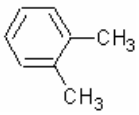
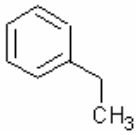
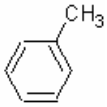
I. Description of TN medium

TN medium

Tryptone	5g
Glucose	1g
Yeast extract	2.5
NaCl	8.5
DI H_2O	1L

J. Synthetic waste

Table 18. List of synthetic waste compounds and their structure

Compound	Structure	Formula	MW
Aniline		Formula: C_6H_7N	MW: 93.13
Me-Naphthalene		Formula: $C_{11}H_{10}$	MW: 142.2
Chloroform		Formula:$CHCl_3$	MW: 119.38
Nitrobenzene		Formula:$C_6H_5NO_2$	MW: 123.11
Chlorobenzene		Formula: C_6H_5Cl	MW: 112.56
Naphthalene		Formula: $C_{10}H_8$	MW: 128.17
O-Xylene		Formula: C_8H_{10}	MW: 106.17
Ethylbenzene		Formula: C_8H_{10}	MW: 106.17
Toluene		Formula: C_7H_8	MW: 92.14