REPUBLIQUE ALGERIENNE DEMOCRATIQUE ET POPULAIRE
MINISTERE DE L'ENSEIGNEMENT SUPERIEUR ET DE LA RECHERCHE SCIENTIFIQUE

UNIVERSITE 8 MAI 1945, GUELMA

FACULTE DES SCIENCES DE LA NATURE, DE LA VIE ET SCIENCES DE LA TERRE ET DE L'UNIVERS

DEPARTEMENT : ECOLOGIE & GENIE DE L'ENVIRONNEMENT

THESE

Présentée en vue de l'obtention du

Diplôme de Doctorat en Biologie

Option : Microbiologie

THEME

Contribution à l'étude bactériologique de la biodégradation et de la bioremédiation des déchets et des produits industriels

Présentée par : Mme. CHEKROUD Zohra

Soutenu devant le jury

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To my Ummah

To my mother, God rest her soul

To my family, may God bless her

For a hope of a healthy and clean world

Thanks to Allah who helped me to succeed in this work

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Chekroud Zohra

Skikda

ملخص

تم اختبار قدرة عزلتان بكتيريتان عزلتا من مناطق ملوثة بالهيدروكربونات في مدبنة سكيكدة إضافة إلى تجمع بكتيري عزل من منطقة ملوثة بالهيدروكربونات في مدينة الإسكندرية (مصر) على تكسير البترول في وسط بحري اصطناعي. العزلات البكتيرية المختارة قامت بتكسير 81 – 90% من البترول بعد 15يوما . العزلتان المحليتان تم تعريفهما على أنهما *Pseudomonas* sp. S و *Rhodococcus* sp. S أما التجمع البكتيري فيضم *Bacillus sp. S, Acinetobacter sp. S* و *Aerobacter sp.* S .

استعمال اليوريا المحلية كمصدر للنيتروجين مع مخصب فوسفوري محلي كمصدر للفوسفور حسن قدرة *Pseudomonas* sp. S و *Rhodococcus* sp. S على تكسير البترول في حين كان له تأثير سلبي بسيط على التجمع البكتيري. تم أيضا دراسة تأثير النسبة نيتروجين\ فوسفور على تكسير البترول , وجد أن النسبة 10\1 لها تأثير ايجابي على تكسير البترول بواسطة *Rhodococcus* sp. S. و التجمع البكتيري في حين لم يكن لها تأثير واضح على *Pseudomonas* sp. S. اختبر تأثير الرقم الهيدروجيني على د رجة تكسير البترول باستخدام العزلات المختارة عند تركيزات مختلفة (4, 6, 7, 8 و 9) و أوضحت النتائج قدرة العزلات قيد الدراسة على النمو الجيد عند الرقم الهيدروجيني 7,6 و 8 و كان للبيئة الحامضية تأثيرا سلبيا على كل العزلات . بإضافة بعض أنواع surfactants تم الحصول على درجة تكسير 88,5-96,5%. تم رفع تركيز البترول من% 1 إلى2 ,3, 4, 5و% 6 في وجود Triton X- 100, وجد أن العزلات البكتيرية استطاعت تكسير ما بين87-%90من 2% من البترول بعد حوالي 15 يوما. تثبيت الخلايا البكتيرية على قش القمح أو تقييدها في الألجينات مكننا من تقليص مدة التكسير من 15 يوما إلى 9 أيام في حالة الخلايا المقيدة في الألجينات و 12 يوما في حالة الخلايا المثبتة على قش القمح.

بما أن التجارب المعملية لا تعكس بالضرورة الظروف الحقلية, تم اختبار قدرة العزلات البكتيريةا على تكسير البترول في مياه البحر الملوثة. تمت دراسة إضافة هذه الكائنات إلى ماء البحر, إضافة المغديات و قدرة بكتيريا البحر على تكسير البترول دون أية إضافات. استخدمت نترات الأمونيوم كمصدر للنيتروجين و فوسفات الصوديوم الأحادي و فوسفات الصوديوم ثنائي الهيدروجين كمصدر للفوسفور . بعد6 أسابيع كانت نسبة تكسيرالبترول تتراوح بين 70,5-95% في كل المعالجات ما عدا في حالة المياه دون أية إضافات (55,5%) .باستعمال اليوريا المحلية كمصدر للنيتروجين مع مخصب فوسفوري محلي كمصدر للفوسفور كانت نسبة التكسير تتراوح بين 77,5و 94% بعد 6 أسابيع. البكتيريا المقيدة على قش القمح لها تأثير أحسن من تأثير البكتيريا الحرة و كان أقصى متوسط تكسير للبترول في حالة البكتيريا الحرة 69,25 % أما في حالة البكتيريا المقيدة فكان 79,08% .تم قياس نشاط إنزيم الديهيدروجيناز الذي يعتبر مؤشرا لنشاط الأكسدة و ذلك في حالة البكتيريا الحرة و البكتيريا المقيدة. ووجد أن نشاط الإنزيم كان أعلى في حالة الخلايا المقيدة منه في حالة الخلايا الحرة . درجة الحرارة 22-26°م لها تأثير أفضل على نشاط الخلايا البكتيرية الحرة و المقيدة مقارنة بدرجة الحرارة 18-20°م. خلايا *Rhodococcus sp.*S و خلايا التجمع البكتيري المثبتة على قش القمح لها القدرة على تكسير(88و%91) على التوالي من 3 % من البترول وهذا في وجود TRITON X-100 وما بين 89,5 و 90% على التوالي في غيابه. قمنا بتحضير أربعة معالجات لتكبير microcosms المعملية. تم الحصول على أفضل النتائج في حالة خلايا التجمع البكتيري المثبتة. ارتفاع نسبة التكسير كان مترافقا مع ارتفاع في نشاط الديهيدروجيناز.

استعمل طحلب ينمو في مياه البحر بمدينة سكيكدة لتقييم درجة سمية البترول ووجد أن درجة السمية تنقص في مياه البحر المعالجة مقارنة مع المياه غير المعالجة.

الكلمات المفتاح: تكسير البترول, *Pseudomonas* sp. S, *Rhodococcus* sp. S , تجمع بكتيري, تثبيت الخلايا, وسط بحري, المعالجة الحيوية.

SUMMARY

Two bacterial strains isolated from hydrocarbon contaminated sites in Skikda, in addition to a consortium isolated from hydrocarbon contaminated site in Alexandria (Egypt) were tested for their capacity to degrade crude oil in a marine medium. The two local strains (*Pseudomonas* sp. S and *Rhodococcus* sp. S.) and the consortium composed of *Bacillus sp. S, Acinetobacter sp. S and Aerobacter sp. S* were able to degrade 81-90% of 1% of crude oil after 15 days of incubation

The use of local urea as nitrogen source with local phosphorus fertiliser slightly stimulated oil biodegradation by *Rhodococcus* sp. S and *Pseudomonas* sp. S and slightly inhibited oil degradation by consortium M. By adding chemical surfactants the percentage of crude oil degradation reached 88.5-96.5% with the tested organisms.

. The concentration of oil was elevated from 1 to 6 % in presence of Triton X-100. The microorganisms were able to degrade about 87-90% of 2% of crude oil after 15 days of incubation. The immobilisation of bacterial strains on wheat straw and in alginate reduced the incubation time to 12 and 9 days respectively.

Because the lab experiments in flasks do not reflect the field conditions, the organisms *Rhodococcus* sp. S, consortium M and *Pseudomonas* sp. S were selected to test their ability to bioremediate crude oil contaminated sea water. Different treatments including bioaugmentation, biostimulation and attenuation were tested. Ammonium nitrate was used as nitrogen source. Dissodium hydrogen phosphate and sodium dihydrogen phosphate were used as phosphorus sources. After six weeks crude oil degradation was between 70.5 and 95% except in the attenuation treatment (55.5%). When we used local urea with local phosphorus fertilizer the degradation percentage ranged between 77.5 and 94% after six weeks. The wheat straw immobilized cells enhanced crude oil degradation in comparison with free cells. Maximum cumulative percent of degradation in case of free cells was 69.25% whereas in case of immobilized cells it reached 79.08%. Dehydrogenase activity (Index of the total oxidative activity) was higher in immobilized cells in comparison with free cells. The temperature range (22-26C°) enhanced the activity of both immobilized and free cells in comparison with the range (18-20C°). The immobilized cells of *Rhodococcus* sp. S and consortium M could degrade 88 and 91% respectively from 3 % of crude oil in presence of Triton X-100 after four weeks. In absence of Triton X-100 they could degrade 89.5 and 90% respectively. The scale-up of lab microcosms revealed that the best results were obtained in bioaugmentation with immobilized cells of consortium M. The increase in biodegradation of crude oil was correlated with an increase in dehydrogenase activity.

The effect of crude oil toxicity was evaluated using marine algae native to Ben Mhidi Beach (Skikda). The results revealed that bioremediated sea water had less toxicity in comparison with untreated one.

Key words: Biodegradation of crude oil, *Pseudomonas* sp. S, *Rhodococcus* sp. S, Bacterial consortium, Immobilisation, Marine medium, Bioremediation

RESUME

L'objectif de ce travail est l'étude de la biodégradation du pétrole par des souches bactériennes dans un milieu marin. Deux Souches bactériennes sont ainsi isolées dans des zones marines polluées par les hydrocarbures de la région de Skikda et un consortium bactérien a été ramené d'Alexandrie(Egypte) et dont leur effet a été testé dans un milieu marin artificiel. Apres 15 jours de traitement, les souches bactériennes locales (*Pseudomonas* sp. et *Rhodococcus* sp.) et le consortium bactérien composé principalement de *Bacillus* sp.S, *Acinetobacter* sp.S et *Aerobacter* sp.S ont dégradé de 81 à 90% du pétrole brut.

L'utilisation de l'urée comme source d'azote en association avec un fertilisant phosphorique (qui constitue la source de phosphore) a augmenté la capacité de *Pseudomonas* sp.S et de *Rhodococcus* sp.S à dégrader, mais n'a point amélioré l'efficacité du consortium bactérien. A des conditions de 10/1 du rapport azote/phosphore, *Rhodococcus* sp. S et le consortium bactérien donnent leurs meilleurs résultats. Ces bactéries sont actives à pH: 6, 7 et 8 et le sont moins à pH acide (pH 4) et basique (pH 9). En ajoutant des surfactants les bactéries ont pu dégrader de 88,5 à 96,5% du pétrole. L'ajout du Triton X-100 a permis d'augmenter la concentration du pétrole de 1 à 6%, où ces souches bactériennes ont pu dégrader de 87- 90% de 2% du pétrole après 15 jours de contact. Enfin, en fixant ou immobilisant les bactéries sur les téguments ou les pailles du blé dur et sur les alginates, la durée de dégradation a été réduite à 12 et à 9 jours respectivement.

Les expérimentations au laboratoire ne reflètent aucunement les conditions du terrain, d'ou l'utilité de tester la capacité des souches bactériennes sur la dégradation du pétrole dans le milieu marin pollué. Différents traitements ont été testés (bioaugmentation, biostimulation et attenuation). NH_4NO_3 a été utilisé comme source de nitrogène, Na_2HPO_4 et NaH_2PO_4 ont été utilisés comme sources de phosphore. Après 6 semaines, la dégradation du pétrole varie de 70,5 à 95% contre 55,5% pour l'atténuation. L'utilisation de l'urée locale en association avec un fertilisant phosphorique local a donné des taux variant entre 77,5 et 94% après 6 semaines. Les bactéries immobilisées sur les téguments (pailles) de blé ont donné de meilleurs résultats (79,08%) par rapport aux bactéries libres (69,25%). *Idem*, l'activité de la déshydrogénase (index de l'activité oxydative totale) était plus élevée chez les bactéries immobilisées par rapport aux bactéries libres. Ces effets sont plus apparents à 22 - 26°C pour les deux types de bactéries. *Rhodococcus* sp.*S et* le consortium bactérien immobilisés ont une capacité de dégradation respective de 88 et 91%(de 3% du pétrole) en présence du Triton X-100. En son absence, on obtient 89,5 et 90%. L'agrandissement des microcosmes était meilleur pour le consortium immobilisé. La dégradation varie proportionnellement avec l'activité des déshydrogénases.

La toxicité du pétrole a été testée sur une algue native de la plage de Ben Mhidi (Skikda). La bioremédiation des eaux marines polluées diminue sa toxicité par rapport les eaux non-traitées.

Mots clés: Biodégradation du pétrole, *Pseudomonas* sp. S, *Rhodococcus* sp. S, Consortium bactérien, Immobilisation, Milieu marin, Biorémediation

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LIST OF ABBREVIATIONS

ALC: Arabian light crude.

API: Kit Approach to Bacterial Identification

Bt: Billion tons

BTEX: Benzene, toluene, ethylbenzene and xylene.

°C: Celsius degree

CD: Cow dung

CDM: Cell dry matter

CFU/g: Colony forming unit per gram of soil

CFU/ml: Colony forming unit per milliliter of sea water

cm: Centimeter

CoA : Co enzyme A

ETS: Electron transport system

g: Gram

h:.Hour

HMW: High molecular weight

kg: Kilogram

L: Liter

M: Molar

mM: Millimol

mg: Milligram

ml: Milliliter

min: Minute

N/P: Nitrogen / phosphorus

NCBI: National Center for Biotechnology Information

nm: Nanometer

OD: Optical density

PAH: Poly aromatic hydrocarbons

p.p.p: Part per million

PUF: Polyutherene foam

PM: Poultry manure

PW: Poultry waste

rpm: Round per minute

SP: Spent oil

T: Ton

TCA: Tricarboxylic cycle acid

TCE: Trichloroethylene

TEM: Total extracted matter

THC: Total hydrocarbons content

TPH: Total poly hydrocarbons

TTC: Triphenyl tetrazolium chloride

µl: microliter

uv: Ultra violet

v/v: Volume/ volume

w/v: Weight per volume

Preface and Objectives

In the last few decades there was a revolution in the petroleum and petrochemical industries in Algeria. This led to many serious environmental problems due to the hazardous use of many petrochemical substances, in addition to the accidental spillage and disposal of crude oil and oily wastes. Skikda is an industrial town located in the North East of Algeria. It is dot of an important petrochemical industrial complex, which constitutes the principal source of hydrocarbons pollution. In fact the town of Skikda knows serious pollution problems which affect the quality of air, water and mainly sea water. It is one of the most oil spills polluted towns.

Bioremediation is being used or proposed as a treatment option at many hydrocarbon-contaminated sites. The effectiveness of bioremediation is often a function of the microbial population or consortium and how it can be enriched and maintained in an environment.

The aim of this work is to investigate the efficiency of oil biodegradation by some microorganisms in marine medium to be used in the bioremediation of oil polluted sea in Skikda, the North-east of Algeria, in case of an oil spill. The optimisation of some parameters which affect the utilisation of oil by the selected strains of bacteria was also done. This work consisted of two experimental tasks. The first part was performed in the laboratory using a) marine medium with crude oil as sole carbon source in shaked flasks and b) bioremediation of crude oil contaminated sea water in lab-scale microcosms.

The objectives of the laboratory study were to:

1. Test the potential of crude oil degradation in an artificial marine medium and in sea water
2. Evaluate the effects of co-substrates, nutrients, pH, surfactants, crude oil concentration and immobilized cells on the biodegradation rates in marine medium.
3. Evaluate the effects of bacterial population, nutrients, temperature, immobilized cells on the degradation of crude oil in contaminated sea water, and
4. Evaluate the microbial activities by dehydrogenase enzyme.

The second part involved the scale-up of the lab-microcosm on a site in the garden of the environmental direction of Skikda.

I. Introduction

I. INTRODUCTION

The quality of life on Earth is linked inextricably to the overall quality of the environment. In early times, we believed that we had an unlimited abundance of land and resources; today, however, the resources in the world show, in greater or lesser degree, our carelessness and negligence in using them. The problems associated with contaminated sites now assume increasing prominence in many countries. Contaminated lands generally result from past industrial activities (Fig.1) when awareness of the health and environment effects connected with the production, use, and disposal of hazardous substances were less well recognized than today (Vidali, 2001). The problem is worldwide, and the estimated number of contaminated sites is significant (Cairney, 1993). It is now widely recognized that contaminated land is a potential threat to human health, and its continual discovery over recent years has led to international efforts to remedy many of these sites, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the site to be redeveloped for use.

Large amounts of spills and leaks of petroleum hydrocarbons such as gasoline, diesel, kerosene, and similar materials have been refined and handled on land every year, and despite careful handling and containment there is the possibility that some may enter the soil environment. The penetration of hydrocarbons from the top of soil into subsoil presents a direct risk of ground water contamination (Morgan and Watkinson, 1989; Margesin and Schinner, 1997a). Although a significant proportion of the compounds in crude oil are relatively innocuous, a number, especially the lower molecular weight compounds are toxic or mutagenic and require remedial action to restrict environmental damage following a spill (Greer et al., 2003).

Petroleum hydrocarbons including polycyclic aromatic hydrocarbons have been categorized as priority pollutants by US Environmental Protection Agency (USEPA), Quebec Ministry of Environment (QMENV) and many other environment and health organizations in the world (Yerushalmi et al., 2003).

Over 3 billion tons of crude oil are extracted annually, and about 0.1% of this finds its way into the sea during the extraction, transportation and consumption of crude oil and petroleum products (Munn, 2004). Most of these contaminated sites are the result of leaking underground storage tanks particularly hydrocarbon storage (Cunningham et al., 2000). Due to systematic accident spills an annual release of oil into the environment in Russia accounts for 205 million tons according to estimation of green peace (Zhanovich et al., 1995). In 1996 the *Sea Empress* tanker accident led to the release of 72,000 tons of crude oil off the coast of Wales, and 85,000

tons of oil spilt in 1993 as a result to the *Brear* tanker accident off the Shetland Isles (Munn, 2004).

Water of the Mediterranean Sea is among the most exposed to hydrocarbons pollution. It is under great pressure because of industrial development. Significant quantities of industrial water loaded with thousands of tons of toxic chemicals (hydrocarbons, organics and bio accumulative toxic heavy metals, phosphates, detergents, etc.) are released annually in the Mediterranean marine ecosystem either directly or through rivers without treatment. (Boudelaa and Medjeram, 2011).

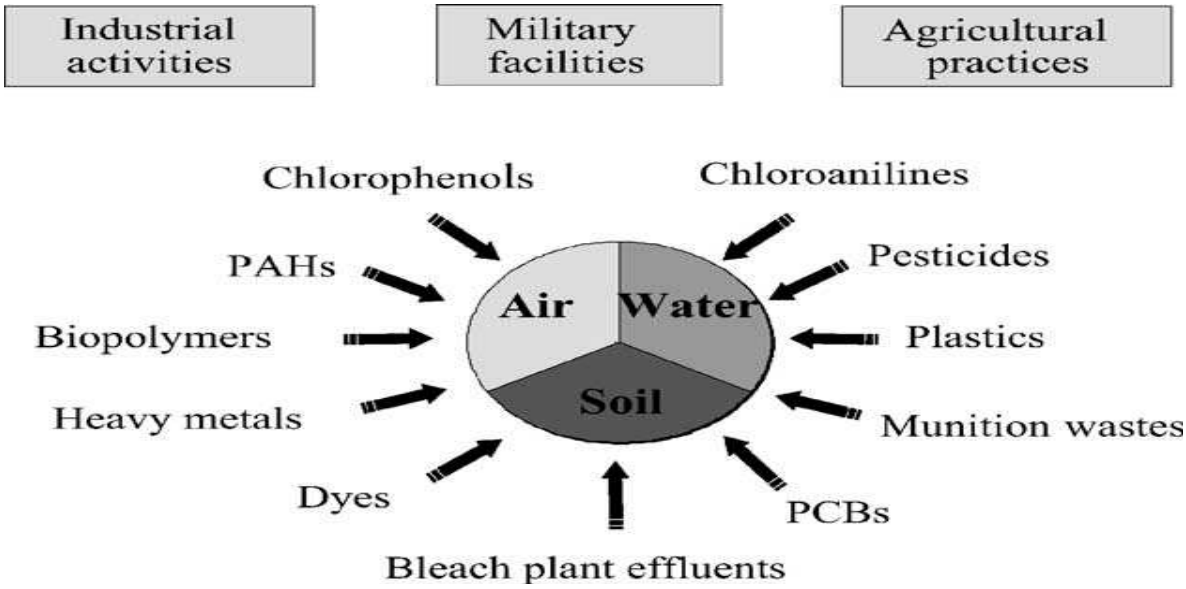

Fig.1: Pollution of the environment by inorganic and organic compounds
(After Gianfreda and Rao, 2004)

1.1. History of hydrocarbons degradation

Miyoshi (1895) published one of the earliest reports concerning the utilization of hydrocarbons. He observed that the fungus *Botrytis cinerea c*ould attack paraffin. Perrier (1913) made the first reference to a yeast utilizing hydrocarbons. He described the oxidation of aromatic compounds by moulds, among which he also classified *Torula* sp.

Bioremediation of petroleum contaminated soils has been investigated since the late 1940s (Margesin and Schinner, 1997b; Jackson and Pardue, 1998). The idea of stimulated oil biodegradation is not new, and Atlas and Bartha (1973) previously reviewed early work in this area. Furthermore, this topic received more extensive treatment in a current review (Atlas,1977). Interest in the field did not become widespread until the *Exxon Valdez* oil spill in 1989; consequently, there has been a number of studies conducted and bioremediation has almost always been found to be an effective treatment of hydrocarbon- contaminated sites (Huesemann and Moore, 1993; Li Y. et al., 1995; Zhou and Crawford, 1995; Lieberg and Cutright, 1999).

The degradation of aliphatic hydrocarbons by microorganisms has been previously reviewed especially by Klug and Markovetz (1971) and Einsele and Fiechter (1971) also the sources and behavior of oil pollutants have been reviewed by Atlas and Bartha (1973) and by the National Academy of Sciences (1975).

Mueller et al. (1989) demonstrated for the first time that the utilization of polyaromatic hydrocarbons (PAH) containing four or more aromatic rings as sole source of carbon and energy by bacteria is possible; they showed that a seven-member bacterial community isolated from creosote-contaminated soil was capable of utilizing fluoroethene. In addition, the community was capable of biotransforming other PAH in a concentration range of 0.3-2.3 mg/l when grown on fluoroethene.

During the ensuing decade, a diverse number of observations regarding the biodegradation of PAH by bacteria were published (Kanaly and Harayama, 2000). Over the past 15 years, biodegradation of high-molecular-weight PAH has been intensively studied (Kim et al., 2005). A diverse number of microorganisms, including algae, fungi, cyanobacteria and heterotrophic bacteria, play a role in PAH degradation (Atlas, 1991; Brennan and Nikaido, 1995; Pothuluri and Cerniglia, 1994; Sutherland et al., 1995).

The clean-up after the *Exxon Valdez* in 1989 allowed the first large-scale evaluation of bioremediation and the lessons learnt from that situation have provided a sound basis for future use of the technology (Munn, 2004).

The intrinsic in situ bioremediation relies on the intrinsic (i.e., naturally occurring) supplies of electron acceptors, nutrients, and other necessary materials to develop a biologically active zone and prevent the migration of contaminants away from the source. It can be used alone or in convert with an engineered bioremediation or other technology (Rittmann and McCarty, 2001).

Engineered in situ bioremediation is used to accelerate biologically driven removal of contaminants trapped in the solid phase; its success depends upon being able to achieve substantially increased inputs of stimulating materials (Rittmann and McCarty, 2001).

In ex situ bioremediation contaminated soils can be excavated and treated in aboveground or ex situ, treatment systems. It is most applicable for small, heavily contaminated sources and when a rapid site clean is desired (Rittmann and McCarty, 2001).

Bioslurry process is a system that consists of a mixture of soil in water maintained in a stirred reactor. The reduction of contaminant concentration in soil is considerably higher with that observed in solid- phase reactors because of the increased solid- liquid mass transfer (Yerushalmi et al., 2003).

The predominant ground water remediation strategy in the USA and Europe has been the application of the so-called “pump –and –treat" technology. This approach uses mainly physico-

chemical techniques to remove the pollutants in the aboveground treatment units, via for example air stripping and activated carbon while biological reactors are used in fewer than 10 % of cases. To date , probably most experience with full scale ex situ and in situ applications of bioremediation has been acquired for the biodegradation of hydrocarbons comprising straight and branched chain, saturated, unsaturated and cyclic aliphatic to mono-, di- and polyaromatic hydrocarbons. Recently, however, new types of bioreactor designs have been developed that eliminate polyaromatic solvents and aromatics as well (Vandevivere and Verstraete, 2001).

Immobilization of hydrocarbon-degrading microorganisms or nutrients in hydrocarbon-adsorbing materials such as granular clay and aquifer sand (Omar and Rehm, 1988), alginate (Li et al., 1994 & 1995), wax (Resnick, 1998), and a microcapsule system (Murakami et al., 1985) has been developed. Oh et al., (2000), tested the use of polyurethane foam (PUF) as a bioremediation technique that can minimize the dilution of applied microorganisms and nutrients in open-water systems.

The use of oleophilic compounds, which stick oil and/ or release nutrients slowly (Munn, 2004) is a strategy used in the bioremediation of oil polluted marines. Inipol^{TM} EPA22 is a microemulsion of urea in brine, encapsulated in an external phase of oleic acid and lauryl phosphate, co-solubilized by butoxy-ethanol. In the bioremediation of the *Exxon Valdez* the Inipol^{TM} EAP22 was combined with a slow-release fertilizer (Customblem^{TM}) which consists of ammonium nitrate, calcium phosphate and ammonium phosphate encapsulated in a coating polymerized linseed oil (Munn, 2004).

Genetically engineered microorganisms are an approach for providing the enzymatic capability to create microorganisms with the capacity to degrade a wide range of compounds. A hydrocarbon-degrading pseudomonad engineered by Chakrabarty was the first organism that the Supreme Court of the United States ruled, in a land mark decision could be patented (Atlas and Unterman, 1999).

1.2. Origin of petroleum hydrocarbons

The generation of the light hydrocarbon gases, methane (C_1), ethane (C_2),propane (C_3) and the butanes (C_4) occurs in three main stages: diagene (<50°C), catagenesis (50-200°C) and metamorphism (>200°C) in which only dry gas and ultimately graphite are formed (Jones III et al., 1999). During the first stage bacteria acting under reducing conditions on organic substrates in sediments form predominantly methane. According to Hunt (1979), about 82% of the methane and practically all the heavier hydrocarbon gases are formed in the next catagenic stage. Ethane, propane and butanes are formed in the temperature range from 70°C and 150°C with peak generation occurring around 120°C. In addition to time, the quantity of gaseous hydrocarbons

formed varies with the type of organic source, material which can be broadly classified as sapropelic (marine) or humic (terrestrial) (Jones III et al., 1999).

1.3. Petroleum composition

Petroleum is a complex mixture of hydrocarbons and other organic compounds, including some organometallo constituents, most notably complexing vanadium and nickel. Petroleum recovered from different reservoirs varies widely in compositional and physical properties (Van Hamme, 2003). Petroleum refinery wastewaters are made up of many different chemicals, which include oil and greases, phenols (cresols and xylenols), sulphides, ammonia, suspended solids, cyanides, nitrogen compounds and heavy metals like chromium, iron, nickel, copper, molybdenum, selenium, vanadium and zinc. Oil consists of five types of components, saturates, non-cyclic hydrocarbons (paraffins), olefinic hydrocarbons (alkenes), aromatics and non-hydrocarbons (sulphur compounds, nitrogen-oxygen compounds and heavy metals) (Cote, 1976).

1.4. The sources of hydrocarbon contamination

Petroleum contamination results from leaking above ground and underground storage tanks, pipelines, spillage during transport of petroleum products , abandoned manufactures gasoline sites, other unplanned releases and current industrial processes (Gallegot et al., 2001; Sarkar et al., 2005).

1.4.1. Tanker operations

The world production of crude oil is about 3bt/year. During unloading of the cargo, a certain amount of oil remains clinging to the walls of the compartment this may amount to as much as 800t in 2000000t tanker. Fortunately, in recent years, greater use of double-hulled tankers has reduced the amount of oil spilt in this way (Munn, 2004).

1.4.2. Marine terminals

Accidents through human error and pipeline failure are an inevitable accompaniment to loading oil on to tankers and discharging it at oil terminals. The small size of this reflects the care taken to reduce such accidents to a minimum.

1.4.3. Tanker accidents

Oil pollution has many sources. Pollution by oil tankers attracts the greatest public concern (Munn, 2004). The wrecks of the tanker *Tampico Maru* near Baja, California, the *Torrey Canyon* southwest of England, the *Ocean Eagle* in San Juan (Puerto Rico) harbor…, all have brought to public attention the ecologic consequences of oil pollution (Bartha and Atlas, 1977).

In spite of the high visibility of these spillages, it is estimated that such accidents form only a small percentage of the annual input of petroleum pollutants into the biosphere (Bartha and Atlas, 1977).

1.4.4. Non-tanker accidents

When a ship is in an accident, its fuel oil may be lost to the sea. Some cargo ships, particularly bulk carriers, are now very large and carry as much fuel as a 1960 crude oil tanker carrier, so this source of oil contamination is not negligible.

1.4.5. Off shore oil production

The oil that is extracted from the seabed invariably contains some water which must be extracted before the oil is transported to the refinery. This is done by oil separators on the platform and the oil concentration in water that is discharged in usually less than 40 parts per million (p.p.m), but in aggregate this amounts to a considerable quantity.

1.4.6. Atmosphere

The incomplete combustion of petrol or diesel in motor vehicles results in petroleum hydrocarbon being released into the atmosphere. They are washed out in rain either directly into the sea or indirectly by contributing to river run-off.

1.4.7. Municipal and industrial wastes

Domestic wastes and sewage contains a quantity of oil and greases and depending on the nature of the industry, its wastes may contain a considerable quantity of petroleum hydrocarbons. In coastal areas, these wastes are often discharged into the sea, even if subjected to treatment; the sewage sludge may retain hydrocarbons, which are then discharged to the sea.

1.4.8. Coastal oil refineries

Refineries require a large volume of water and total discharge of oil is not negligible, especially as it is continuously discharged into the same body of water.

1.4.9. Urban and river run –off

Every time it rains, iridescence caused by oil and petrol can be seen on the roads. This is washed down drains and into water courses and eventually reaches the sea. Garage forecourts sustain a large amount of spilled oil, which is washed into the drains.

1.4.10. Licensed dumping at sea

Shipping channels in estuaries and ports commonly need regular dredging. The dredging spoil, which is usually dumped at sea, is contaminated with oil. Various kinds of solid municipal and industrial wastes that are dumped at sea may also contain petroleum hydrocarbons.

1.4.11. Natural sources

Oil deposits to the earth’s surface seep out and have done so for millennia. The pitch lakes of Trinidad are the product of natural seepage and coastal oil seeps occur in many parts of the world.

1.4.12. Biosynthesis

Oil deposits are produced by plant remains that have become fossilized under marine conditions. Recent and fossil hydrocarbons have different constituents and may well have different effects on marine ecosystems but the dominating inputs of hydrocarbons from plants need to be borne in mind when assessing the effect of petroleum hydrocarbons.

1.5. Effects of hydrocarbons

1.5.1. Public health risk from oil pollution

Some petroleum hydrocarbons are toxic to humans and there are few cases on record of children being made seriously ill or even dying after inadvertently swallowing kerosene. As petroleum contains hazardous chemicals such as benzene, toluene, ethyl benzene, xylene and naphthalene. This contamination can be hazardous to the health of plants, animals, and humans (Lieberg and Cutright, 1999; Vasudevan and Rajaram, 2001; Zhou and Crawford, 1995). These chemicals pose serious health and ecological problems due to their toxicity and mutagenicity (Yerushalmi et al., 2003), some of which are known carcinogens. In 1970s there was a fear that (PAH) beheaded in much the same way as chlorinated hydrocarbons like DDT that they were resistant to bacterial attack. These compounds might concentrate in the tissues of marine organisms with the concentration increasing up to the food chain to reach the highest levels in carnivorous fish. Human consumers of these fish might therefore be exposed to relatively large amounts of these carcinogens even in the absence of over oil pollution.

1.5.2. Effect on water

It is well known that oil film contamination can cause serious damage to aquatic life as oil films retard the penetration of oxygen into water. Oil washed onto beaches also destroys foreshore marine life. If the oil is not collected and removed within a certain time after the spill, evaporation of volatiles contained in the oil will enhance the concentration of non-volatiles and thus increase the density and viscosity of the oil causing it to sink. It will then associate irreversibly with sediments including those, which support populations of economy or ecological significance (Oh et al., 2000).

1.5.3. Effect on soil structure

Hydrocarbons tend to accumulate in the pores between soil particles (Morgan and Watkinson, 1989). The sorption of chemicals into the soil is strongly dependent on the hydrophobic/hydrophilic properties of the chemical as well as on its concentration in the water phase (Verstraete and Devliegher, 1996). This results in reduced O_2 and water permeability through the soil. Viscous hydrocarbon mixtures may coat the surface of soil particles and significantly alter the binding properties of the clay minerals present and the water-holding capacity of the soil (Morgan and Watkinson, 1989).

1.5.4. Effect on soil biology

Major inputs of petroleum may limit plant growth and animal activity and this may in turn affect microorganisms: the penetration of soil by plant roots, earth worms, and burrowing animals may also transport organic material to the biologically more active surface layers of soil (bioturbation) (Morgan and Watkinson, 1989).

1.5.5. Effect on soil microbiology

Direct effects of hydrocarbons on soil microbiology have been widely studied (Morgan and Watkinson, 1989). There is normally rapid increase in the size of the hydrocarbon-metabolizing portion of the community and an increase in the number of microorganisms capable of utilizing metabolites produced by the hydrocarbon –utilizing microorganisms (Bartha and Atlas, 1977; Lode, 1986; Sextone and Atlas, 1977a).This effect is far more marked for the bacterial than the fungal population. Indeed, an equivalent rapid increase in fungal numbers is frequently not observed and although longer term population growth may occur, a short term increase biomass, rather than in individuals may be the result of hydrocarbon contamination (Loynachan, 1978; Pfaender and Buckley, 1984; Sextone and Atlas, 1977b).

1.5.6. Effect on phytoplanktons and algae

There are few studies that look at the effects of refinery effluents or its components on algae. Saha and Konar (1985) used 90 days toxicity tests on phytoplanktons. It was found that the highest concentration tested (5.48% refinery effluent) decreased the phytoplankton's growth. The refinery effluent inhibited the growth of the algae *Salarolstron apricorntum* and the duckweed *Lemma gibba*. It also reduced the germination in *Luctuca* seed by 15%. Reduced productivity of phytoplankton and/ or algae will have a knock on effect to the other organisms in the environment such as *Crustaceans* and fish (Wake, 2005).

1.5.7. Effect on the invertebrates

Many studies have used freshwater and marine invertebrates as test organisms to observe the effects of refinery effluent and its individual components. *Crustaceans* seem to be more sensitive than other aquatic organisms. Sublethal toxicity tests on invertebrates have concentrated on the changes in reproductive success (Wake, 2005). Norbert-King and Mount (1986) observed that *Ceriodaphnia* in diluted refinery wastewater produced fewer young per female than the controls.

1.5.8. Effect on fish

Fish have been used for the toxicity testing of oil refinery effluent in many different studies, most of which have looked at sub lethal effects. Many different species of fish have been tested over the years (Wake, 2005). Irwin (1965) used acute toxicity tests to determine the sensitivity of 57 species of fish to refinery wastewater. It was discovered that there was a variation

both within and between species. The guppy (*Libestes reticulates*) was the most resistant of the 57 species that were tested.

1.6. Methods of sea water remediation

In recent years, oil remediation technologies have gained considerable development. Bioremediation has appeared to be promising because it is more effective and economic with lesser undue damages to environment in comparison to several physical and chemical approaches. Bioremediation uses microorganisms to mitigate or eliminate environmental hazards and attempts to accelerate the natural biodegradation rates by modifying environmental factors. Generally, the degradation of hydrocarbon pollutants depends on the composition of the oil, on the nature of the microbial consortium, and on environmental factors that influence microbial activities (Si-Zhong et al.,2009)

1.6.1. Bioremediation

Bioremediation is a pollution treatment technology that uses biological systems to catalyze the destruction or transformation of various chemicals to less harmful forms (Atlas and Unterman, 1999; Hamman, 2004). As such, it uses relatively low-cost, low-technology techniques, which generally have a high public acceptance and can often be carried out on site. Bioremediation methods have focused on the addition of microorganisms or nutrients (Oh et al., 2000). There are different techniques of bioremediation.

1.6.1.1. In situ bioremediation

These techniques are generally the most desirable options due to lower cost and less disturbance since they provide the treatment in place avoiding excavation and transport of contaminants (Gruiz and Kriston, 1995).

1.6.1.2. Ex situ bioremediation

Bioremediation may be applied *ex situ* after contaminants and/or accompanying soils, sediments, or waters are removed from the contaminated sites (Si-Zhong et al., 2009).

1.6.1.3. Intrinsic bioremediation

It is a process whereby the natural microflora and environmental conditions exist for natural attenuation of pollutants to safe levels within an acceptable period. This is generally the first choice for biological treatment because it requires no engineered measures to increase the supply rates of oxygen, nutrients, or other stimulants (Atlas and Unterman, 1999).

1.6.1.4. Engineered bioremediation

This technique involves the introduction of engineered modified processes such as adding microorganisms and supplying nutrients. The principle of engineered remediation is to change environmental conditions for accelerating microorganisms' activity.

1.6.1.4.1. Biostimulation

Often natural microbial communities will not be able to carry out biodegradation processes at a desired rate due to limiting physical or nutritional factors (Prescott et al., 2002).

Most of the early efforts to stimulate the degradative activities of microorganisms involved the modification of oxygen, temperature or nutrients (Korda et al., 1997; Prescott et al., 2002) in the form of organic and/ or inorganic fertilizers into the contaminated site (Pankrantz, 2001), now called engineered bioremediation. Often it is found that the addition of easily metabolized organic matter such as glucose increases biodegradation of recalcitrant compounds that are usually not used as carbon and energy source (Prescott et al., 2002). Urea, sawdust, compost, manure and biosolids have been used in biostimulation (Cho et al., 1997; Namkoong et al., 2002; Rosenberg et al., 1992; Walworth and Reynolds, 1995; Williams et al., 1999).

1.6.1.4.2. Bioaugmentation

Both laboratory and field studies made to speed up existing microbiological processes by adding known active microorganisms to soils, waters, and other complex systems, in addition to stimulating indigenous microbial populations to degrade the contaminants. The microbes used in these experiments have been isolated from contaminated sites, taken from culture collections that have been previously proven to degrade hydrocarbons or derived from uncharacterized enrichment cultures (Prescott et al., 2002; Sarkar et al., 2005). For specific applications, bioaugmentation can be carried out with genetically engineered microorganisms (Vandevivere and Verstraete, 2001).

1.6.2. Laboratory studies

Laboratory studies are necessary for assessing the biodegradation potential of a site prior to initiating the process at full-scale. Laboratory studies are conducted in various ways. Generally, three kinds of tests are used: (1) pan studies, which stimulate solid-phase bioactivity; (2) flask studies that perform liquid-phase and slurry-phase biological process; (3) column studies which represent in situ bioremediation (Nelson et al., 1994).

Laboratory tests can be used to select optimal conditions for bioremediation. Several conditions are usually tested including unmodified microorganisms, nutrient amended microorganisms, and biologically inhibited conditions. These tests can measure the rate of change on the microbial populations. They provide data on the rate and extent of conversion of contaminants (Britto et al., 1994).

1.6.3. Factors affecting bioremediation

Bioremediation normally takes place in the active layer, which is on the top of permafrost; therefore, the bioremediation effectiveness mainly depends on some limitations (Si -Zhong et al., 2009).

1.6.3.1. Hydrocarbon variety and concentration

Hydrocarbon variety and concentration are factors that affect biodegradation. Hydrocarbons with low molecular weight are relatively easy to biodegrade. Branched hydrocarbons degrade more slowly than the corresponding straight-chain hydrocarbons (Baker and Herson, 1994). The concentration of hydrocarbon can affect the bioactivity and be toxic to the microorganisms (US. Environmental Protection Agency, 1985). High concentration of hydrocarbons can be inhibitory to microorganisms, thus slowing down the remediation rate.

1.6.3.2. Temperature

Temperature has a profound influence on the rate of all biochemical processes, and affects the biodegradation of hydrocarbons directly as well as indirect ways (Atlas, 1991; Bartha and Atlas, 1977). The optimal temperature for biodegradation of petroleum has generally been found to be 30-40°C (Morgan and Watkinson, 1989). However, local environmental conditions may select for a population with a lower optimal temperature (Morgan and Watkinson, 1989, Margesin and Schinner, 1997a). Zobell (1973) reported mineral oil degradation at temperatures below 0°C in low- temperature marine environment.

1.6.3.3. Oxygen

The availability of molecular oxygen has a profound effect on the biodegradation of various compounds. Oxygen is the most troublesome problem facing the in situ bioremediation for hydrocarbons and other pollutants that are biodegradable aerobically (Atlas and Unterman, 1999). Metabolism of both aliphatic and aromatic hydrocarbons normally requires the presence of molecular oxygen since the initial biochemical step is oxygenase-catalysed reaction to produce alcohol or phenol (Morgan and Watkinson, 1989). Dineen et al. (1990) reported that the requirement of oxygen to degrade hydrocarbon is 3.1 g of oxygen for 1.0 g of hydrocarbon.

1.6.3.4. pH value

It has been shown that mineralization of hydrocarbons proceeds most rapidly at pH values between 6.5 and 8.0. (Dibble and Bartha, 1979; Jones et al., 1970). According to Munro (1970) many microbes have an optimum pH for growth around 7, and most preferring the pH range 5-8 although there are many exceptions of these trends.

1.6.3.5. Bioavailability

Bioavailability is the tendency of individual oil components to be taken up by microorganisms. As for the microbial aspects, difficulties in bioavailability result from the obstacles for hydrocarbons transferring into cellulous enzymes and from limitations in energy for maintaining degradation (Si-Zhong et al., 2009).

1.6.3.6. Inorganic nutrients

Biodegradation rates can be limited by the available concentrations of various nutrients. Since microorganisms require nitrogen and phosphorus for incorporation into biomass, the availability of these nutrients within the same area as the hydrocarbons is critical (Atlas and Unterman, 1999). Consequently, it is necessary to look into the environment in which the biodegradation takes place to provide the other required nutrients (Bartha and Atlas, 1977). Commercial inorganic nitrogen-phosphorus fertilizers and defined inorganic mixtures have been employed and have been generally found to enhance degradation of spilled oils (Bossert and Bartha, 1984; Nawar, 1997; Raymond et al., 1976). Westlake et al. (1978) examined the in situ degradation of oil in a soil of the boreal region of Canada, where fertilizer containing nitrogen and phosphorus was applied to the soil; there was a rapid increase in bacterial numbers. This was followed by a rapid disappearance in n-alkane and isoprenoids.

1.6.3.7. Microbial metabolic versatility

Many microorganisms are able to degrade petroleum hydrocarbons. They are present in contaminated soil and water; most of them are aerobic organisms and can make use of organic contaminants for their growth. Metabolic activity and adaptability may range from almost zero to highly active (Morgan and Watkinson, 1989). Metabolic limitations may result from the enzyme-substrate interaction and the energy needed to activate metabolism. If the proper enzyme already exists, the degradation rate may then be determined by specific interactions of the compound with the enzyme (Schwarzenbach *et al*., 1993). Generally, the enzymatic limitations result from substrate recognition and steric hindrance of substrate because the recognition and approach are required for enzymatic catalyzing. The larger the compound size, the bigger the steric hindrance, and the more difficult the compound interaction with the active center of the enzyme (Bressler and Gray, 2003).

1.7. Metabolism of hydrocarbons

Many different microbes (at least 160 genera) capable of degrading petroleum hydrocarbons have been isolated; most of the hydrocarbon-degrading isolates are heterotrophs belonging to the *Proteobacteria* (eg. *Pseudomonas, Acinetobacter, Cycloclasticus and Alcanivorax*). Yeasts, filamentous fungi and some alga such as *Ochromonas* and *Cyanobacteria* have also been linked to hydrocarbon degradation (Munn, 2004).

The metabolism of hydrocarbons presents a variety of fermentation problems. Among these are the solubility of hydrocarbons in aqueous systems, diffusion of hydrocarbons in media, how to achieve proper mixing and emulsification and complexity of hydrocarbons (Einsele and Fiechter, 1971; Zajic, 1964).

The degradation of hydrocarbons by microorganisms occurs quite specifically. Zobell (1946) tried to formulate this specificity by four means:

1. Aliphatic compounds are more readily attacked by microorganisms than aromatic compounds.

2. Long chains are degraded preferentially as compared with short chains.

3. Unsaturated compounds are degraded more readily than straight chains.

4. Branched chains are degraded more readily than straight chains.

1.7.1. Degradation of aliphatic hydrocarbons

1.7.1.1. Oxidation of alkanes

The n-alkanes are generally considered to be the most readily degraded compounds in a petroleum mixture. Biodegradation of n-alkanes with molecular weight up to C_{44} has been demonstrated (Haines and Alexander, 1974; Omar and Rehm, 1988; Omar et al., 1990; Nawar, 1997). Three steps are involved in degradation of aliphatic hydrocarbons (Gaudy Jr. and Gaudy, 1980; Fukui and Tanaka, 1981; Einsele, 1983). The initial step is an oxidation reaction that involves molecular oxygen, and oxidation is catalyzed by an enzyme (mono-oxygenase) and leads to the formation of alcohol. The terminal methyl group is first oxidized to a primary alcohol. The alcohol then undergoes successive oxidation to form an aldehyde which is then converted to fatty acid (Fig.2). The oxidation of hydrocarbons by microbes, like other types of organic oxidation under aerobic conditions, is linked to the electron transport system of the cell. The enzymes of the electron transport system (ETS) include a number of dehydrogenases, thus dehydrogenase activity can be used as an overall measure of activity in the contaminated environment.

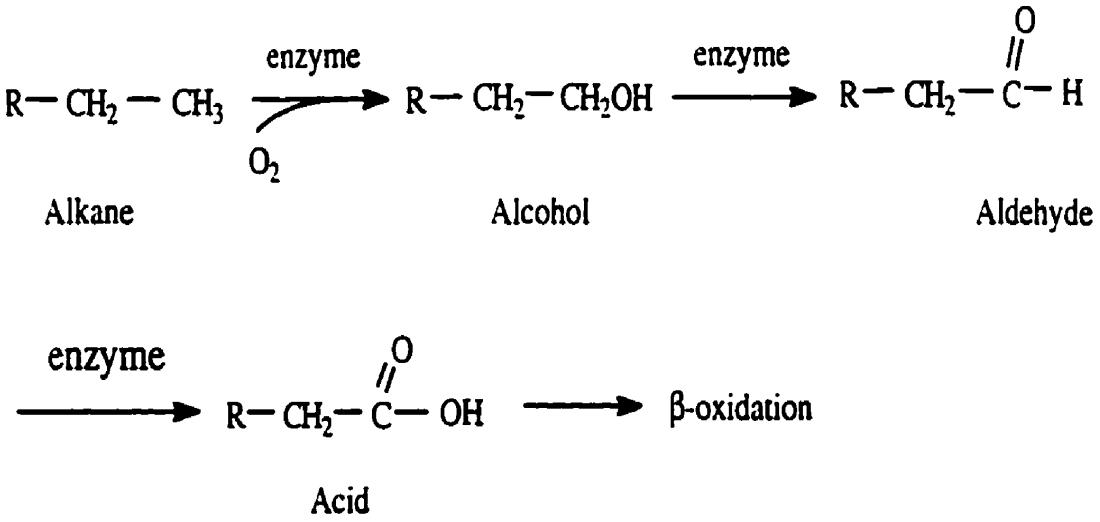

Fig.2. Degradation of aliphatic hydrocarbon (After Gaudy Jr. and Gaudy, 1980)

1.7.1.2. β-oxidation

β-oxidation (Fig.3) is an oxidized process in which the beta carbon (second carbon from the carboxyl carbon) is oxidized. The first step involved in the β-oxidation reaction is the conversion of fatty acid into acyl-CoA with an enzyme catalyzing the reaction. The acyl-CoA is converted into an unsaturated acyl-CoA by the enzyme. The unsaturated acyl-CoA is then converted into β-hydroxyacyl-CoA and then to β-ketoacyl-CoA with the mediation of the enzymes. The product is now cleaved into acetyl-CoA and fatty acid acyl-CoA by the enzyme thiolase. The fatty acid acyl-CoA, which is shorter than the original fatty acid by two carbon atoms, now goes through the same series of reaction, loosing the next two carbon atoms as acetyl-CoA. Repetition of this reaction sequence converts a fatty acid with an even number of carbon atoms totally to acetyl-CoA which enters the tricarboxylic cycle acid (TCA) (Gaudy Jr. and Gaudy, 1980; Omar and Rehm, 1980; Munn, 2004).

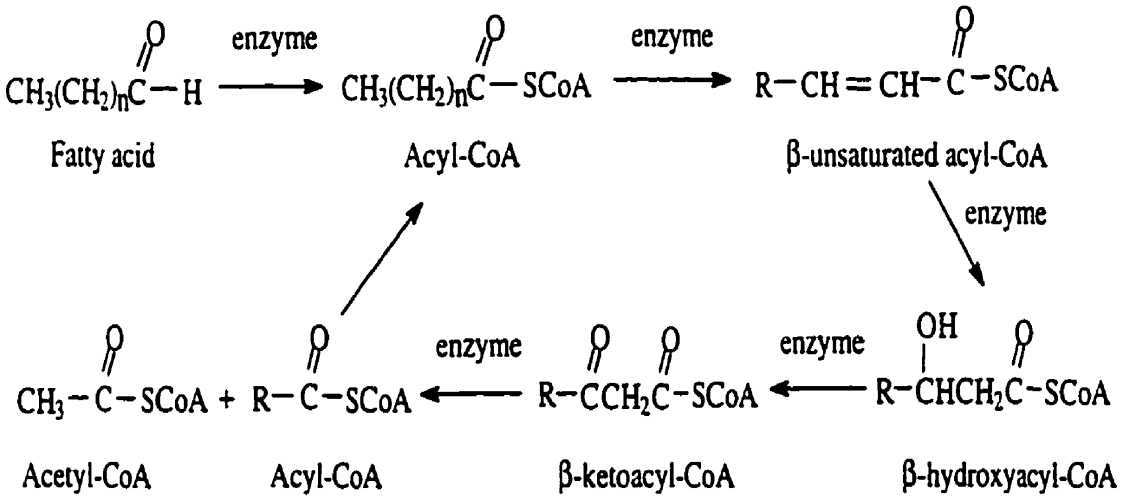

Fig.3: Degradation of fatty acid by β-oxidation (After Gaudy Jr. and Gaudy, 1980)

1.7.2. The metabolism of polycyclic aromatic hydrocarbons

Microbial degradation of (PAH) and other hydrophobic substrates is believed to be limited by the amounts dissolved in the water phase (Bosma et al., 1997; Harms and Bosma, 1997; Ogram et al., 1985; Rijnaarts et al., 1990; Volkering et al., 1992). Since bacteria initiate PAH degradation by the action of intracellular dioxygenases, the PAH must be taken up by the cells before degradation can take place (Johnsen et al., 2005). It is understood that the initial step in the aerobic catabolism of a PAH molecule by bacteria occurs via oxidation of the PAH to dihydroxydiol by a multi component enzyme system (Kanaly and Harayama, 2000). These dihydroxylated intermediates may then be processed through either an ortho cleavage type pathway or a meta cleavage type pathway by the enzyme dehydrogenase, leading to central intermediates such as

protocatechuates and catecols, which are further converted to TCA (Fig.4) (Van der Meer et al., 1992).

The degradation of PAH can serve three different functions:

1. Assimilative biodegradation that yields carbon and energy for the degrading organism and goes along with the mineralization of the compound or part of it.
2. Intracellular detoxification processes where the purpose is to make the PAH water soluble as a pre-requisite for excretion of the compounds.
2. Co-metabolism, which is the degradation of PAH without generation of energy and carbon (Johnsen et al., 2005).

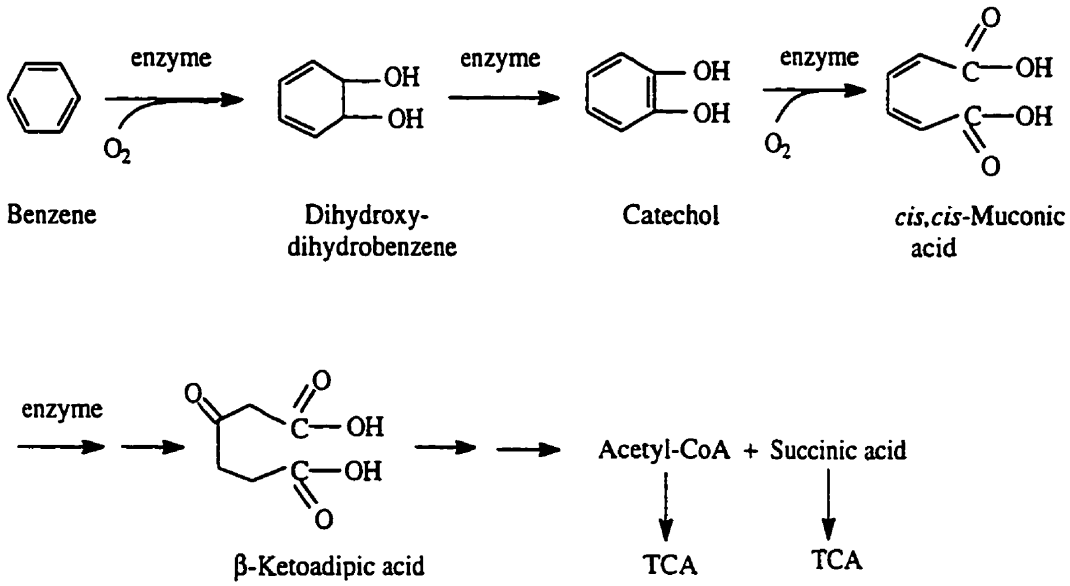

Fig.4: Degradation of typical aromatic hydrocarbon (After Gaudy Jr. and Gaudy, 1980)

1.8. Effects of surfactants on petroleum hydrocarbons biodegradation

Surfactants can affect hydrocarbon solubilization and mobilization, and influence the success of bioremediation, since the physical state of a hydrocarbon can determine its rate of biodegradation. Surfactants can increase the bioavailability and improve microbial utilization rates.

Surfactants may be added in order to facilitate the mass transfer of poorly soluble hydrocarbons into the water phase where the microorganisms live (Vandevivere and Verstraete, 2001).

Surfactants can be chemical surfactants or biosurfactants, which are produced by some microorganisms when grown on a specific substrate. These particular microorganisms enhance the bioavailability of both organic and inorganic compounds through producing biosurfactants (Champion et al., 1995).

1.9. Successful cases

A well-known example of bioremediation, which highlighted the usefulness of this treatment strategy and accelerated its development, was in the biological cleanup in the large accidental oil spill by the tanker *Exxon Valdez* in Alaska in March 1989(Si-Zhong et al., 2009). The accident spilled approximately 41 000 m3 of crude oil and contaminated about 2 000 km of coastline. Bioremediation was extensively used. Nutrient addition was used in coastal environments including beaches and marshes (Bragg et al., 1994; Wright et al., 1997). Fertilizers were typically applied on the surfaces of sand and sediments contaminated with oil, but the application was not feasible for large areas of contamination because it required huge quantities of nutrients. The study of using fertilizers in one shoreline following the *Valdez* spill resulted in a five fold increase in oil degradation (Bragg et al., 1994). Recent Alaskan bioaugmentation projects suggest that commercially available fertilizers are as or more effective than commercial bioproducts (Braddock et al., 1997).

II.

MATERIALS and METHODS

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2.1. Materials

2.1.1. Microorganisms

The microorganisms used in this research were isolated from hydrocarbon contaminated sites in Skikda: fuel station, Stora beach, Stora port, Ben Mhidi beach (post 3) and from wastes of the refinery complex (Sonatrach) in addition to a consortium of bacteria and a bacterial strain isolated from hydrocarbon contaminated sites in Alexandria (Egypt).

2.1.2. Chemicals

2.1.2.1. Crude oil

Crude oil was provided by the surveillance station; of the environmental agency (Skikda). Its source is Hassi Messaoud Petroleum Complex. Sea water was collected from Ben Mhidi beach.

2.1.2.2. Fertilisers

Urea 46% was obtained from Abou Quir fertilisers and chemical industries Company, Alexandria, Egypt. Super Phosphate 15.5% was obtained from Suez Company, Egypt. Local nitrogen and phosphorus fertilisers (local calcium ammonium nitrate 27%N and local super phosphate 20% P) in addition to local urea 32% N were obtained from ASMIDAL Company, Algeria.

2.1.2.3. Surfactants

Igepal was purchased from biomedical INC.,Tergitol from Sigma Company, Triton X-100 from BDH laboratory supplies, whereas Tween 20 and Tween 80 were obtained from Acros Organic Company.

2.2. Methods

2.2.1. Isolation and enrichment of microorganisms

For the isolation and culture of crude oil degrading microorganisms the mineral medium of Moran et al. (2000) was prepared. It consisted of a filtered sea water supplemented with the following (per litter of sea water): NH_4NO_3, 1g ; yeast extract, 0.2g ; and 4 ml of a phosphate solution containing (g/l) Na_2HPO_4, 25 ; NaH_2PO_4, 3.6.

For enrichment, 0.1% of glucose was added to the above described medium. The medium was inoculated with 1g or 1ml from the hydrocarbon contaminated samples, collected from different sites in Skikda and Alexandria (Egypt) and then incubated under shaked conditions at 120 rpm and 30°C. One ml from each flask was transferred weekly to 50 ml fresh medium with 1% (v/v) crude oil as the sole carbon source. The process was repeated for one month. At

the end of the enrichment period, serial dilutions were made for each sample, then spread on the surface of nutrient agar plates and incubated at 30°C.

2.2.2. Maintenance of stock cultures

The isolated bacteria were subcultured on nutrient agar slants. The subcultures were incubated at 30°C for 48 hours, then maintained in a refrigerator at 4°C and subcultured monthly.

2.2.3. The inoculum

Inocula were prepared by adding 5ml of 1% sterile saline solution to the bacterial cultures.

2.2.4. Cultivation

The organisms under test were cultivated in 50 ml of the sterile medium (2.2.1.) with 1% (v/v) crude oil as the sole carbon source. With the aid of a sterile pipette, 1 ml of the inoculum (2.2.3.) was transferred to each flask. In all the experiments, the flasks were incubated on a rotary shaker at 30°C and 120 rpm for the desired time mentioned for each experiment.

2.2.5. Identification of microorganisms

The bacterial strains able to degrade crude oil in a marine medium were tested for Gram reaction by Gram stain. Different standard morphological, physiological and biochemical tests were performed using API 20 kits.

2.2.6. Determination of residual crude oil in liquid medium

The residual crude oil was extracted from the flasks using the acetone- hexane (1:1) solvent (Dionex, 2004) according to the standard method for the determination of oils, grease and hydrocarbons in water as described by the American Society for Testing and Materials (D 3921-96) (1996) as follows: Ten ml of the solvent was added to 50 ml sample in 250 ml capacity capped Erlenmeyer flask, and shaked for 30 min then allowed to stand until the contents settle and the bubbles disappear. The cap of the flask was opened carefully to release any pressure build up and the contents were immediately transferred to a clean separating funnel and let to settle. The flask was washed with 10 ml of the solvent; the bottom layer was transferred into a clean 100 ml volumetric flask containing about 2g of sodium sulfate on a filter paper to absorb water. The extract was analyzed by injecting microliter volumes of extracted crude oil into a split/splitless injector of a 8000 gas chromatograph fitted with a rubber septa silver aluminum capillary column and equipped with a Flame Ionization Detector (FID). The oven temperature was kept initially at 250 ◦C for 5 min, followed by an increase to 300 ◦C at a rate of 10 ◦C /min for 20 minutes. Injector and detector temperatures were 350 ◦C. The carrier gas was nitrogen.

The degradation percent was determined using the following formula according to Bento et al., (2005).

% degradation = [(TO control – TO treatment) / TO control] x100.

Where TO control = mineral medium with crude oil and without inoculation.

TO treatment = total crude oil after degradation.

2.2.7. Determination of cell dry matter (CDM)

The cells pellets were separated from the medium by centrifugation at 14,000 rpm for 15 min, washed with hot distilled water several times, and finally dried in an oven at 70°C over night. The CDM was then determined gravimetrically.

2.2.8. Determination of bacterial growth

The bacterial growth in crude oil contaminated marine medium under different conditions was determined using two approaches:

a. measuring bacterial density as increase in optical density at 600nm using a UV mini 1240, Shimadzu spectrophotometer

b. determination of protein content

2.2.9. Determination of protein content

Cells pellets were suspended in 300 ml of 1 M NaOH. The mixture was centrifuged at 14,000 rpm for 10 min. 100 µl of the supernatant were used for protein determination according to Lowry et al. (1951) using the following reagents:

Lowry A: 20 g Na_2CO_3 and 0.55 g Na-tartarate in 900 ml distilled water + 100 ml (1M NaOH)

Lowry B: 0.1 % $CuSO_4.5\ H_2O$

Lowry C: 9 ml Lowry A + 1 ml Lowry B

Lowry D: 1:2 folin reagent in distilled water

Procedure:

100µl of the sample was mixed with 1 ml of Lowry (C) for 10 min at 30°C. 100µl of Lowry (D) were added and incubated for 30 min at the same temperature. The absorbance of the reaction was measured at 690 nm. The concentration of protein in the sample was determined from the slope of a standard curve, which was previously estimated using bovine serum albumin.

2.2.10. Determination of nitrogen and phosphorus content of manure:

The nitrogen and phosphorus content of manure was determined at the laboratory of the surveillance station, the direction of environment (Skikda) according to Rodier (1996).

2.2.11. Chemical composition of sea water

The chemical composition of sea water was determined in the laboratory of the surveillance station, the direction of environment according to Rodier (1996).

2.2.12. Microbiological characteristics of sea water

The total heterotrophic microorganisms existing in sea water were enumerated by the spread plate method. This procedure can be conducted by preparing a serial dilution of sea water and spreading an aliquot of dilution on the surface of marine agar plates. Agar plates were incubated at 30°C for a week and the number of microorganisms present was expressed as (CFU/ml). On the other hand the total degrading microorganisms were enumerated by spreading an aliquot of dilutions on the surface of marine agar containing crude oil as the sole carbon source.

2.2.13. Bioremediation treatments of crude oil contaminated sea water:

Three treatments were carried out to evaluate the efficiency of crude oil degradation in sea water, using sterilized sea water as control. The treatments were: 1) natural attenuation (sea water natural ability to degrade the contaminant; 2) biostimulation (adding nutrients to improve the natural biodegradation rate) and 3) bioaugmentation (addition of a specific bacterial strain isolated from hydrocarbon contaminated site plus nutrients).

2.2.14. Microcosm description

The experiment was performed in plastic pots (23 cm x 17cm x 12 cm). Each pot containing 1 L of filtered sea water was supplemented with 10 ml of crude oil. (Fig.5).

The bacterial isolates *Pseudomonas* sp. S and *Rhodococcus sp.S* and consortium M which had the ability to degrade crude oil in marine medium were tested. These organisms were cultivated in the marine medium with 10 g/l glucose as carbon source and incubated for 24h under shaked conditions at 30°C and 120 rpm. At the end of incubation period, the cultures were centrifuged at 1400 rpm for 10 minutes.

For bioaugmentation treatments the pots previously described received 80 x 10^5 cell/ml of bacterial cells. A consortium of the bacterial cells was also prepared. Each pot was supplemented with ammonium nitrate, disodium hydrogen phosphate and sodium dihydrogen phosphate as the following: NH_4NO_3, 1g; and 4 ml of a phosphate solution containing (g/l) Na_2HPO_4, 25 ; NaH_2PO_4, 3.6. N/P ratio was adjusted to 10/1.

For biostimulation test, pots containing 1 L contaminated sea water with natural flora were supplemented with nitrogen and phosphorus sources as the following (per litter of sea water): NH_4NO_3, 1g; and 4 ml of a phosphate solution containing (g/l) Na_2HPO_4, 25 ; NaH_2PO_4, 3.6. The N/P ratio was adjusted to 10/1.

For attenuation of the natural flora, pots containing 1 L of sea water supplemented with 10 ml of crude oil were only aerated.

Sterile crude oil contaminated sea water (autoclaved at 121°C for 30 min) was performed to test the abiotic effect on crude oil degradation, which served as control. In all the treatments the pots were continuously aerated to provide sufficient oxygen.

Sampled were taken after 1, 2, 3, 4, 5 and 6 weeks to measure the residual crude oil, pH, temperature, the total heterotrophic bacteria and the total crude oil degrading bacteria.

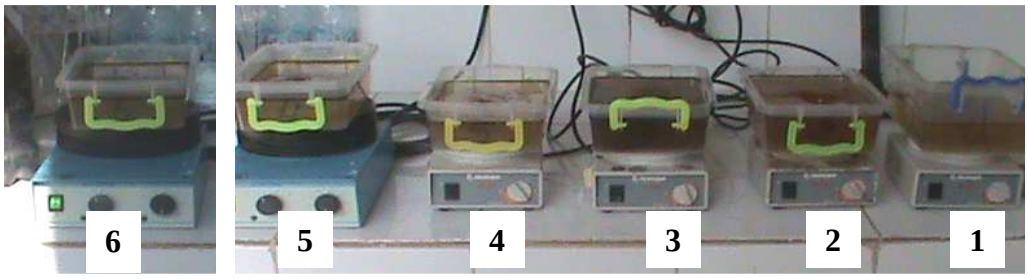


Fig. 5: Microcosms containing 1 L of sea water and 1% (v/v) of crude oil

1) Bioaugmentation by *Rhodococcus* sp. S; 2) Bioaugmentation by consortium M ; 3)Bioaugmentation by *pseudomonas* sp. S; 4) Biostimulation; 5) Attenuation; 6) Control

2.2.15. Immobilisation of cells on wheat straw:

Immobilised cells on wheat straw were prepared according to the method of Gouda et al. (2007a&b) with some modifications. Wheat straw (2, 20 and 200g) cut into small pieces (2 cm) and nutrient broth (50, 500 and 1000ml) were sterilized at 120°C for 20 min and then inoculated with 5, 50 and 100 ml of 24 h old seed culture (cultivated on nutrient broth medium). The flasks were incubated for 48h. At the end of incubation time, the nutrient broth was decanted and the straw immobilized cells was washed with sterile distilled water 2-3 times. The wheat straw immobilised cells (2 g in each flask) were then used to inoculate 50 ml of marine medium containing 2% (v/v) of crude oil and 1% of the surfactant Triton X-100 and incubated for different incubation periods (3, 6, 9, 12 and 15 days). Control uninoculated flasks were also prepared to test the probability of crude oil adsorption on straw. For microcosms experiment 20 g of rice straw with immobilized cells were mixed with 1L of crude oil contaminated sea water. The scale- up of lab microcosms was performed using 200 g of rice straw with immobilized cells.

2.2.16. Immobilisation of cells by entrapment in alginate

3% of alginate was prepared by dissolving 3g sodium alginate in 85 ml distilled water and sterilised at 100-110◦C for 10 minutes. After cooling at room temperature, 15 ml bacterial suspension was added to the sterilized alginate solution, mix well. 10 ml of the alginate -

bacterial suspension was added dropwise to 100ml of sterilized calcium chloride. The beads were left for 1 hour in calcium chloride solution, then filtered and the alginate beads were washed by sterilized distilled water several times. The alginate beads of each flask were added to the marine medium, which contain 2% of crude oil and 1% of Triton X-100. The flasks were incubated at different incubation periods (3, 6, 9, 12 and 15 days). A control uninoculated flask was also prepared.

2.2.17. Determination of dehydrogenase activity

To determine dehydrogenase activity in sea water samples the method of Admaski et al. (2000) was used with some modifications: 10 ml of crude oil contaminated sea water samples were incubated with TTC (1.5 g/100 ml buffer phosphate, pH 7.5, 0.2 M) for 24 h. After incubation the sea water samples with TTC were centrifuged. The supernatant containing the red triphenyl formazan was decanted. The samples containing the red triphenyl formazen were suspended in ethanol to extract the red triphenyl formazen (Alef and Nannipieri, 1995). The sea water was centrifuged and the supernatant added to the previously decanted supernatant containing the red triphenyl formazan and measured at 546 nm using a UV mini 1240, Shimadzu spectrophotometer.

2.2.18. Scale-up of lab microcosms

The scale-up of the lab microcosms was performed in the garden of the surveillance station, the direction of environment (Fig.6). Sterilized contaminated sea water without any addition was used as control and four treatments were prepared in pots of 40 cm of diameter as follows:

1) Bioaugmentation with immobilized cells of *Rhodococcus* sp. S, 2) bioaugmentation with immobilized cells of the consortium M, 3) biostimulation and 4) attenuation. Local urea and local phosphorus fertilizer were used as nitrogen and phosphorus sources, N/P ratio was adjusted to 10/1. Each treatment was performed with 10L of contaminated sea water supplemented with 300 ml of crude oil (3% V/V).

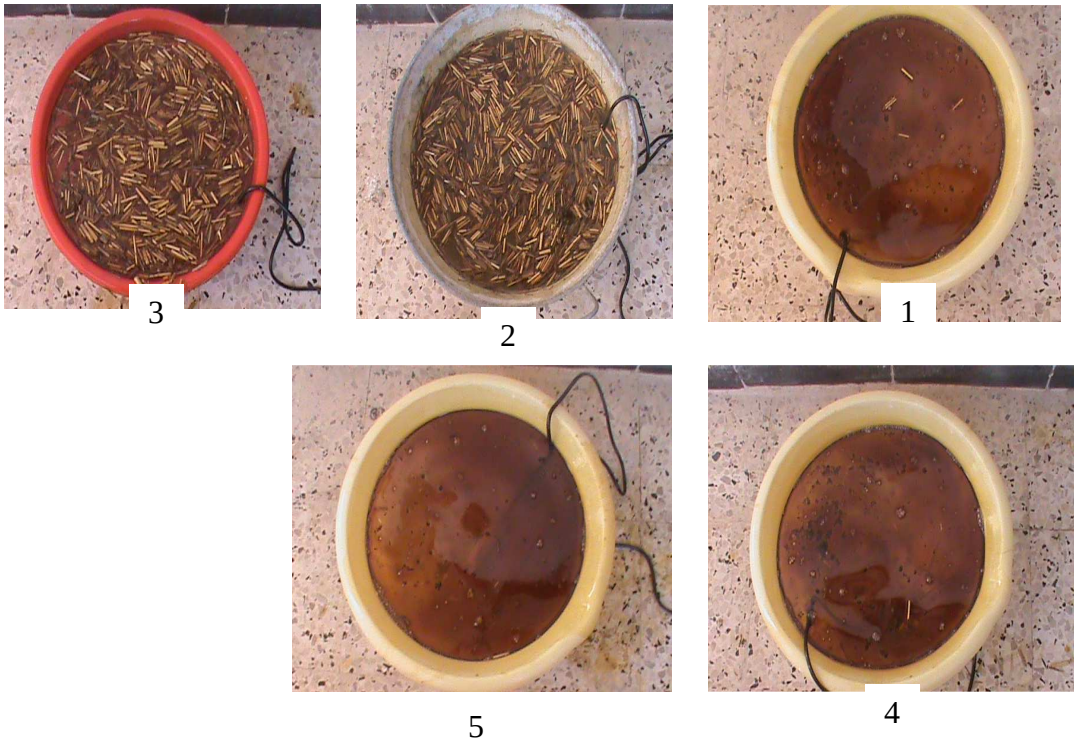


Fig.6: Scale-up of lab microcosms.

1) Control, 2) Bioaugmentation by *Rhodococcus* sp. S, 3) Bioaugmentation by consortium M, 4) Biostimulation, 5) Attenuation

2.2.19. Cultivation of *Algae* in crude oil contaminated and bioremediated sea water for determination of crude oil toxicity

The experiment was performed over a period of seven days in the surveillance station at the environmental agency. A local algae growing in Ben Mehidi beach at Sikkda was collected, washed with distilled water and put on a filter paper to absorb water. About ten grams of the algae was cultivated in each of the four pots containing 1L of crude oil contaminated sea water; after treatment for five weeks, in addition to a pot with untreated sea water. A control sample was also prepared by cultivating ten grams of the same algae in a pot containing the algae's native source seawater. The pots were continuously aerated and exposed to sun light. The growth of the algae was evaluated by dry weight determination at the end of the experiment.

2.2.20. Statistical analysis of data

Experiments were conducted using two independent replicates. Data were subjected to analysis of variance (ANOVA) at $p \leq 0.01$ according to Steel and Torrie (1980)

III.
RESULTS
PART I: Flasks Study

III. RESULTS

Part I. flasks study

3.1. Isolation of crude oil degrading microorganisms

Thirty microorganisms were isolated from different hydrocarbon contaminated sites in Skikda (2.1.1.). Twenty eight were bacteria (B), and two fungi (F) in addition to a natural consortium (M) and a bacterial strain (VA) isolated from hydrocarbon contaminated site in Alexandria (Egypt).

3.2. Selection of the best crude oil degrading organisms

To select the best organisms, which have the ability to degrade crude oil in marine medium, all isolates, were cultivated in 50 ml of artificial marine medium supplemented with 1 (v/v) crude oil in Erlenmeyer flasks (250 ml capacity). The cultures were incubated at 30°C for 15 days. From all isolates, three bacteria (O, R and V) were able to grow in the presence of crude oil, in addition to the consortium (M) and the bacterial strain (VA) isolated from Alexandria. The four isolates, in addition to consortium M and a new prepared consortium C (consisted of isolates R, O and V) were recultured on the same medium with 1% (v/v) crude oil for 7 and 15 days. The results presented in Table 1 and Fig.7 showed that the highest degradation was recorded for consortium M, followed by isolate O, then isolate V and finally consortium C after 15 days. Moderate degradation was obtained in the presence of organisms R and VA after the same time. It was also observed that the degradation rate decreased with increasing the time of incubation from 7 to 15 days all the isolates.

Fig. 8 presents the chromatographic patterns of crude oil biodegradation in a marine medium by the tested organisms. The sharp peaks shown in the crude oil (Fig. 8) representing the n-alkanes and the peaks between them comprise the naphthenes and aromatics having similar molecular weight to the adjacent n-alkanes. It was noted that the chromatographic profiles of n-alkanes had a different degradation pattern after 15 days in comparison with the zero time and after 7 days of incubation. The fraction C4- C13 is rapidly degraded by all organisms. Above C13 the degradation pattern differs according to the organisms under test. The consortium M showed the highest degradation pattern of the fraction above C13, where as the lowest one was recorded in case of the organism R. In general we observed that the degradation pattern envisaged that n-alkanes are easily degraded at higher rate than both naphthenes and aromatics. In addition, the lighter part of crude oil was also found to be degraded faster than the heavier one.

The growth of the tested organisms was evaluated in terms of optical density (OD) and intracellular protein content. The results presented in Table 2 showed that the highest optical

density was recorded for consortium M after 15 days of incubation (0.57) whereas the lowest value was measured for consortium C after 7 days of incubation (0.24). It was also observed that the OD values increased by increasing the incubation time.

The results presented in Table 3 showed that the consortium M had the highest protein content after 15 days of incubation (2.55mg). Increasing the incubation time increased the protein content of the tested organisms, except for isolate R which showed the lowest protein (0.90 mg) content after the same incubation time.

Generally, it was noted that the percent of degradation after 15 days of incubation was correlated with the total protein content with some fluctuations for isolate R (Fig. 9).

Table 1: Effect of incubation periods on crude oil biodegradation using different organisms

Incubation time Organism	7 days	15 days
	% crude oil degradation	
VA	45.00% ± 1.00 **D** **(6.43)***	60.00% ± 2.00 **E** **(4.00)**
R	35.00% ± 1.00 **E** **(5)**	53.50% ± 0.50 **F** **(3.7)**
O	50.00% ± 1.00 **B** **(7.14)**	86.75% ± 1.25 **AB** **(5.78)**
Consortium M	60.75% ± 1.25 **A** **(8.68)**	90.00% ± 1.00 **A** **(6.00)**
V	48.25% ± 0.5 **C** **(6.89)**	81.00% ± 0.50 **CD** **(5.40)**
Consortium C	46.25% ± 0.75 **CD** **(6.61)**	79.50% ± 0.75 **BC** **(5.30)**

Same capital letters are statistically not different among treatments at $p<0.01$± standard error (n=2)
*The degradation rate = % degradation / time of incubation

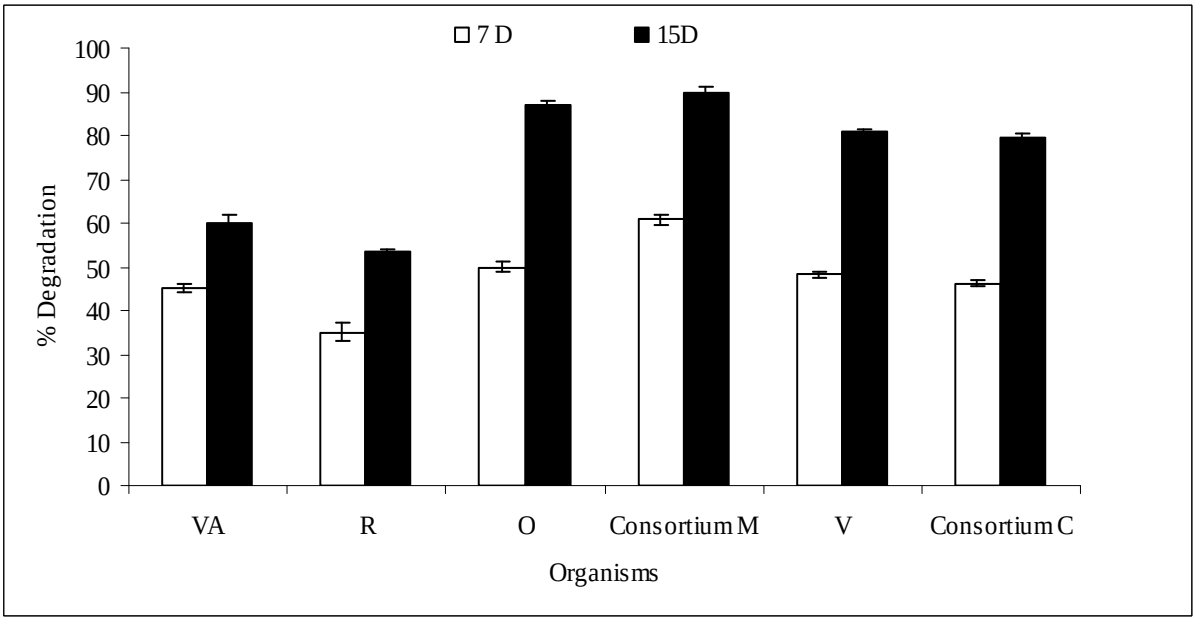
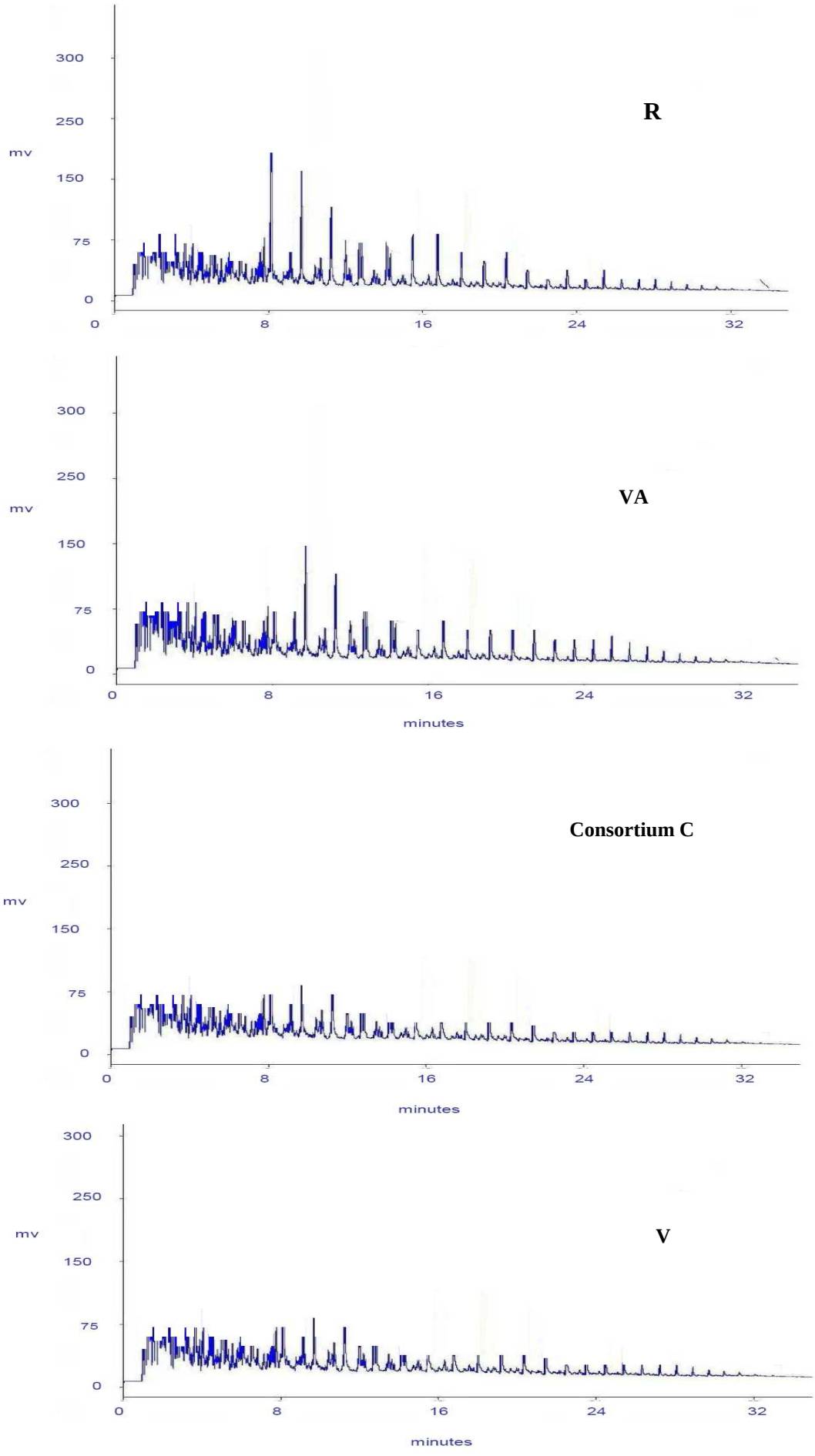


Fig.7**:** Effect of incubation periods on crude oil biodegradation using different organisms


R
VA
Consortium C
V
mv
300
250
150
75
0
0
8
16
24
32
minutes

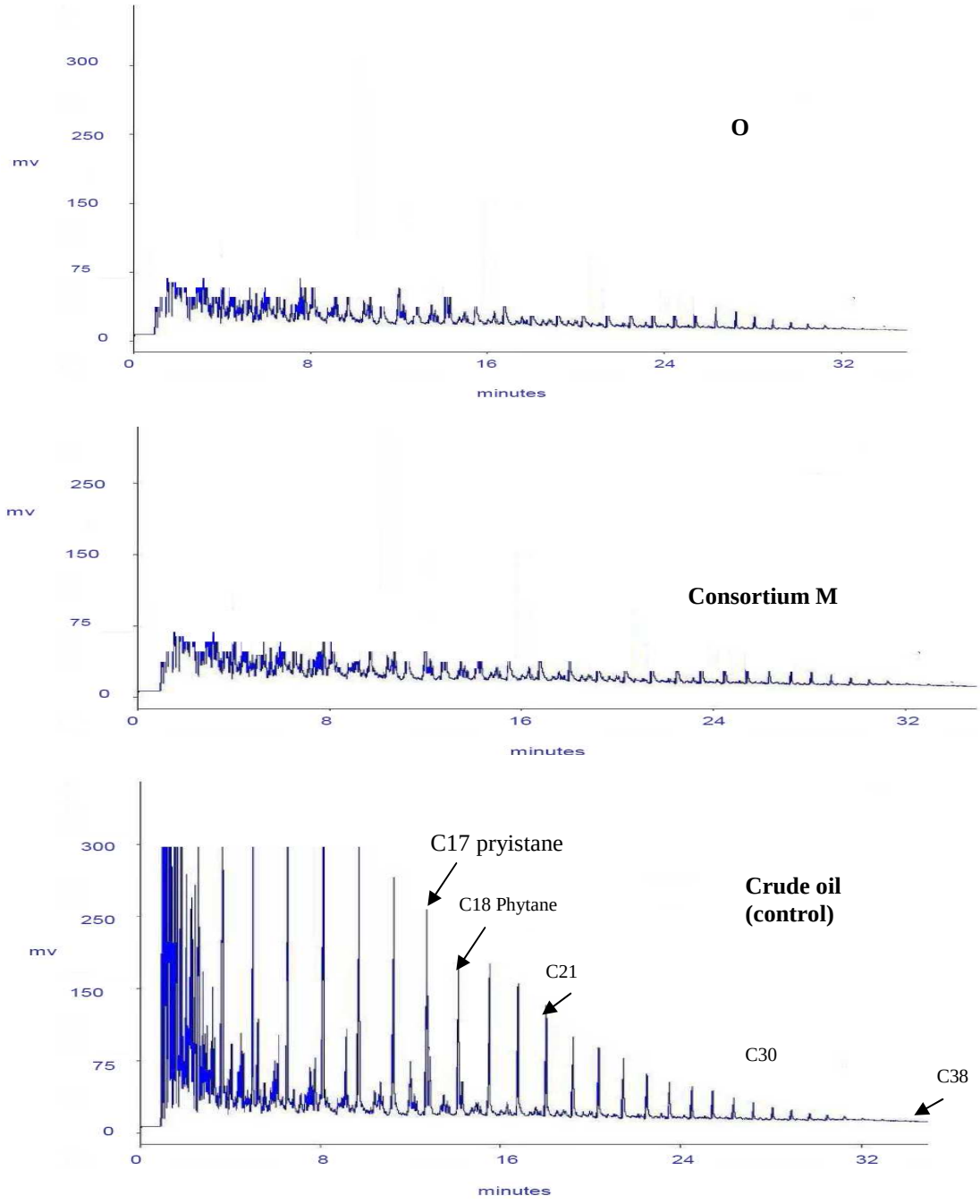


Fig.8: Gas chromatograms of the residual crude oil in marine medium using different organisms after 15 days of incubation

Table 2: Effect of incubation periods on the growth (OD) of the different organisms

Incubation time / Organism	7 days	15 days
	OD	OD
VA	0.255	0.326
R	0.263	0.328
O	0.345	0.565
Consortium M	0.368	0.572
V	0.345	0.488
Consortium C	0.238	0.314

Table 3**:** Effect of incubation periods on the protein content of the different organisms

Incubation time / Organism	7 days		15 days	
	Protein content (mg/ml)	Total protein content (mg)*	Protein content (mg / ml)	Total protein content (mg) *
VA	0.23	1.15	0.24	1.20
R	0.24	1.20	0.18	0.90
O	0.26	1.30	0.42	2.10
Consortium M	0.25	1.25	0.51	2.55
V	0.28	1.40	0.36	1.80
Consortium C	0.27	1.35	0.43	2.15

*Protein content of the total dry weight resulted from the growth of the organism in 50 ml medium containing 1% (v/v) of crude oil.

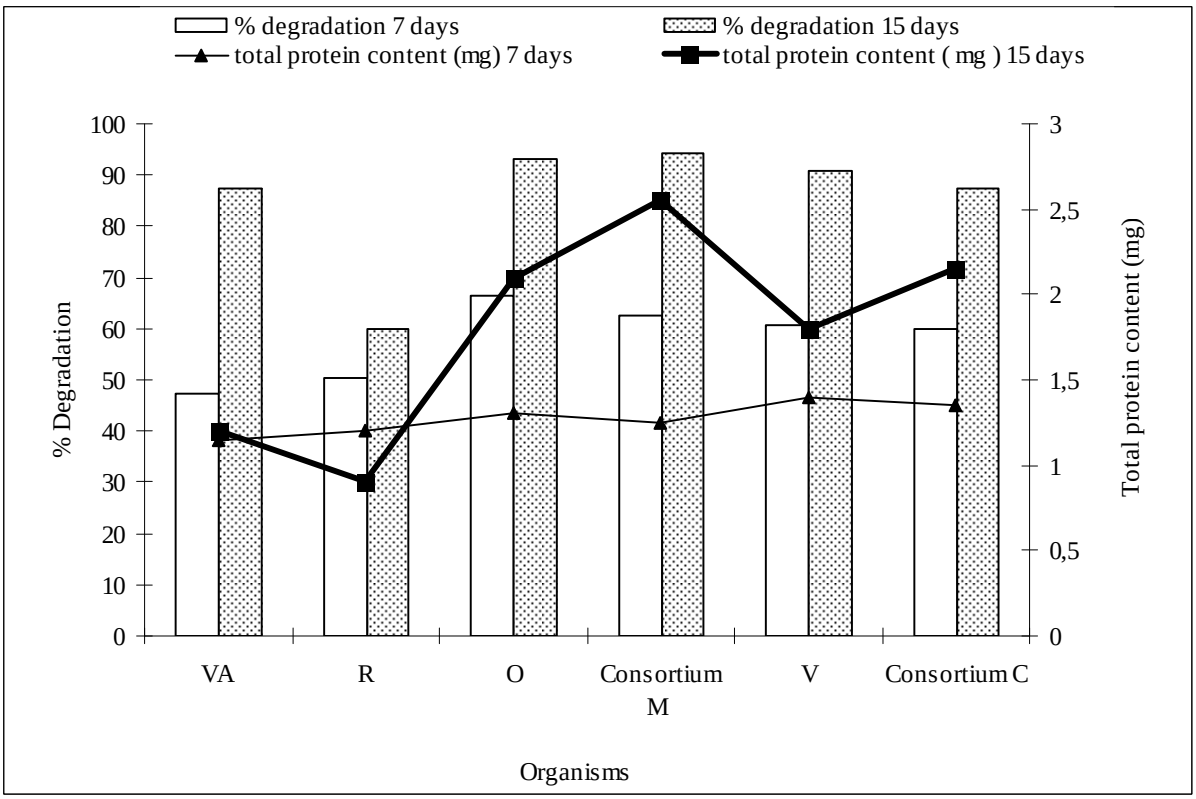


Fig. 9. Relation between the degradation rate and the protein content of the tested organisms after different incubation periods.

Two microorganisms (O and V) in addition to the consortium M were selected to complete this research. The selected isolates (O and V) were identified using gram staining and API 20 kits as *Rhodococcus* sp. S and *Pseudomonas* sp. S. The consortium is composed of *Bacillus sp. S, Acinetobacter sp. S and Aerobacter sp. S* (Table 4).

Table 4: Morphological, physiological and biochemical characteristics of the selected isolates

Isolates characteristics	*Pseudomonas* sp.S (V)	*Rhodococcus* sp. S (O)	Consortium M		
			Bacillus sp. S	*Acinetobacter* sp.S	*Aerobacter* sp. S
Morphology	Rods	Coccobacilli	Rods	Rods	Rods
Colony	Green	Orange	White	White	Creamy
Gram	-	+	+	-	-
Urea	-	+	-	-	-
Indol	-	-	-	-	-
TDA	-	-	-	-	-
Mannitol	-	+	-	-	-
Nitrite	-	+	+	-	-
Glucose	-	+	-	+	+
Lactose	-	+	-	-	-
Saccharose	-	+	-	-	-
H_2S	-	+	-	-	-
Gas	-	-	-	-	-
Citrate	+	-	-	+	+
RM	-	-	+	-	-
VP	-	-	-	-	-
LDC	-	-	-	-	-
ODC	-	-	+	-	-
Oxydase	+	-	-	-	-
Catalase	+	+	+	+	+
Gelatine	+	-	-	-	-
ONPG	-	-	-	-	-
ADH	+	-	-	-	-
Mobility	+	-	+	-	-

+ : a positive reaction

-: a negative reaction

3.3. Optimization of the factors affecting crude oil degradation in marine liquid medium

3.3.1. Effect of co-substrates:

The effect of addition of 0.5% of different co-substrates such as molasses glucose, wheat bran, yeast extract, and peptone on crude oil degradation in marine medium was investigated, using the basal medium described under (2.2.1.) with 1% crude oil. The results demonstrated in Table 5 and Fig. 10 showed that the glucose had strongly inhibitory effect on crude oil biodegradation by all tested microorganisms with a maximum inhibition in case of *Rhodococcus* sp. S (15.5% in comparison with its absence; 86.75%). The yeast extract had no significant effect on all organisms, while the addition of peptone had a slight stimulating effect on *Pseudomonas* sp. S and an inhibitory effect on *Rhodococcus* sp. S and consortium M. It was also observed that the supplementation of wheat bran stimulated the biodegradation of crude oil by all tested microorganisms. On the other hand, molasses slightly inhibited the biodegradation by *Rhodococcus* sp. S and inhibit the biodegradation by consortium (M) and *Pseudomonas* sp. S by about 19 and 16% respectively.

The results mentioned in Table 6 demonstrated that the addition of glucose, peptone and molasses decreased the OD values of all microorganisms under test with the maximum inhibitory effect on *Rhodococcus* sp. S cultivated in presence of glucose (0.12 in comparison with 0.49 in its absence). On the other hand, the supplementation of wheat bran increased the OD values for *Rhodococcus* sp. S and *Pseudomonas* sp. S and had no effect on consortium M. Yeast extract had no effect on the OD of *Rhodococcus* sp. S and showed a slight stimulating effect in OD values of consortium M, while negative effect in the OD values of *Pseudomonas* sp. S was observed.

According to the results presented in Table 7 it was found that the addition of glucose to the marine medium decreased the protein content of the selected microorganisms. Wheat bran highly increased the total protein content of all tested organisms by about 43, 30 and 35% for *Rhodococcus*, consortium M and *Pseudomonas* respectively. These results were followed by addition of peptone, which increased the total protein content by about 33, 12 and 16% in the same order. The use of yeast extract or molasses as co-substrate in the basal medium had more or less the same effect on the total protein content by the tested bacterial isolates and resulted in slight increase in the protein content of *Rhodococcus*, while no significant effect in the case of consortium and *Pseudomonas*.

The results mentioned in Fig. 11 showed that the protein content of the organisms under test is correlated with the percentage of degradation with some exceptions. Maximum protein contents (2.55, 3 and 2.45mg) were recorded when the degradation rate reached its maximum (6.5, 6.3 and 6.2 respectively) whereas the lowest value (0.6 mg) was obtained when the degradation rate was only 1.03 (Table 5).

Table 5: Effect of co-substrates on crude oil biodegradation by the selected bacterial isolates after 15 days of incubation

Co-Substrate / Organism	Basal medium[1]	Glucose[2]	Yeast extract[2]	Peptone[2]	Molasses[2]	Wheat bran[2]
	% degradation					
Rhodococcus sp. S [b]	86.75%± 1.25 **C** **(5.78)[3]**	15.50%± 1.00 **H** **(1.03)**	87.00%± 1.00 **C** **(5.8)**	75.00%± 2.00 **E** **(5.00)**	82.50%± 0.50 **CD** **(5.50)**	95.25%± 0.75 **A** **(6.5)**
Consortium M [a]	90.00%± 1.00 **B** **(6.00)**	61.75%± 1.75 **I** **(4.17)**	92.75%± 0.75 **AB** **(6.18)**	84.00%± 2.00 **B** **(5.60)**	73.00%±1.50 **EF** **(4.87)**	94.00%± 1.00 **A** **(6.27)**
Pseudomonas sp. S [ab]	81.00%± 0.50 **D** **(5.40)**	70.50%± 1.00 **F** **(4.70)**	83.25%± 2.25 **CD** **(5.55)**	84.75%± 1.75 **CD** **(5.65)**	68.25%%± 0.75 **G** **(4.55)**	93.50%± 1.00 **A** **(6.23)**

[1] Without addition of co-substrate.
[2] Co-substrates were added at 0.5%.
Incubation time = 15 days.
[3] Degradation rate**.**

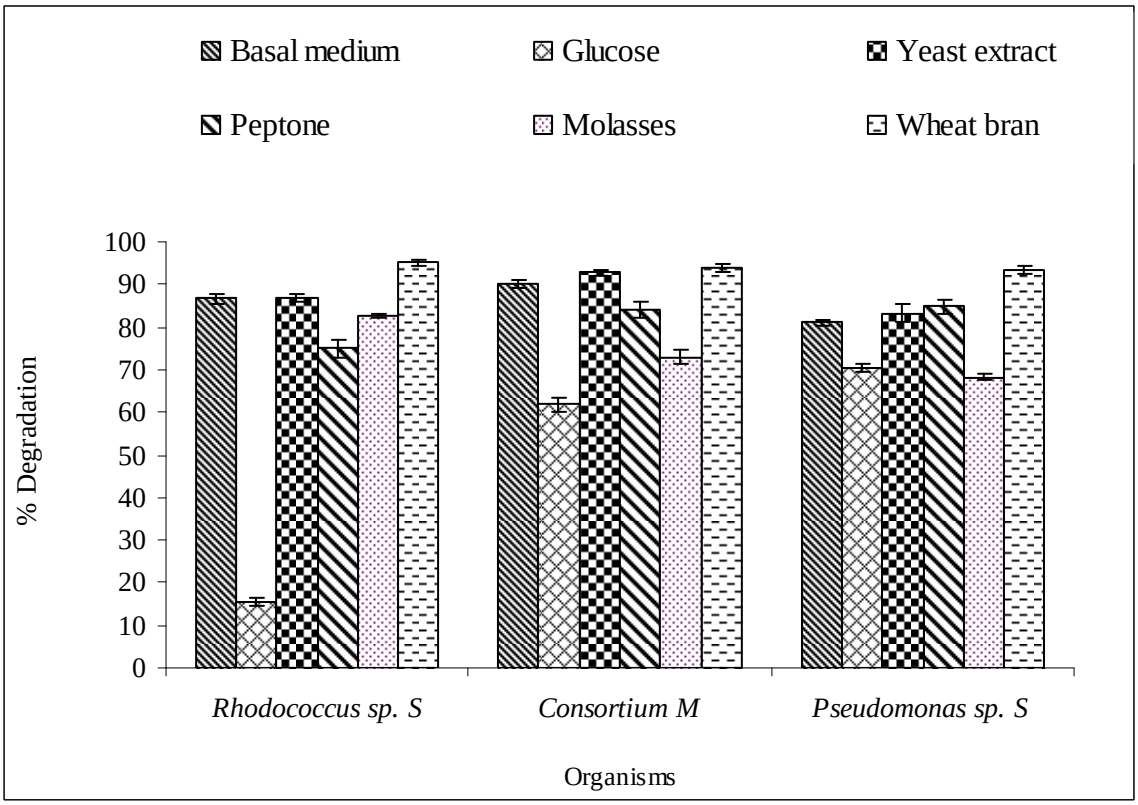


Fig.10: Effect of co-substrates on crude oil biodegradation by the selected bacterial isolates after 15 days of incubation

Table 6: Effect of co-substrates on the growth (OD) of the selected bacterial isolates after 15 days of incubation

Co-substrate / Organisms	Basal medium*	Glucose	Yeast extract	Peptone	Molasses	Wheat bran
	OD					
Rhodococcus sp. S	0.49	0.12	0.49	0.34	0.4	0.53
Consortium M	0.52	0.31	0.54	0.47	0.37	0.52
Pseudomonas sp. S	0.36	0.30	0.31	0.33	0.29	0.40

* without co-substrate

Table 7: Effect of co-substrates on the protein content of the selected bacterial isolates

Co-substrates / Organism	Basal medium*		Glucose		Yeast extract		Peptone		Molasses		Wheat bran	
	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total Protein content (mg)	Protein content (mg/ml)	Total Protein content (mg)	Protein content (mg/ml)	Total Protein content (mg)	Protein content (mg/ml)	Total Protein content (mg)	Protein content (mg/ml)	Total Protein content (mg)
Rhodococcus sp.S	0.29	1.45	0.12	0.6	0.38	1.90	0.43	2.15	0.38	1.9	0.51	2.55
Consortium M	0.42	2.10	0.20	1	0.41	2.05	0.48	2.40	0.42	2.1	0.6	3
Pseudomonas sp. S	0.32	1.60	0.16	0.80	0.34	1.70	0.38	1.9	0.34	1.7	0.49	2.45

* without co-substrates.

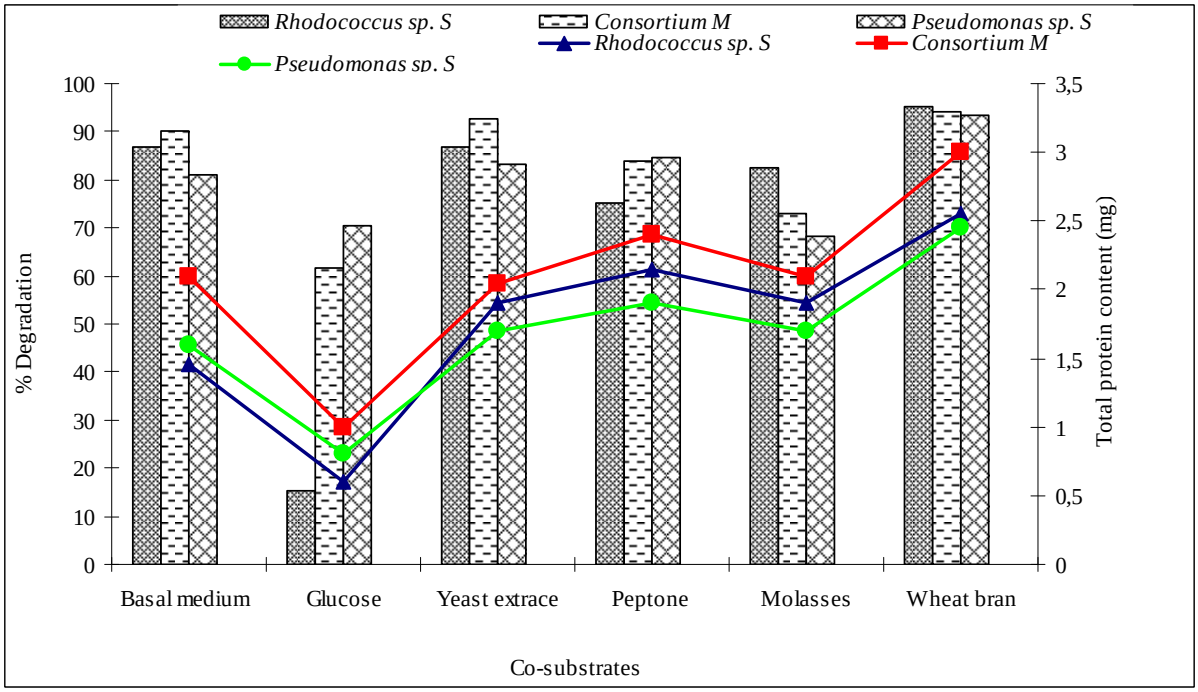


Fig.11. Relation between the degradation rate and the protein content of the selected bacterial isolates using different co- substrates

3.3.2. Effect of nitrogen sources

To study the effect of different nitrogen sources on the degradation of crude oil in marine medium, ammonium sulfate, sodium nitrate, urea 46%, local urea, local nitrogen fertilizer and manure were used. Ammonium nitrate in the basal medium was substituted with the above mentioned nitrogen sources on equal nitrogen basis. Chicken manure, which contain (0.175 g/100 ml nitrogen and 0.03 g/100 ml phosphorus), was added at a concentration of 5% (w/v). 1% of crude oil was used as the sole carbon source. The flasks were incubated for 15 days at 30°C and 120 rpm.

The results presented in Table 8 and Fig. 12 shows that ammonium sulfate had an inhibitory effect on crude oil biodegradation by all bacterial isolates, while the addition of manure gave the maximum biodegradation (94-97%). The use of urea 46% had no effect on crude oil biodegradation by the consortium and slightly increased the degradation rate by *Rhodococcus* sp. S and *Pseudomonas* sp. S. The use of local urea instead of ammonium nitrate had no significant effect on *Rhodococcus* sp. S and slightly increased the biodegradation by consortium M and *Pseudomonas* sp. S. The results also show that the substitution of ammonium nitrate in the basal medium by sodium nitrate decreased the degradation of crude oil by *Rhodococcus* sp. S and consortium and slightly increased the degradation by *Pseudomonas* sp. S. The local nitrogen fertilizer decreased the degradation of crude oil by *Rhodococcus* sp. S and *Pseudomonas* sp. S whereas it had a stimulating effect on degradation by consortium.

From the results mentioned in Table 9, using ammonium sulphate as nitrogen source decreased the OD values of all tested organisms, while local urea had a stimulating effect on the OD of the selected bacterial isolates. The use of urea 46% decreased the OD of the consortium M, increased the OD of *Pseudomonas* sp. S and it had no significant effect on the OD of *Rhodococcus* sp. S. Sodium nitrate had a decreasing effect on the OD of *Rhodococcus* sp. S and the consortium, while it had no effect on the growth of *Pseudomonas* sp. S. The substitution of ammonium nitrate in the basal medium by a local nitrogen fertilizer slightly stimulated the growth of the consortium (0.55 in comparison with 0.52 in the basal medium).

According to the results showed in Table10, it was observed that the use of ammonium sulphate as nitrogen source decreased the protein content of all bacteria under test in comparison with ammonium nitrate in the basal medium. On the other hand, urea 46%, local urea, sodium nitrate, and the local nitrogen fertilizer increased the protein content of the tested microorganisms with a maximum value in the case of the consortium cultivated in presence of local urea as nitrogen source (2.80 mg).

Fig. 13 revealed that the increase in degradation was correlated with the protein content of the tested bacterial isolates with some fluctuations. The maximum protein content (2.8 mg) was

obtained when the degradation reached its maximum value (94.5%), while the lowest degradation (68.25%) was recorded at the lowest protein content (1.05 mg).

The local urea was used as nitrogen source in the subsequent experiments due to its low cost and its good results with *Rhodococcus* sp. S, consortium M and *Pseudomonas* sp. S. Manure was not applied, in spite of its excellent degradation rate of crude oil due to hygiene purposes.

Table 8: Effect of some nitrogen sources on crude oil biodegradation by the selected bacterial isolates after 15 days of incubation

Nitrogen source / Organism	Basal medium[1]	Ammonium sulfate	Urea 46%	Local urea	Sodium nitrate	Nitrogen fertiliser	manure
	% Degradation						
Rhodococcus sp. S[ab]	86.75% ± 1,25 **AB** (5.78) [2]	75.00% ± 1.00 **D** (5.00)	88.25% ± 0.75 **ABC** (5.88)	85.50% ± 0.50 **ABC** (5.70)	79.75% ± 1.25 **CD** (5.32)	77.75% ± 1.25 **CD** (5.18)	95.00% ±1.00 **B** (6.33)
Consortium M[a]	90.00% ± 1.00 **ABC** (6.00)	78.50% ± 1.50 **CD** (5.23)	90.00% ± 1.00 **AB** (6.00)	94.50% ± 1.50 **AB** (6.30)	85.75% ± 1.00 **ABC** (5.72)	93.75% ± 0.75 **AB** (6.25)	94.25%±00. 75 **AB** (6.28)
Pseudomonas sp. S[b]	81.00% ± 0,5 **C** (5.40)	68.25% ± 1.25 **E** (4.55)	86.75% ± 1.25 **ABC** (5.78)	84.00% ± 1.00 **ABC** (5.60)	83.00% ± 2.00 **C** (5.33)	79.00% ± 1.00 **CD** (5.27)	97.00% ± 1.00 **A** (6.47)

[1]Ammonium nitrate was used as nitrogen source
[2] Degradation rate (% degradation/ incubation time)
Same capital letters are not statistically different among nitrogen sources at $p<0.01$± standard error (n=2)
Same small letters are not statistically different among organisms at $p<0.01$± standard error (n=2)

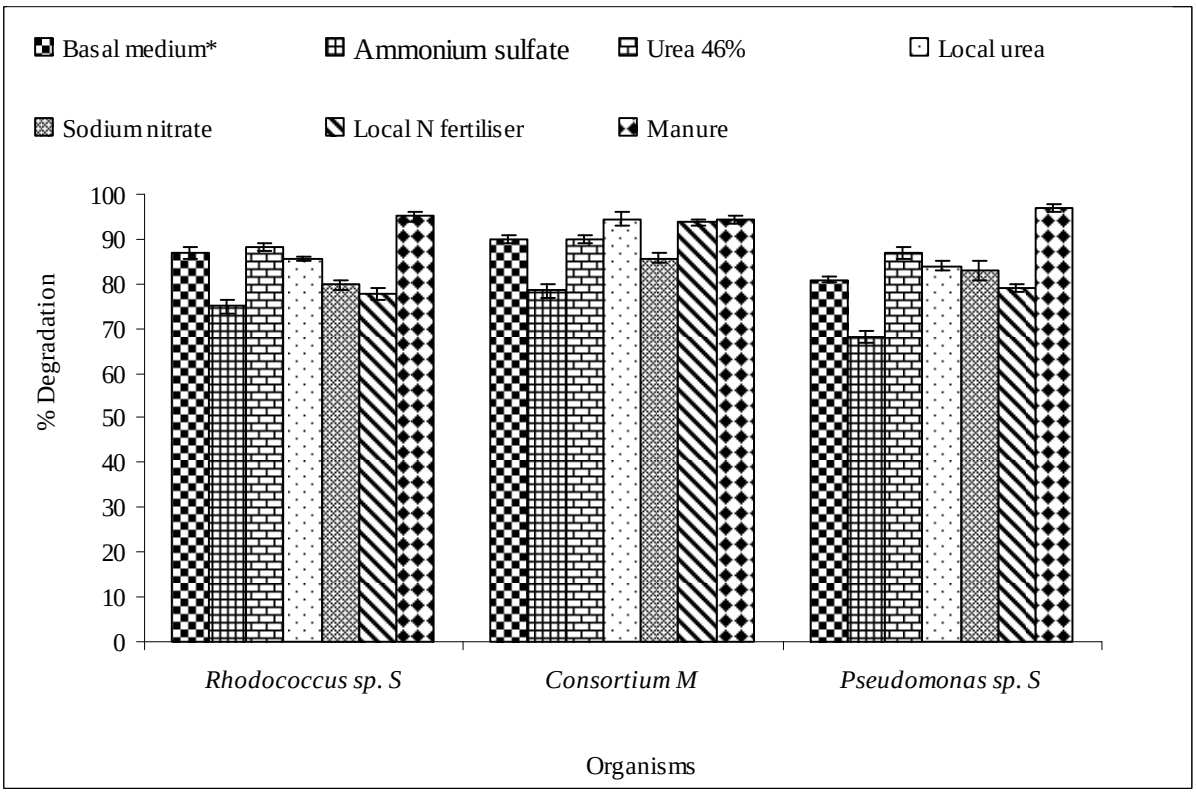


Fig. 12. Effect of some nitrogen sources on crude oil degradation by the selected bacterial isolates after 15 days of incubation

Table 9: Effect of different nitrogen sources on OD of the selected bacterial isolates after 15 days of incubation

Nitrogen source / Organism	Basal medium	Ammonium sulfate	Urea 46%	Local urea	Sodium nitrate	Nitrogen fertiliser	Manure
	OD						
Rhodococcus sp. S	0.49	0.43	0.49	0.5	0.46	0.42	ND*
Consortium M	0.52	0.44	0.5	0.57	0.49	0.55	ND
Pseudomonas sp. S	0.36	0.32	0.38	0.38	0.36	0.33	ND

ND*: not determined due to high turbidity of manure

Table 10 : Effect of some nitrogen sources on the protein content of selected bacterial isolates

Nitrogen source / Organism	Basal medium		Ammonium sulfate		Urea 46%		Local urea		Sodium nitrate		Local nitrogen fertilizer	
	Protein content (mg /ml)	Total protein content (mg)	Protein content (mg /ml)	Total protein content (mg)	Protein content (mg /ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg /ml)	Total protein content (mg)
Rhodococcus sp.S	0.29	1.45	0.25	1.25	0.48	2.40	0.47	2.35	0.39	1.95	0.38	1.90
Consortium M	0.42	2.10	0.27	1.35	0.50	2.50	0.56	2.80	0.52	2.6	0.53	2.65
Pseudomonas sp. S	0.32	1.60	0.21	1.05	0.45	2.25	0.38	1.9	0.4	2	0.41	2.05

Note: In case of manure, the cells cannot be separated from the manure therefore the protein content cannot be calculated.

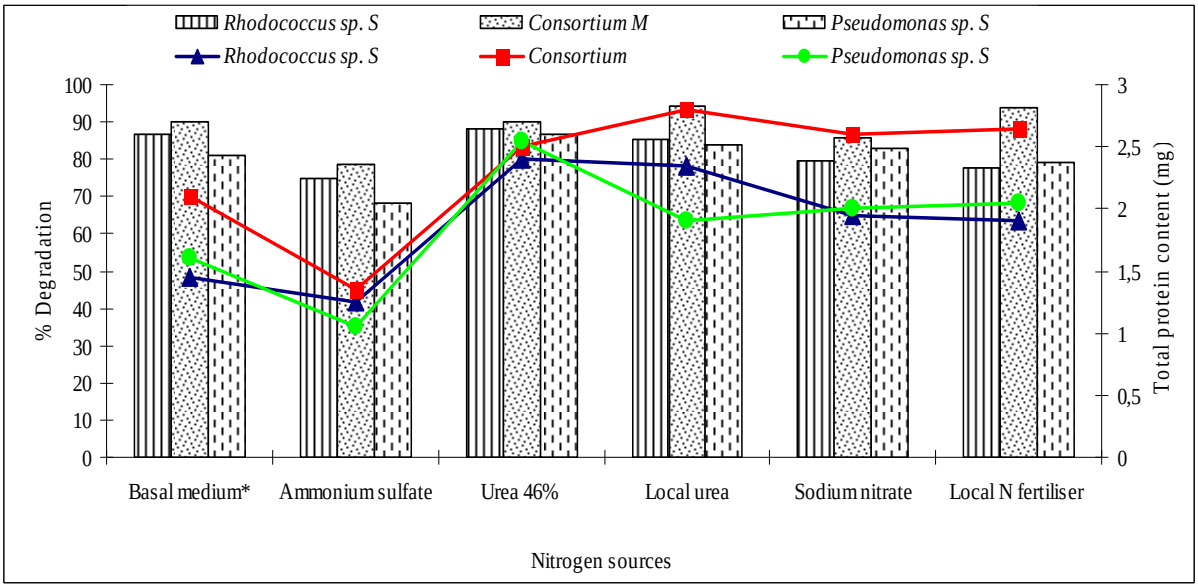


Fig. 13 Relation between the degradation and the protein content of the selected bacterial isolates using different nitrogen sources.

3.3.3. Effect of phosphorus sources

In this experiment, Na_2HPO_4 and NaH_2PO_4 used in the basal medium were substituted with super phosphate 15.5%, $(NH_4)_2PO_4$ and a local phosphorus fertilizer one at a time. When $(NH_4)_2PO_4$ was used as phosphate source, the nitrogen content of the medium was optimized to give the same ratio in the basal medium by subtracting the amount of nitrogen in ammonium phosphate from the amount of urea which was used as nitrogen source. The cultures were incubated for 15 days.

The results are shown in Table 11 and Fig. 14. The use of super phosphate 15.5% had a stimulating effect on *Rhodococcus* sp. S and consortium (91.75% and 97% respectively) in comparison with the basal medium (85.5% and 94. 5% respectively). On the other hand, it had no effect on crude oil degradation by *Pseudomonas* sp. S. Local phosphorus fertilizer slightly stimulated crude oil degradation by *Rhodococcus* sp .S and *Pseudomonas sp*. S by about 2 and 4% respectively. On the other hand, the addition of ammonium phosphate increased the degradation by *Rhodococcus* sp. S by about 8% and decreased it by consortium and *Pseudomonas* sp. S by about 7 and 14 % respectively. The chromatogram pattern of crude oil degradation using different phosphorus sources is presented in Fig. 15

From the results presented in Table 12, using super phosphate 15.5% as phosphorus source increased the OD values of all tested bacterial isolates. The local phosphorus fertilizer decreased the OD of *Rhodococcus* sp. S and consortium M, while it increased the OD of *Pseudomonas* sp. S. On the other hand, the use of ammonium phosphate as phosphorus source decreased the OD of all tested bacteria with a maximum reduction (0.3) for *Pseudomonase* sp. S in comparison with (0.38) with the basal medium.

The results presented in Table 13 showed that the use of super phosphate 15.5% as phosphorus source increased the protein content of *Rhodococcus* sp. S and the consortium M, which gave the highest protein content (3.95 mg), while it decreased the protein content of *Pseudomonas* sp. S. The local phosphorus fertilizer had a stimulating effect on the protein content of all organisms. The results revealed that ammonium phosphate increased only the protein content of *Rhodococcus* sp. S, while it decreased the protein content of the consortium and *Pseudomonas* sp. S.

According to the results demonstrated in Fig. 16 it was observed that the protein content of the bacterial isolates under test is correlated with the biodegradation of crude oil degradation with some fluctuations.

The subsequent experiments were performed using the local phosphorus fertilizer as phosphorus source due its low cost and availability as local commercial fertilizer.

Table 11: Effect of some phosphorus sources on crude oil biodegradation by the selected bacterial isolates after 15 days incubation

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Phosphorus source organism	Basal medium [1]	Super phosphate 15.5% [2]	Local phosphorus fertilise [2]	Ammonium phosphate[2]
	% Degradation			
Rhodococcus sp.S [ab]	85.50% ± 0.50 **BC** (5.70)[3]	91.75% ± 1.50 **AB** (6.12)	87.00% ±1.00 **ABC** (5.8)	93.00% ± 1.00 **AB** (6.20)
Consortium M [a]	94.50% ± 1.50 **AB** (6.30)	97.00% ± 1.00 **A** (6.47)	90.00% ± 2.00 **B** (6.00)	87.25% ± 0.75 **ABC** (5.82)
Pseudomonas sp. S [b]	84.00% ± 1.00 **C** (5.60)	84.50% ± 1.50 **C** (5.63)	88.25% ± 1.25 **ABC** (5.88)	70.25% ± 0.75 **D** (4.68)

[1] Local urea was used as nitrogen source

[2] Phosphorus sources in the basal medium were substituted by the sources mentioned in table 3

[3] Degradation rate

Same capital letters are not statistically different among phosphorus sources at $p<0.01$± standard error (n=2)

Same small letters are not statistically different among microorganisms at $p<0.01$± standard error (n=2)

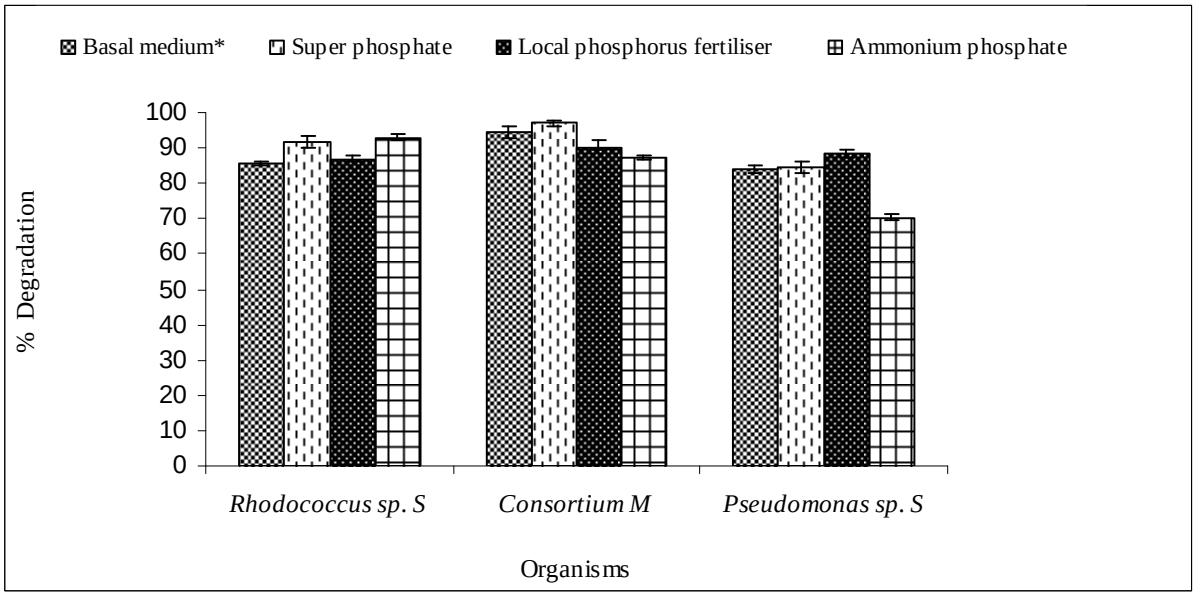
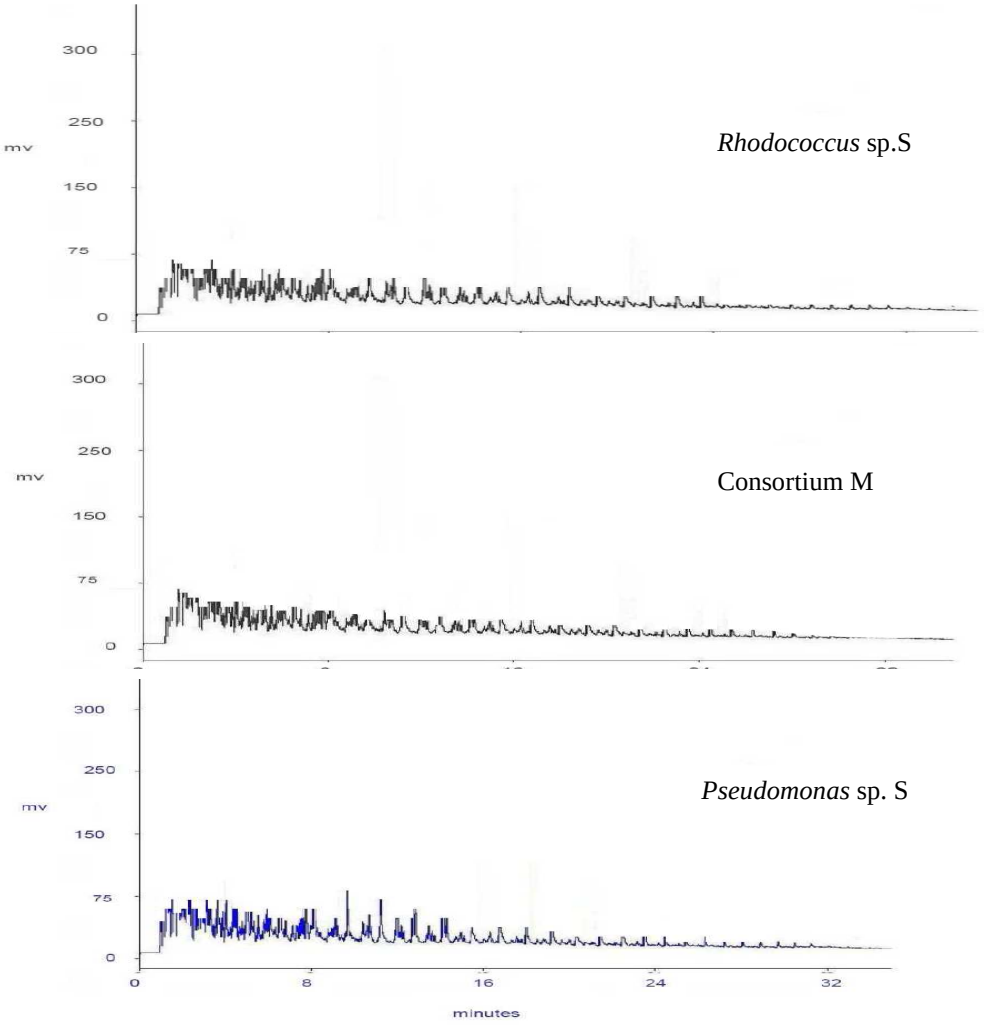
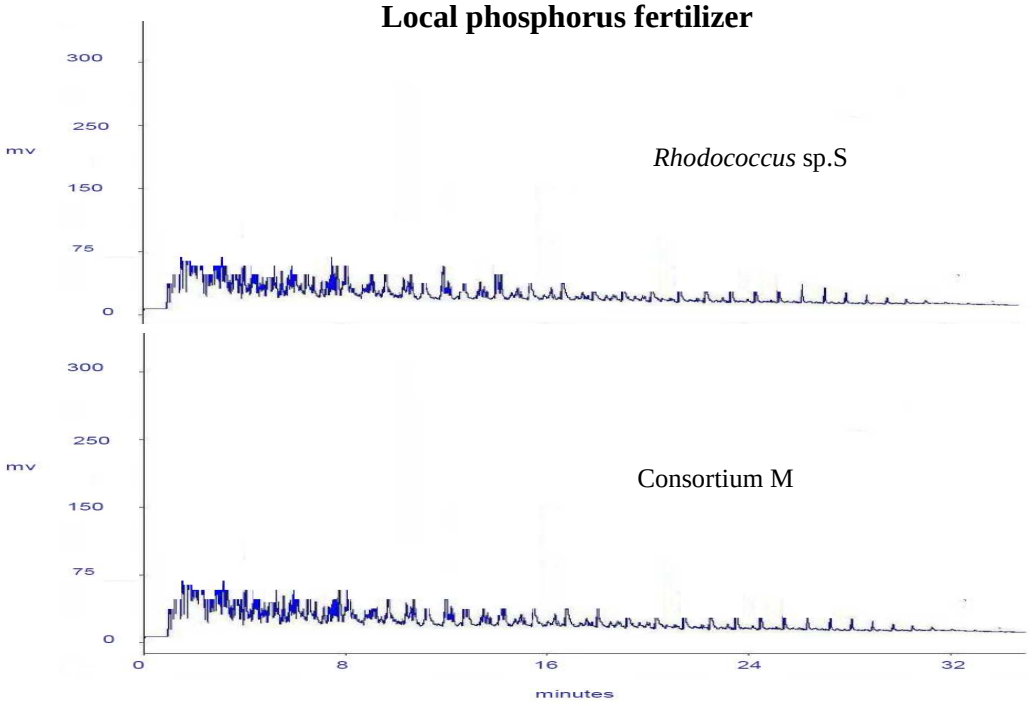


Fig. 14. Effect of some phosphorus sources on crude oil biodegradation by the selected bacterial isolates after 15 days incubation

Super phosphate 15.5%



Local phosphorus fertilizer



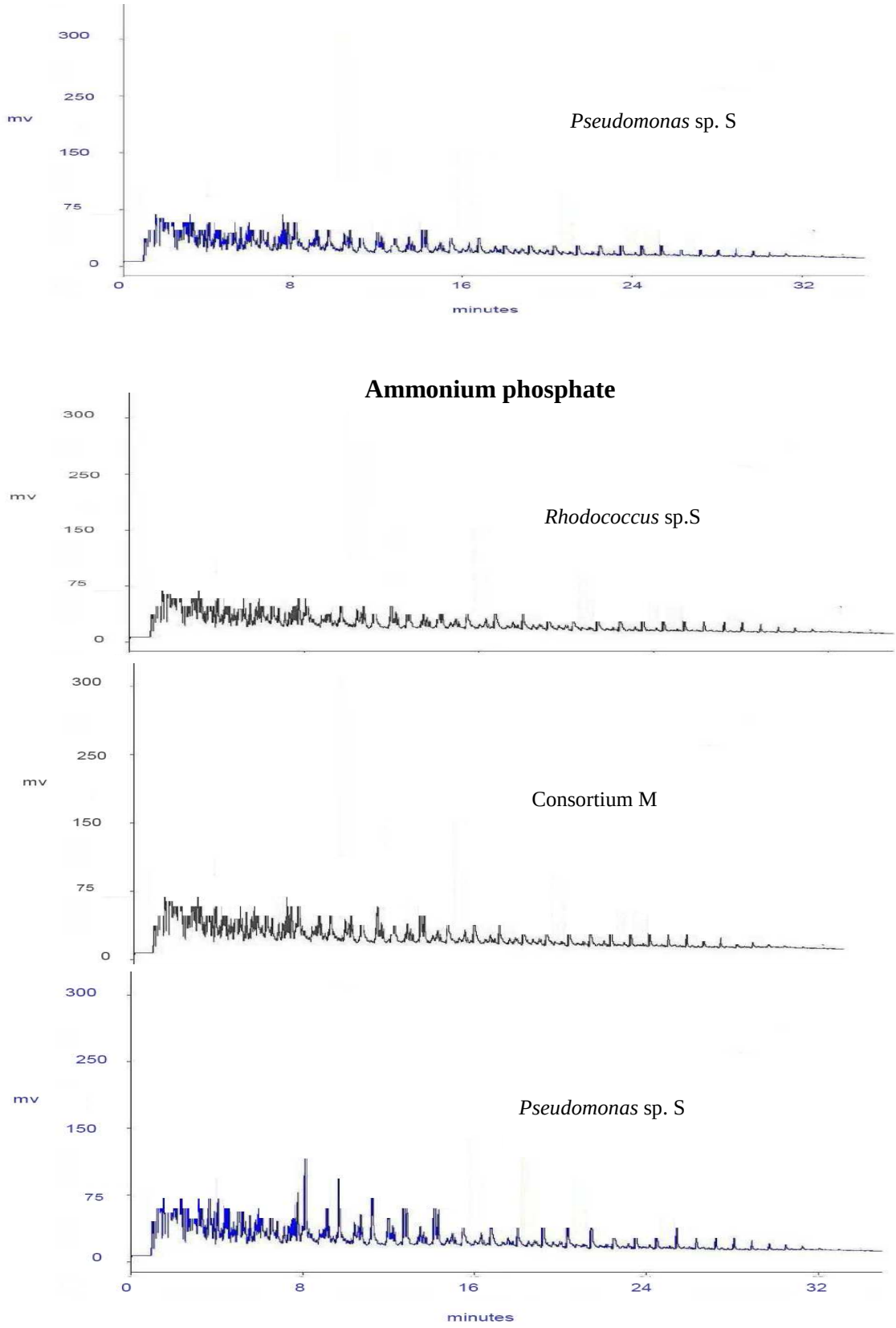


Fig. 15. Gas chromatogram of crude oil biodegradation with the tested bacterial isolates using different phosphorus sources.

Table 12:Effect of some phosphorus sources on OD of the selected bacterial isolates

Phosphorus source organism	Basal medium	Super phosphate 15.5%	Local phosphorus fertilise	Ammonium phosphate
	OD			
Rhodococcus sp.S	0,5	0.56	0.47	0.5
Consortium M	0.57	0.6	0.49	0.46
Pseudomonas sp. S	0.38	0.40	0.41	0.3

Table 13: Effect of some phosphorus sources on the protein content of the selected bacterial isolates

Phosphorus source / Organism	Basal medium*		Super phosphate 15.5%		Local phosphorus fertilizer		Ammonium phosphate	
	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total Protein content (mg)	protein content (mg/ml)	Total Protein content (mg)	protein content (mg/ml)	Total Protein content (mg)
Rhodococcus sp.S	0.47	2.35	0.57	2.85	0.60	3.00	0.56	2.8
Consortium M	0.56	2.80	0.79	3.95	0.64	3.20	0.49	2.45
Pseudomonas sp. S	0.38	1.9	0.35	1.75	0.58	2.90	0.34	1.7

* Local urea was used as nitrogen source

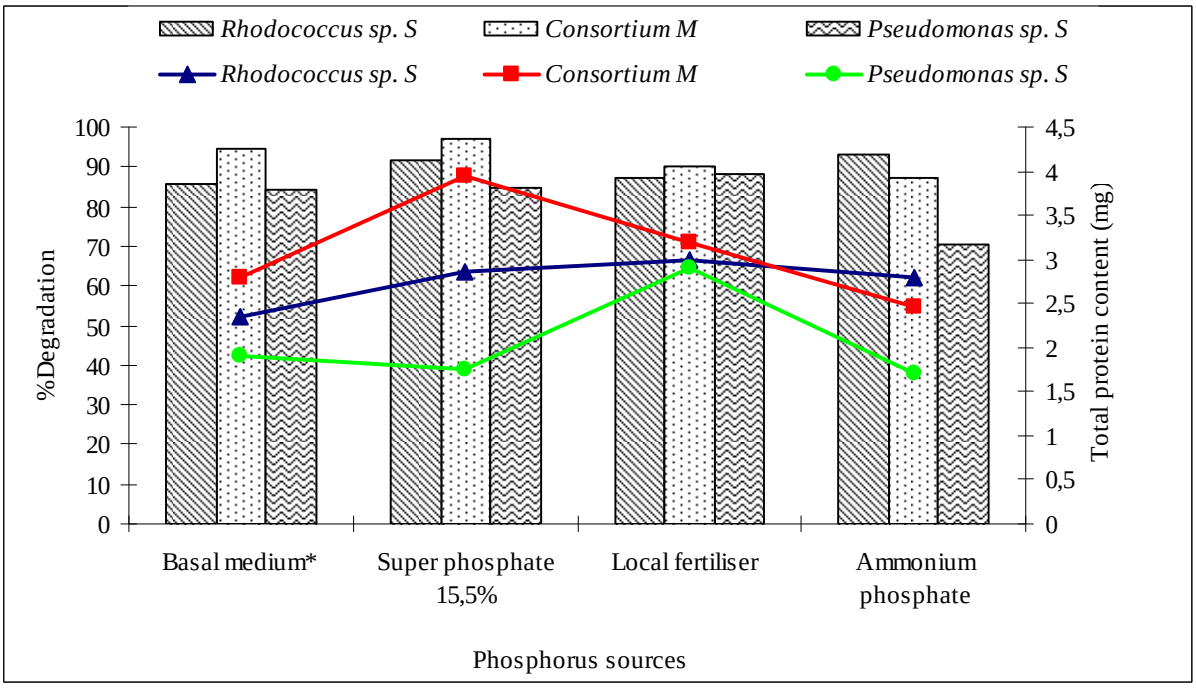


Fig. 16. Relation between the degradation and the protein content of the selected organisms using different phosphorus sources

3.3.4. Effect of nitrogen/phosphorus (N/P) ratios

To study the effect of different nitrogen/phosphorus ratios on the degradation of crude oil, the ratios 1/1, 3/1, 7/1, 10/1, and 20/1, were tested. Local urea and local phosphorus fertilizer were used as nitrogen and phosphorus sources. According to the results shown Table 14 and Fig. 17 it was found that the ratios 1/1, 3/1 and 20/1 reduced the activity of all the bacteria isolates under test by about 2-20% with a maximum inhibition in case of *Pseudomonas* sp. S at the ratio 1/1 (68.25%). Although the ratio 7/1 had no significant effect on consortium M, it decreased the activity of *Rhodococcus* sp. S and *Pseudomonas* sp. S by about 11 and 8% respectively . The ratio 10/1 stimulated the activity of *Rhodococcus* sp. S and consortium M by about 7and 3% respectively whereas it had no significant effect on *Pseudomonas* sp. S.

The effect of different N/P ratios on the OD of the tested bacterial isolates is demonstrated in Table 15. It was observed that the ratios 1/1 and 3/1and 20/1 decreased the OD of all the tested organisms. The minimum OD was recorded in case of *Pseudomonas* sp. S cultivated at the N/P ratio 1/1 (0.26). The ratios 7/1 and 10/1 stimulated the OD of *Rhodococcus* sp. S and the consortium M while they decreased the OD of *Pseudomonas* sp. S

The results presented in Table 16 show that the ratios 1/1 , 3/1 and 20/1 decreased the protein content of all the bacterial isolates with the maximum inhibitory effect on Pseudomonas sp. S cultivated at the N/P ratio 1/1. The ratios 7/1 had more or the same results. The ratio 10/1 increased the protein content of all bacterial isolates with the highest value in case of consortium M at the ratio 10/1 (4.45mg).

According to Fig. 18 it was found that the protein content more or less correlated with the crude oil biodegradation with some fluctuations.

Table 14: Effect of N/P ratios on crude oil biodegradation by the selected bacterial isolates after 15 days of incubation

N/P ratio / Organism	Basal medium *	N/P=1/1	N/P=3/1	N/P=7/1	N/P=10/1	N/P=20/1
	% Degradation					
Rhodococcus sp. S [b]	87.00%± 1.00 **BC** (5.8)[1]	85.00%± 0.50 **C** (5.67)	76.50%± 2.00 **E** (5.1)	75.50%± 1.50 **E** (5.03)	94.00%± **A** 2.00 (6.27)	84.25%± **C** 0.75 (5.62)
The consortium M [a]	90.00%± 2.00 **A** (6.00)	78.75%± 1.00 **DE** (5.25)	82,25%± 1,25 **A** (5,48)	90.75%± 0.75 **A** (6.25)	93.25%± 1.25 **A** (6.22)	88.25%± 0.75 **B** (5.88)
Pseudomonas sp. S [b]	88.25%± **B** 1.25 (5.88)	68.25%± 0.75 **F** (4.55)	80.00%± **D** 1.00 (5.33)	80.25%± 0.25 **AB** (5.35)	87.50%± 1.50 **BC** (5.83)	77.00%± **E**1.00 (5.13)

*Local Urea and local phosphorus fertilizer were used as nitrogen and phosphorus sources**.**

Incubation time = 15 days,

[1] Degradation rate

Same capital letters are not statistically different among N/P ratios at $p< 0.01$.

Same small letters are not statistically different among organisms at $p< 0.01$,

± Standard error (n=2).

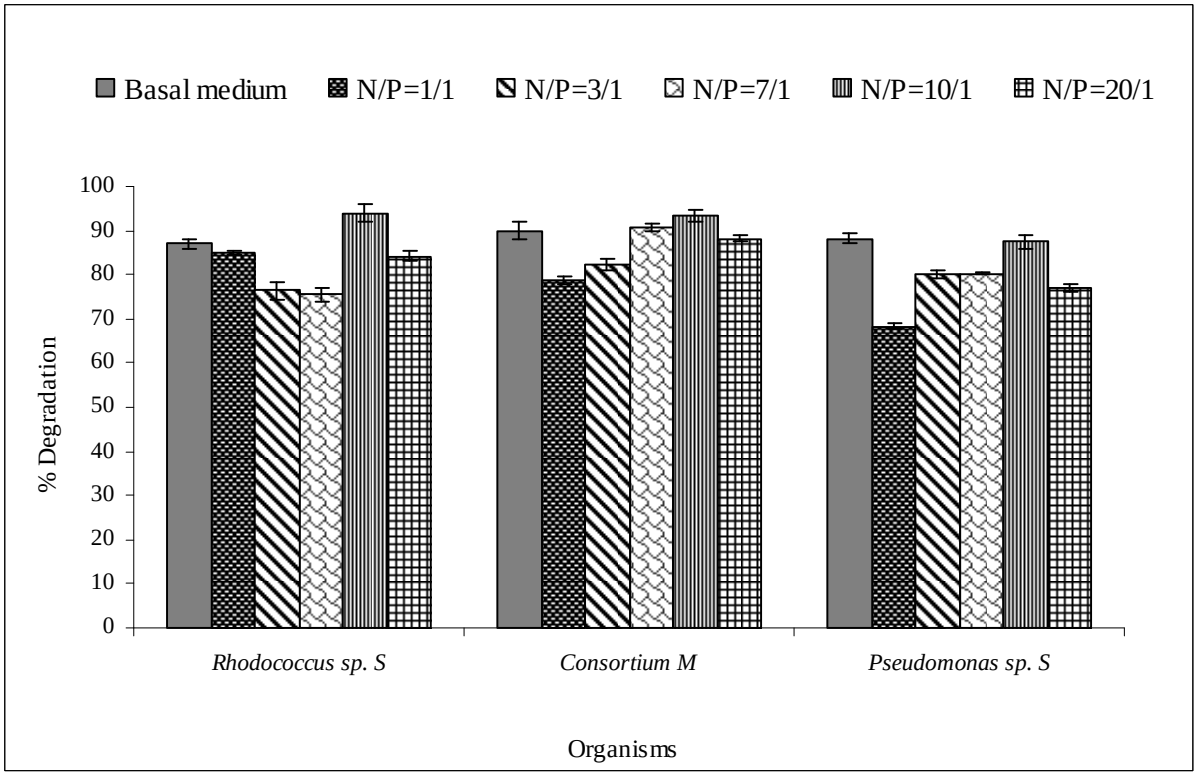


Fig. 17 : Effect of N/P ratios on crude oil biodegradation by the selected bacterial isolates after 15 days of incubation

Table 15: Effect of the N/P ratios on OD of the selected bacterial isolates

N/P ratio	Basal medium *	N/P=1/1	N/P=3/1	N/P=7/1	N/P=10/1	N/P=20/1
	OD					
Rhodococcus sp.S	0.47	0.46	0.35	0.52	0.52	0.43
The consortium M	0.49	0.36	0.46	0.55	0.58	0.46
Pseudomonas sp. S	0.41	0.26	0.3	0.35	0.36	0.28

Table16: Effect of different N/P ratios on the protein content of the selected bacterial isolates

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N/P ratio / Organism	Basal medium		N/P=1/1		N/P=3/1		N/P=7/1		N/P=10/1		N/P=20/1	
	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)
Rhodococcus sp. S	0.6	3.00	0.50	2.50	0.42	2.1	0.60	3.00	0.73	3.65	0.48	2.40
The consortium M	0.64	3.2	0.41	2.05	0.61	3.05	0.83	4.15	0.89	4.45	0.60	3.00
Pseudomonas sp. S	0.58	2.90	0.25	1.25	0.53	2.65	0.58	2.90	0.62	3.1	0.52	2.6

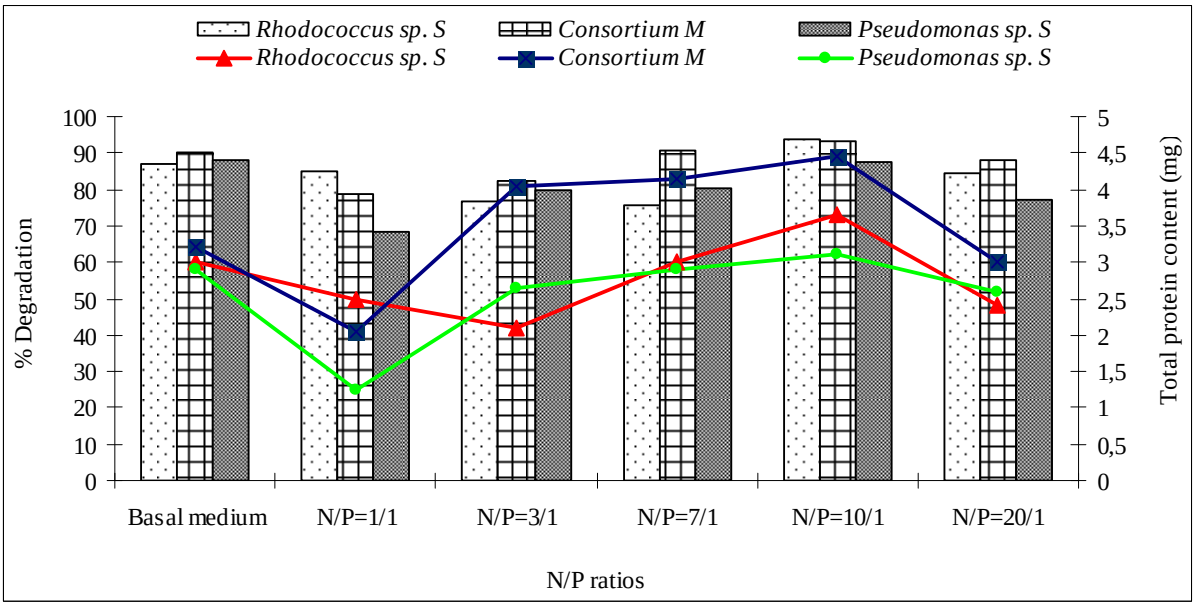


Fig.18. Relation between the degradation rate and the protein content of the tested bacterial isolates cultivated at different N/P ratios.

3.3.5. Effect of pH

In this experiment different pH values (4, 6, 7, 8 and 9) were used for the cultivation of the organisms under test. 1% crude oil was added as carbon source and the experiment was performed under the cultivation conditions mentioned under 2.2.1. for 15 days. It was observed that the acidic and alkaline pH (4 and 9) reduced the activity of the two bacteria and the consortium by about 6-27%. pH 6, 7 and 8 gave the best biodegradation results, which ranged between (86.5-95%) as shown in Table17 and Fig.19. The growth of the tested organisms was evaluated as OD. The results presented in Table 18 revealed that the maximum growth of all tested bacterial isolates was recorded at neutral pH with the best growth for consortium (0.58).

The growth of the organisms was also evaluated as intracellular protein content. The results are demonstrated in Table 19. It was found that maximum protein content was obtained in neutral pH (4.45 mg in case of the consortium). Drastic decrease in protein content was observed at pH 4 and pH 9.

Generally, it was observed that the biodegradation of crude oil at different pH values was correlated with the protein content of the tested organisms (Fig.20). Maximum protein content (4.45 mg) was obtained at the maximum degradation (93.25%) by consortium at pH 7. On the other hand, maximum protein content (4.20 mg) and maximum degradation (95%) was recorded by *Pseudomonas* at slightly alkaline pH (pH 8).

Table 17: Effect of the medium pH on crude oil degradation by the tested bacterial isolates

pH / Organism	pH 4	pH 6	pH 7	pH 8	pH 9
	% Degradation				
Rhodococcus sp. S[ab]	67.00%± 2.00 **E** (4.47)[1]	89.00%± 1.00**BC** (5.93)	94.00%± 2.00 **AB** (6.27)	83.00%± 1.5**C** (5.53)	79.00%± 1.00 **CD** (5.23)
Consortium M[a]	74.00%± 1.50 **D** (4.93)	90.00%± 2.00 **B** (6.00)	93.25%± 1.25 **AB** (6.22)	91.00%± 1.00 **AB** (6.07)	87.00%± 1.00 **BC** (5.80)
Pseudomonas sp. S[ab]	65.00%± 2.50 **E** (4.33)	92.00%± 2.00 **AB** (6.13)	87.50± 1.50 **BC** (5.77)	95.00%± 2.00 **A** (6.33)	75.50%± 1.50 **D** (5.03)

Incubation time= 15 days
[1] degradation rate

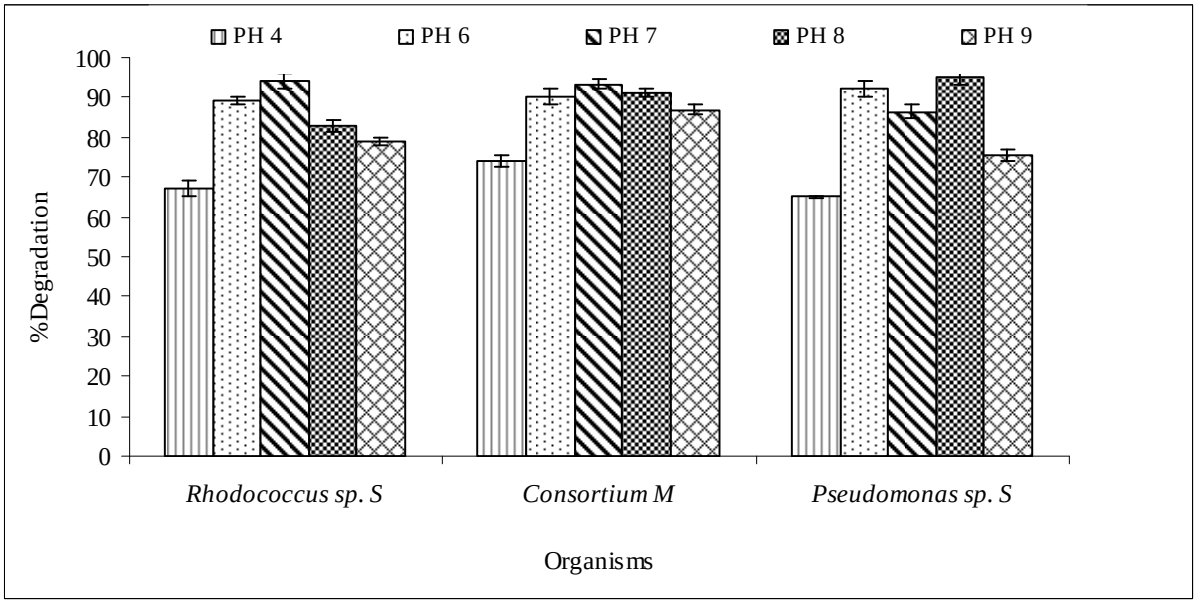


Fig. 19: Effect of the medium pH on crude oil degradation by the tested bacterial isolates

Table 18: Effect of the medium pH on OD of the tested bacterial isolates

pH / Organism	pH 4	pH 6	pH 7	pH 8	pH 9
	OD				
Rhodococcus sp. S	0.35	0.42	0.52	0.49	0.42
Consortium M	0.42	0.51	0.58	0.53	0.31
Pseudomonas sp. S	0.22	0.36	0.36	0.33	0.29

Table 19: Effect of the medium pH on the protein content tested bacterial isolates

pH / Organism	pH 4		pH 6		pH 7		pH 8		pH 9	
	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)
Rhodococcus sp. S	0.24	1.2	0.55	2.75	0.73	3.65	0.40	2.00	0.23	1.15
Consortium M	0.40	2.00	0.76	3.80	0.89	4.45	0.75	3.75	0.49	2.45
Pseudomonas sp. S	0.29	1.45	0.57	2.85	0.62	3.10	0.84	4.20	0.25	1.25

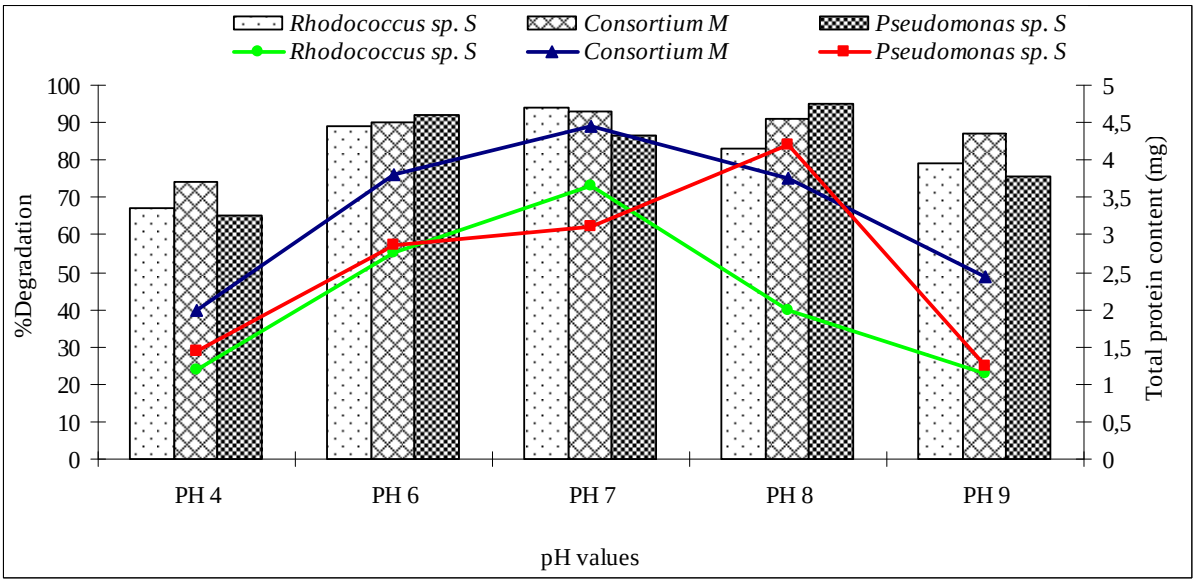


Fig. 20: Relation between the degradation and the protein content of the tested bacterial isolates cultivated under different pH values.

3.3.6. Effect of surfactants

Different surfactants (Igepal, Tergitol, Triton X-100, Tween 20 and Tween80) were used to increase the emulsification of crude oil with the medium. Each surfactant was added at 1% and the pH of the medium was adjusted to 7. The cultures were incubated for 15 days. The addition of Tergitol decreased the biodegradation of crude oil by *Rhodococcus* sp. S by about 5%. The results presented in Table 20 and demonstrated in Fig. 21 revealed that the addition of Igepal and Tween 80 increased the biodegradation of crude oil by all tested organisms. Although Triton X-100 and Tween 20 showed no significant effect on crude oil biodegradation by *Rhodococcus* and consortium M, it had a stimulating effect on the degradation by *Pseudomonas*. Triton X-100 was selected to complete the rest of the experiments due to its low cost.

Statistical analysis showed that there was no significant difference between the surfactants except in case of *Rhodococcus* sp. S cultivated in presence of Tergitol (Table 20).

Table 21 showed the effect of the addition of surfactants on the OD of the tested organisms. It was observed that the use of different surfactants stimulated the OD of all the organisms except in the case of the consortium M where the OD slightly decreased form 0.58 in absence of the surfactants to 0.56 in presence of Triton X-100 and 0.54 in presence of Tween 80 (Table 21).

It was found that the effect of the surfactants on the protein content depends on the organism under test. Maximum values of the protein content (4.85 and 4.7 mg) were obtained by *Pseudomonas* sp. S cultivated in presence of Tween 20 and Tween 80 respectively, while the maximum protein content (4.1 mg) was recorded by *Rhodococcus* sp.S in presence of Triton X-100 as surfactant (Table 22).

The results demonstrated in Fig. 22 revealed that the protein content more or less correlated with the crude oil biodegradation with some fluctuations. It was observed that in most cases the increase in protein content is correlated with an increase in the biodegradation.

Table 20: Effect of surfactants on crude oil biodegradation by the tested bacterial isolates

Surfactant / Organism	Basal medium *	Igepal**	Tergitol**	Triton X-100 **	Tween 20**	Tween 80**
	% Degradation					
Rhodococcus sp. S[a]	94.00%± 2.00 **AB** (6.27)[1]	95.00%±2.00 **AB** (6.33)	88.50%± 2.50 **ABC** (5.9)	94.25%± 1.75 **AB** (6.28)	92.00%± 2.00 **AB** (6.13)	95.50%± 1.50 **AB** (6.37)
Consortium M[a]	93.25%± 1.25 **AB** (6.22)	96.50%± 2.50 **A** (6.43)	92.25%± 0.75 **AB** (6.15)	95.00%± 2.50 **AB** (6.33)	93.50%± 2.50 **AB** (6.23)	96.00%± 2.50 **A** (6.4)
Pseudomonas sp. S[a]	87.50± 1.50**C** (5.77)	90.50%± 2.50 **AB** (6.03)	92.25%± 0.25 **AB** (6.15)	95.00%± 2.00 **AB** (6.33)	93.50%± 2.50 **AB** (6.23)	96.00%± 2.00**A** (6.4)

*

The basal medium without addition of surfactants and with local urea and local phosphorus
** Surfactants were added to the medium at 1%
[1] Degradation rate
Incubation time = 15 days

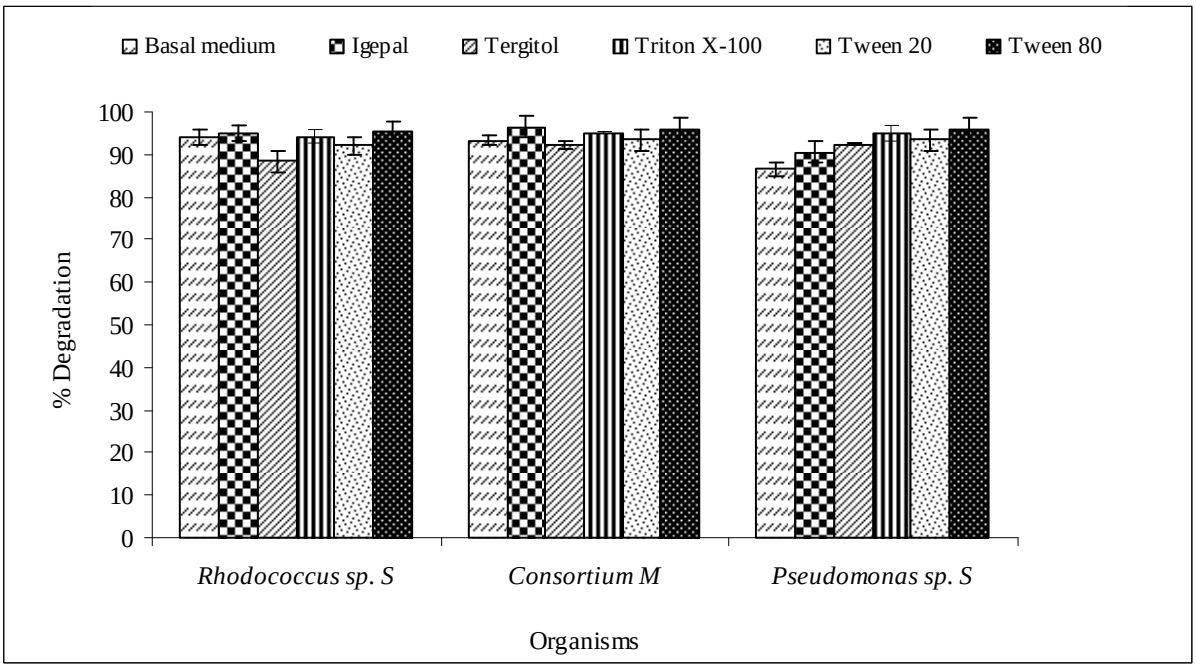


Fig. 21: Effect of surfactants on crude oil biodegradation by the tested bacterial isolates

Table 21: Effect of surfactants on OD of the tested bacterial isolates

Surfactant / Organism	Basal medium	Igepal	Tergitol	Triton X-100	Tween 20	Tween 80
	OD					
Rhodococcus sp. S	0.52	0.56	0.54	0.53	0.55	0.56
Consortium M	0.58	0.61	0.57	0.56	0.57	0.54
Pseudomonas sp. S	0.36	0.54	0.55	0.54	0.56	0.55

Table 22: Effect of different surfactants on the protein content of the tested bacterial isolates

surfactant / Organism	Basal medium *		Igepal		Tergitol		Triton X-100		Tween 20		Tween 80	
	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Protein content (mg)	protein content (mg/ml)	Total protein content (mg)	protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)
Rhodococcus sp.S	0.73	3.65	0.77	3.85	0.67	3.35	0.82	4.1	0.73	3.65	0.71	3.55
Consortium M	0.89	4.45	0.7	3.5	0.73	3.65	0.90	4.5	0.77	3.85	0.8	4
Pseudomonas sp. S	0.62	3.10	0.64	3.2	0.79	3.95	0.6	3	0.97	4.85	0.94	4.7

* The basal medium without addition of surfactants and with local urea and local phosphorus fertilizer as phosphorus source

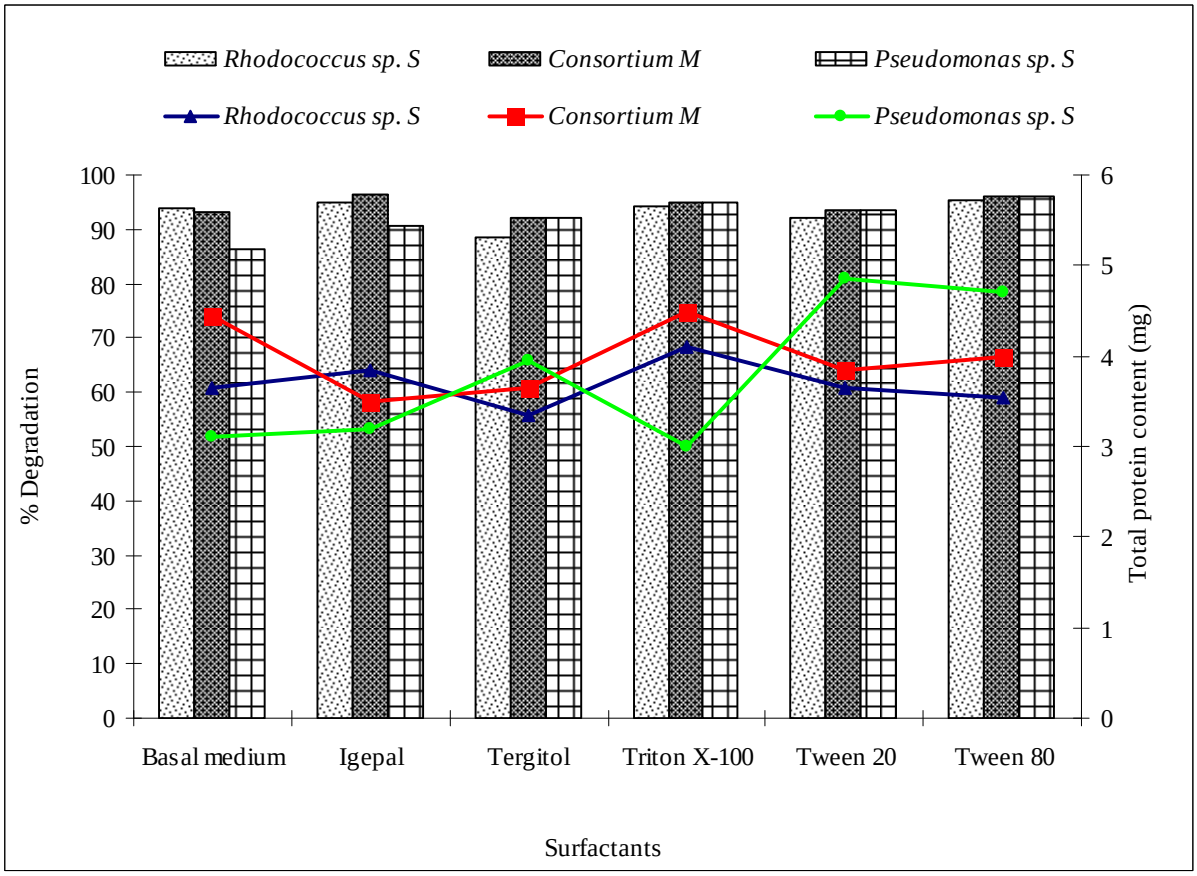


Fig.22. Relation between the degradation and the protein content of the tested bacterial isolates cultivated using different surfactants

3.3.7. Effect of crude oil concentration

The concentration of oil was elevated from 1% to 6%. Triton X-100 was added at 1%. The results presented in Table 23 and demonstrated in Fig. 23 showed that *Rhodococcus* sp .S could degrade 94% from 1% crude oil, while the degradation ability was reduced by about 30% at 5% crude oil. The degradation ability of the consortium and *Pseudomonas* sp. S was also reduced by about 22 and 40% respectively at 5% in comparison with the degradation at 1%. Increasing the crude oil concentration decreased the degradation rate by all tested organisms with the maximum reduction at 6%.

The OD values at different crude oil concentration are presented in Table 24. It was observed that the maximum OD values were obtained at 1% of crude oil concentration .The OD values decreased by increasing the concentration of crude oil except in the case of the consortium M where the OD slightly increased from 0.56 at 1% to 5.7 at 2% . The lowest OD values were observed at 6% crude oil.

The results of total protein content are presented in Table 25. It was found that the protein content of *Rhodococcus* sp. S, consortium M and *Pseudomonas* sp. S slightly increased at 2% in comparison with 1%. Increasing the crude oil concentration above 2% decreased the protein content of the tested bacteria with a minimum value (0.7 mg) for *Rhodococcus* sp.S growing at 6%.

It was also observed that the decrease in the degradation ability of the tested organisms was correlated with a decrease in protein content (Fig. 24). The lowest degradation value (43.5%) was obtained at the lowest protein content (0.7 mg).

Table 23: Effect of crude oil concentration on the biodegradation by the tested bacterial isolates

Crude oil concentration / Organism	1%	2%	3%	4%	5%	6%
	% Degradation					
Rhodococcus sp. S [b]	94.25%± 1.75 **A** (6.28)	89.00%± 1.75 **AB** (5.93)	74.50%± 2.50 **D** (4.97)	75.50%± 1.00 **CD** (5.03)	66.75%± 0.75**DE** (4.45)	43.50%± 2.00 **G** (2.90)
Consortium M[a]	95.00%± 2.50 **A** (6.33)	90.50%± 1.50 **AB** (6.03)	83.25%± 1.25 **BC** (5.55)	81.50%± 1.50 **C** (5.43)	73.50%± 1.50 **D** (4.90)	45.75%± 1.75 **I** (3.05)
Pseudomonas sp. S [b]	95.00%± 2.00 **AB** (6.33)	87.50%± 2.00 **B** (5.83)	84.75%± 2.25 **BC** (5.65)	63.25%± 1.75 **E** (4.22)	55.25%± 1.25 **F** (3.68)	48.00%± 2.00 **H** (3.2)

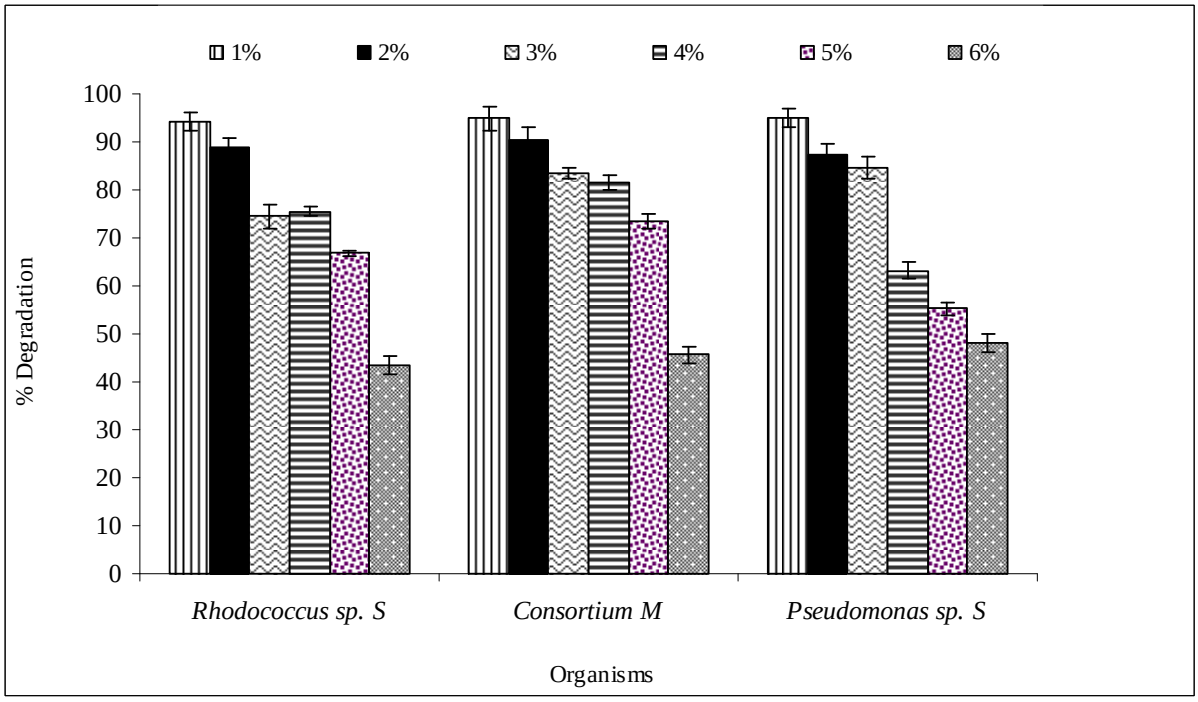


Fig.23: Effect of crude oil concentration on the biodegradation by the tested bacterial isolates

Table 24: Effect of crude oil concentration on OD of the tested bacterial isolates

Crude oil concentration / Organism	1%	2%	3%	4%	5%	6%
	OD					
Rhodococcus sp. S	0.53	0.52	0.48	0.45	0.35	0.23
Consortium M	0.56	0.57	0.56	0.54	0.45	0.37
Pseudomonas sp. S	0.54	0.54	0.53	0.42	0.4	0.21

Table 25: Effect of crude oil concentration on the biodegradation by the selected bacterial isolates

Crude oil concentration / Organism	1%		2%		3%		4%		5%		6%	
	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Protein content (mg)	protein content (mg/ml)	Total protein content (mg)	protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)	Protein content (mg/ml)	Total protein content (mg)
Rhodococcus sp.S	0.82	4.1	0.9	4.5	0.45	2.25	0.23	1.15	0.32	1.6	0.14	0.7
Consortium M	0.9	4.5	0.95	4.75	0.67	3.35	0,54	2.7	0.5	2.5	0.28	1.4
Pseudomonas sp. S	0.6	3	0.63	3.15	0.52	2.6	0,25	1.25	0.21	1.05	0.19	0.95

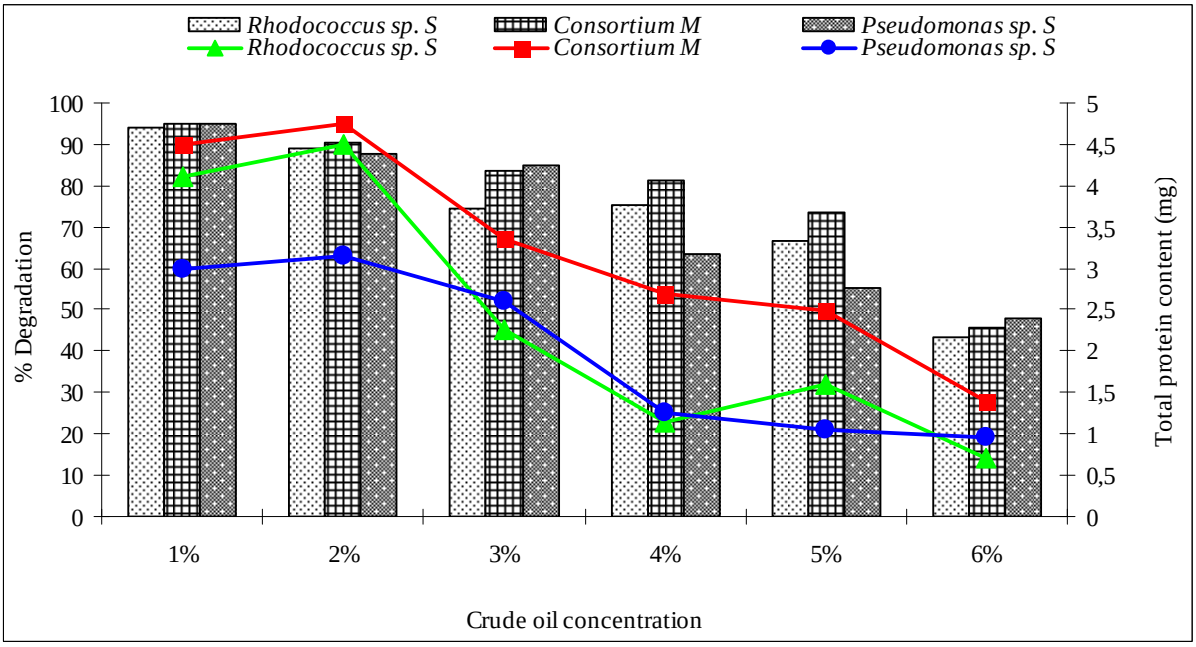


Fig. 24: Relation between the degradation and the protein content of the tested bacterial isolates at different crude oil concentrations

3.3.8. Effect of free and immobilized cells on crude oil degradation in marine medium

Crude oil biodegradation using free and immobilised cells on wheat straw and entrapment in alginate was performed in liquid medium containing local urea as nitrogen source and local phosphorus source as phosphorus source. The surfactant Triton X-100 was supplied at 1% (Fig.25). The experiment was realised in presence of 2% of crude oil. The results demonstrated in Table 26 and Fig. 26 revealed that the minimum biodegradation values were recorded after 3 days of incubation in all treatments, which did not exceed 51.5% by the cells immobilised on wheat straw. After 6 days, incubation the degradation was increased in all treatments to reach 83.5% by consortium cells immobilised on wheat straw. After 9 days, the results showed that the immobilised cells by entrapment in alginate stimulated oil biodegradation in comparison with free cells and immobilised cells on wheat straw. The immobilised cells by entrapment in alginate could degrade 87.75-92% from the 2% crude oil after 9 days, while immobilised cells on wheat straw had the capacity to degrade 88-90% of oil after 12 days. It was also found that wheat straw absorbed about 8-10% of crude oil.

d c b a

Immobilized cells on wheat straw

d c b a

Immobilized cells in alginate

Fig.25: Biodegradation of crude oil using immobilized cells

a) Control; b) Immobilized cells of *Rhodococcus* sp. S after 15 days; c) Immobilized cells of consortium M after 15 days; d) Immobilized cells of *Pseudomonas* sp. S after 15 days

Table 26: Effect of free and immobilised bacterial cells on crude oil biodegradation in marine medium after different incubation periods

Time (Days) / Organism	Free cells					Immobilized cells on wheat straw					Immobilized cells by entrapment in alginate				
	3	6	9	12	15	3	6	9	12	15	3	6	9	12	15
	% Degradation					% Degradation					% Degradation				
Rhodococcus sp. S	42.50	51.25	78.25	80.50	89.00	40.00	61.25	77.50	89.75	88.00	35.00	67.25	87.75	92.75	92.50
Consortium M	47.00	60.50	70.50	84.75	90.50	51.50	83.50	92.50	90.00	93.00	45.50	80.00	92.00	94.50	95.00
Pseudomonas sp. S	33.75	65.00	80.00	83.50	87.50	36.75	60.00	74.00	88.00	90.50	47.25	56.75	89.25	93.00	90.00

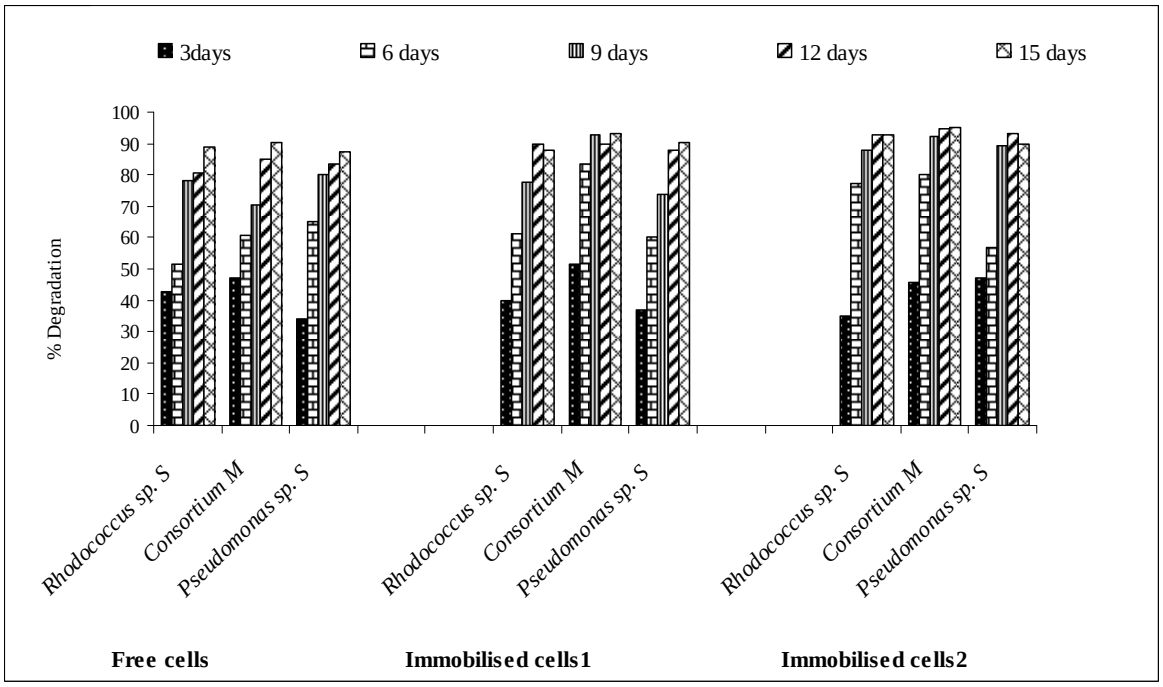


Fig. 26. Effect of free and immobilised bacterial cells on crude oil biodegradation in marine medium after different incubation periods

Immobilised cells1: Immobilized cells on wheat straw
Immobilised cells2: Immobilized cells by entrapment in alginate

III.
RESULTS
PART II: LAB MICROCOSMS

Part II. Lab microcosms

3.4. Bioremediation of crude oil contaminated sea water under different conditions

Bioremediation of sea water contaminated with crude oil was tested using different nitrogen, phosphorus sources at different temperatures. In addition, the effect of immobilization and free cells was also examined. Table 27 illustrates the chemical analysis of sea water collected from Ben Mhidi beach (post 3).

The number of the heterotrophic bacteria present in sea water, presented as Log CFU/ml, was 6.7- 6.9 while the number of crude oil degrading bacteria was 1.3-1.49 Log CFU/ml

Table 27: Chemical composition of sea water collected from Ben Mhidi beach (post 3)

Salinity (mg/l)	SO_4 (mg/l)	Mg (mg/l)	Ca (mg/l)	K (mg/l)	Zn (mg/l)	Fer (mg/l)	Cu (mg/l)	NH_4 (mg/l)	NO_3 (mg/l)	$P O_4$ (mg/l)	Total hydrocarbons (mg/l)
36.5	2600	1325	420	405	traces	traces	traces	0.002	0.003	0.005	0.1

3.4.1. Bioremediation of crude oil contaminated sea water in lab- scale microcosms at room temperature

The organisms *Rhodococcus* sp.S and *Pseudomonas* sp. S and the consortium M which showed high degradation rate in marine medium were selected to test their ability to bioremediate the crude oil contaminated sea water (Fig.27).

In this experiment the effect of bioaugmentation of the tested organisms (alone or in consortium), was tested. Attenuation of the natural flora or its biostimulation was also tested. A control sample was made by sterilizing 1L crude oil contaminated sea water at 120°C for 30 min. The results obtained were illustrated in Table 28 and Fig. 28. It was found that the degradation dependent on the time and the type of treatment. After one week the degradation rate ranged between 25.5 to 55% with the highest value in presence of consortium M (55%) . After 6 weeks the degradation rate was about 55.5-95% with the lowest value in case of attenuation (55.5%) and the highest value in case of consortium M (95%). The use of consortium C, which consisted of a mixture of the tested organisms did not have a significant effect on crude oil biodegradation in comparison with the use of the organisms one at a time. The results also show that the crude oil degradation by consortium C reached 88% after 6 weeks in comparison with 93% by *Rhodococcus* sp. S, 95% by consortium M and 89.5 % by *Pseudomonas* sp. S.

The biostimulated treatments gave maximum degradation (70.5%) after 6 weeks of incubation. The sterilized sample (control) showed no change in its crude oil content after 6 weeks.

The effect of the bioremediation treatments on the cumulative percent of degradation was calculated and presented in Table 28 . After six weeks of incubation, the highest percentage of degradation (76.5%) was observed when the sea waster was bioaugmented with the consortium (M). The lowest percentage of degradation was found upon natural attenuation of the contaminated sea water (43.2%). For the other treatments (biostimulation, bioaugmentation with *Rhodococcus* sp. S or with *Pseudomonas* sp. S), the cumulative percentage of degradation was between 59.33-70.33%. Fig. 29 shows the chromatographic patterns of crude oil biodegradation in sea water.

The number of total aerobic heterotrophic bacteria and crude oil degrading bacteria present in the crude oil contaminated sea water upon the bioremediation treatments is presented in Table 29 and illustrated in Fig. 30. The results showed that there was an increase in the aerobic heterotrophic bacterial population after the first week. It was found that the

population of heterotrophic bacteria at zero time was 6.9 CFU/ml which increased to reach the value between 7.2 – 8.79 Log CFU/ml after the first week in the different treatments. Increasing the incubation time, increased the population density with some fluctuations in some treatments to reach its maximum value (9.4 Log CFU/ml sea water) in case of bioaugmentation using consortium M. The lowest number was recorded in case of attenuation treatment (8.01 Log CFU/ml after six weeks)

The increase of heterotrophic bacteria was accompanied with an increase in the number of crude oil degrading bacteria. The results showed that the number of crude oil degrading bacteria was very low at zero time (1.49 Log CFU/ml) and increased after the first week to reach 2.5-3.73 Log CFU/ml. The population density of crude oil degrading bacteria increased with increasing the time with some fluctuations in some cases. It reached 5.1 Log CFU/ml in bioaugmentation treatment by consortium M after six weeks of incubation, while the lowest number was obtained in attenuation treatment after the same period (3.85 Log CFU/ml)

From the results demonstrated in Fig. 28 and 30, it was found that the increase in the degradation rate was correlated with an increase in the number of crude oil degrading bacteria with some fluctuations. The highest degradation percentages (93 and 95%) were accompanied with the highest number of crude oil degrading bacteria (5.11 and 5.02 respectively)

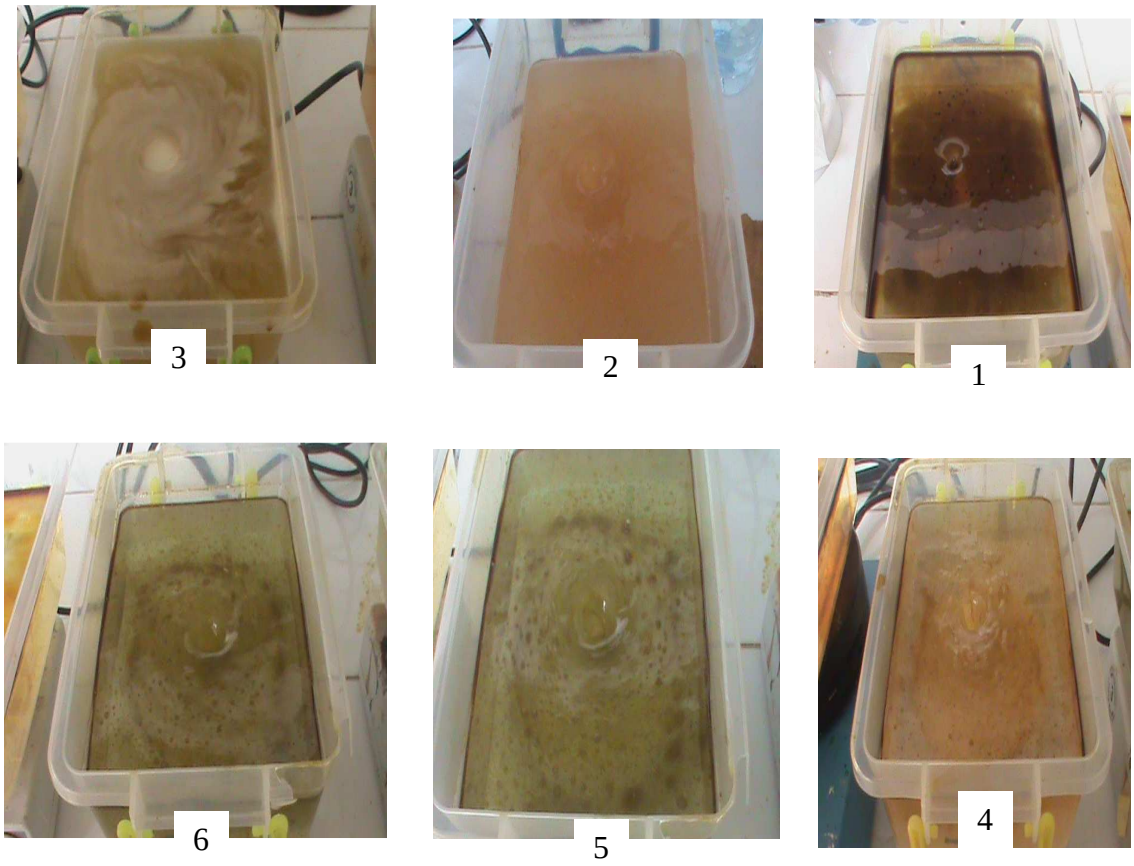


Fig.27. Bioremediation of crude oil contaminated sea water in lab- microcosms after six weeks. 1)Control; 2)Bioagmentation by *Rhodococcus* sp. S; 3) Bioaugmentation by consortium M; 4) Bioaugmentation by *Pseudomonas* sp. S; 5) Biostimulation 6) Attenuation

Table 28: Bioremediation of crude oil contaminated sea water

Time (week) / Treatment	1	2	3	4	5	6	Cumulative % of degradation*
	% Degradation						
Rhodococcus sp.S	48	52	62	775	89.5	93	70.33
Consortium M	55	61.5	75.5	83.5	88.5	95	76.5
Pseudomonas sp. S	35	50.5	65	76.5	81	89.5	66.25
Consortium C	38	49.5	54.5	73	71.5	88	62.42
Biostimulation	40.5	45.5	57	72.5	70	70.5	59.33
Attenuation	25.5	35	41.5	48	66	55.5	43.2

pH: 7.4- 7.6
Temperature: 20-24 ºC
* Cumulative % = ∑ % of degradation / time

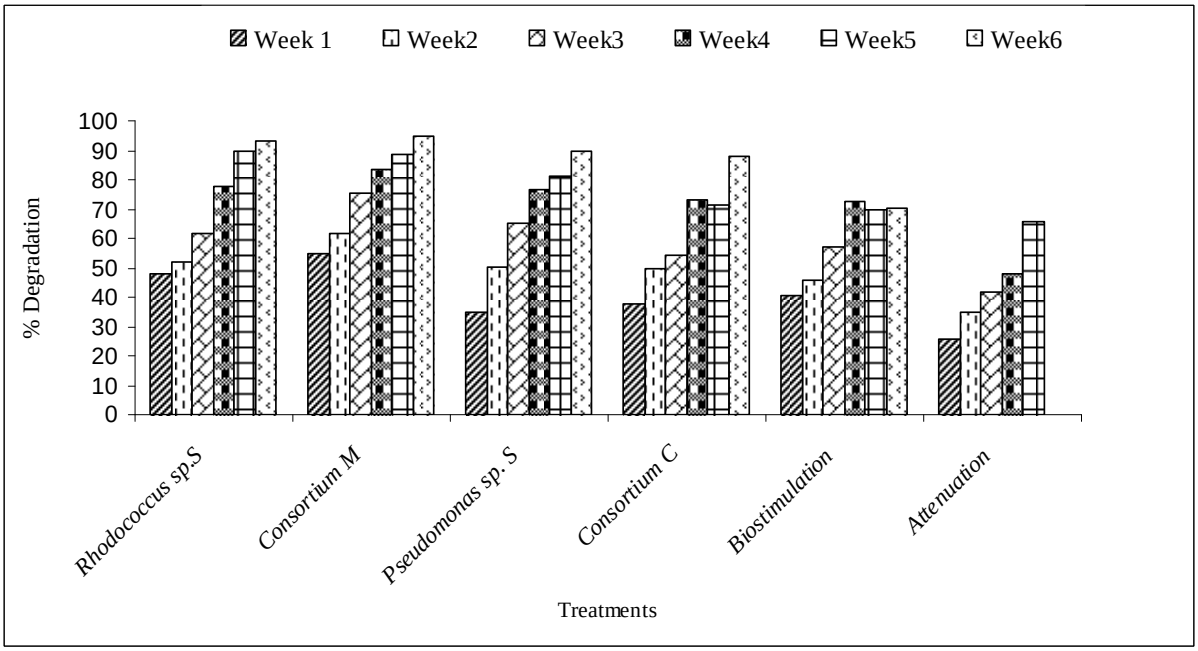
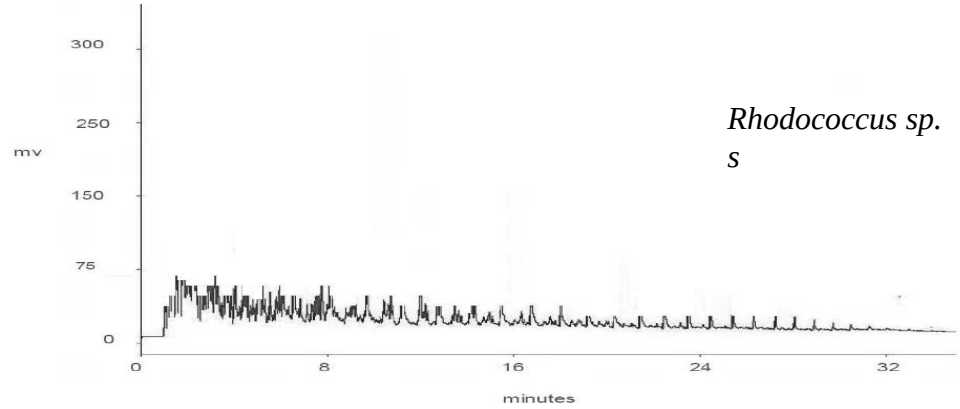
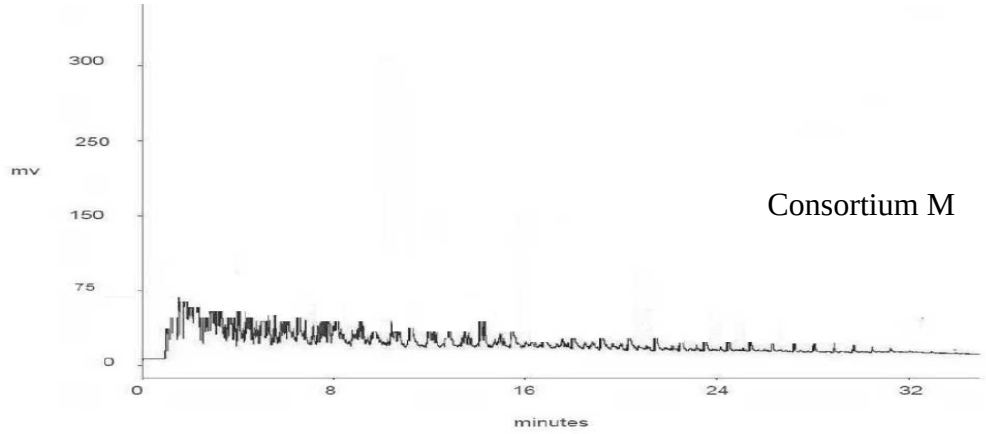


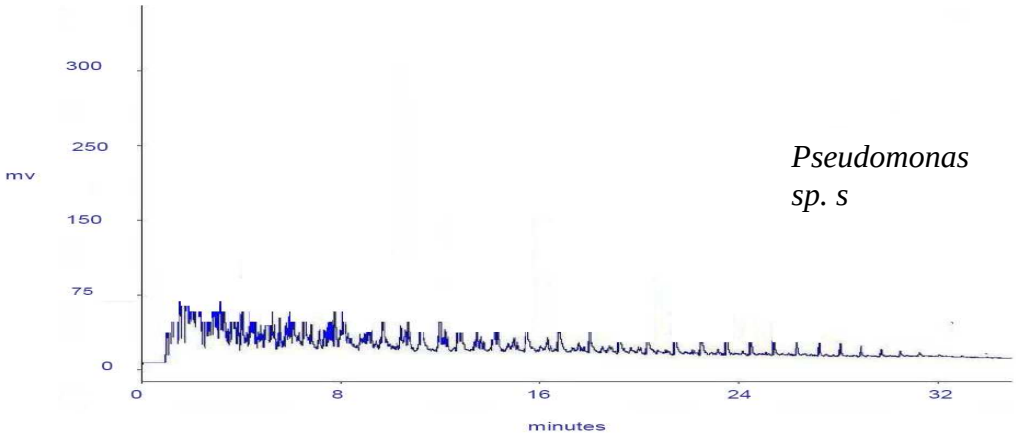
Fig. 28. Bioremediation of crude oil contaminated sea water



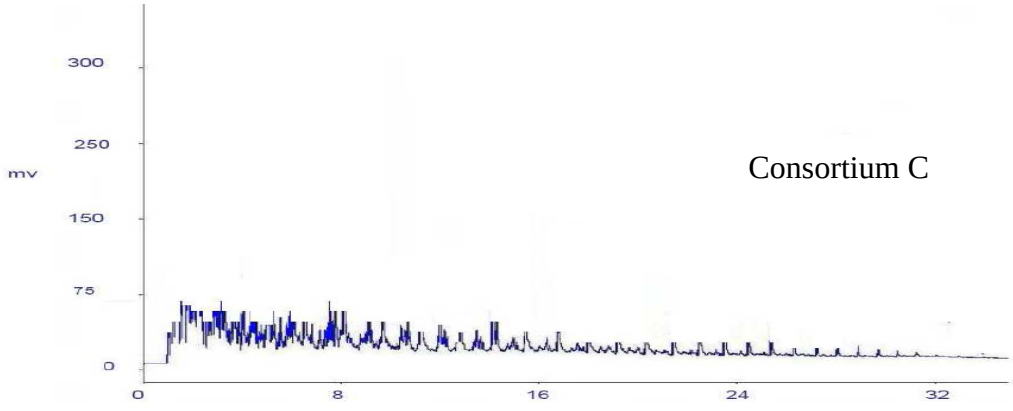
Rhodococcus sp. s



Consortium M



Pseudomonas sp. s



Consortium C

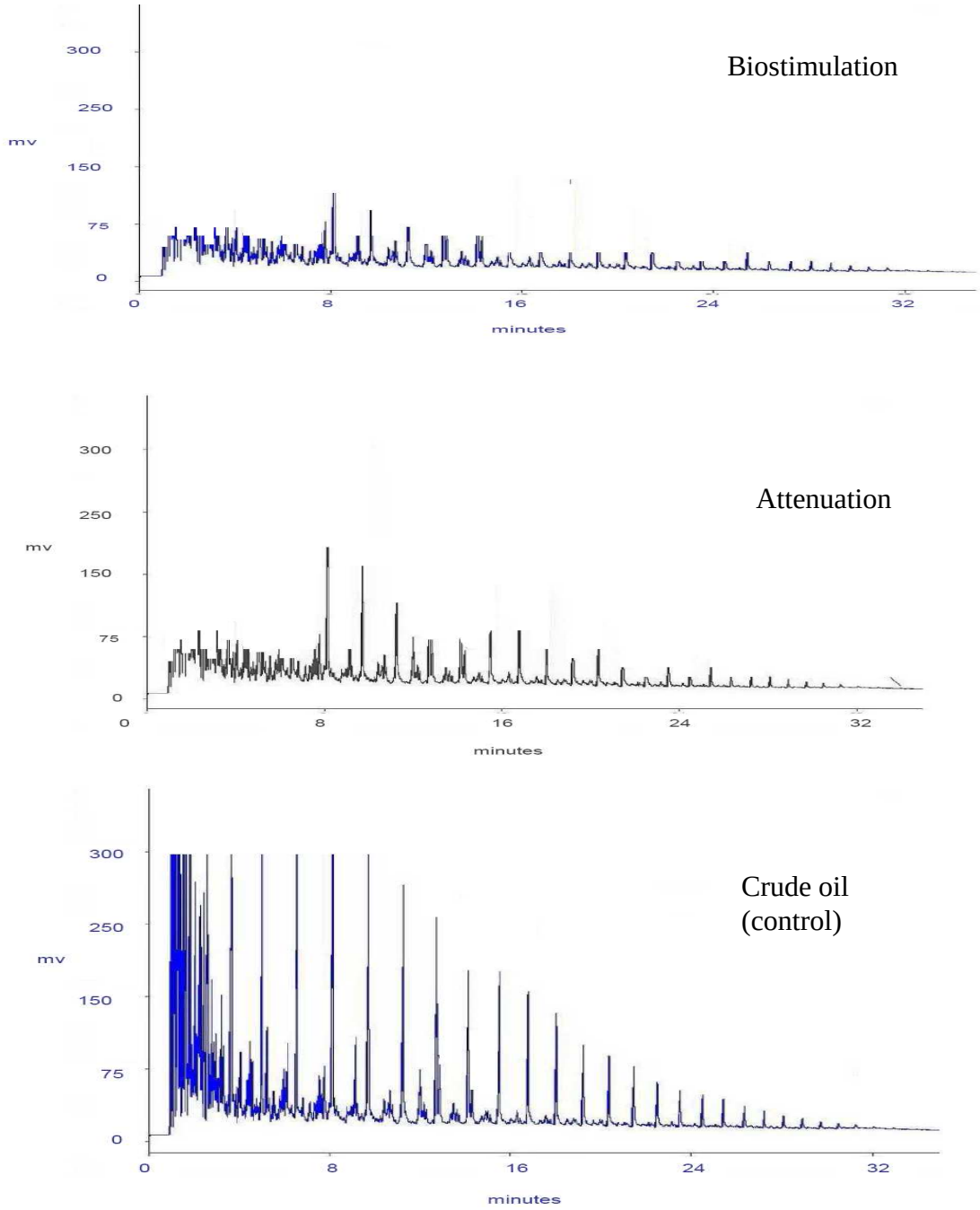


Fig 29. Gas chromatogram of crude oil biodegradation in sea water after six weeks

Table29. The total number of aerobic heterotrophic bacteria and crude oil bacteria during the bioremediation experiments

Treatment / Time (Week)	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
	H	C	H	C	H	C	H	C	H	C	H	C
Rhodococcus sp.S	8.57	3.73	8.89	4.2	8.96	4.6	8.91	4.5	9	4.75	9.22	5.1
Consortium M	8.79	3.71	9.2	3.97	9.01	4.62	9.08	4.6	9.3	4.8	9.4	5.02
Pseudomonas sp. S	8.67	3.7	8.75	4.35	8.88	4.45	9.06	4.5	9.1	4.65	9.2	4.75
Consortium C	8.5	3.54	8.67	4.5	8.71	4.58	8.89	4.49	9.06	4.66	9.05	4.85
Biostimulation	7.47	3.3	8.65	4.43	8.81	4.38	8.75	4.6	8.89	4.4	8.81	4.55
Attenuation	7.2	2.5	7.87	3.42	7.85	3.5	7.72	3.4	7.89	3.5	8.01	3.85

H: The number of aerobic heterotrophic bacteria (Log CFU/ml)
C: The number of crude oil degraing bacteria (Log CFU/ml)
At zero time the number of heterotrophic bacteria was 6.9 and the number of crude oil degradaing bacteria was 1.49

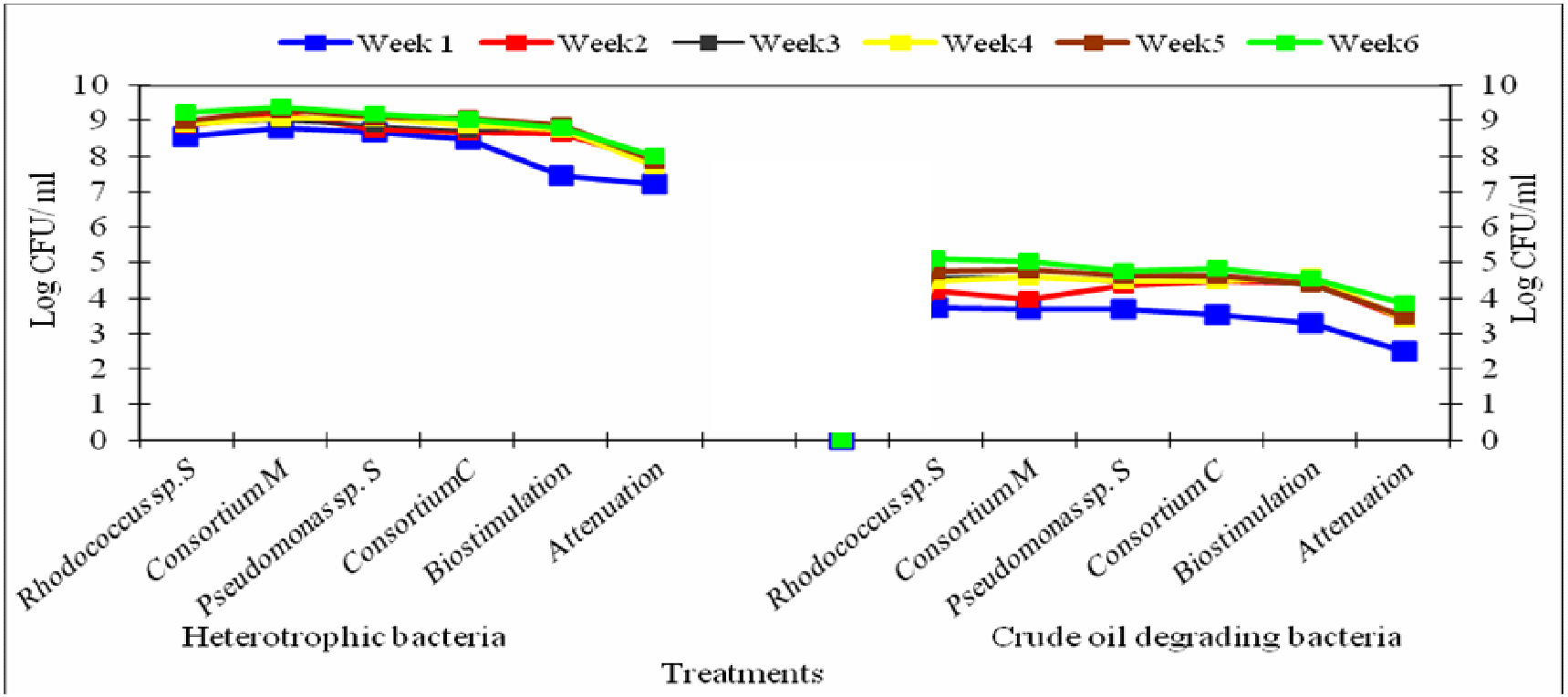


Fig.30. The total number of aerobic heterotrophic bacteria and crude oil bacteria during the bioremediation experiments
At zero time the number of heterotrophic bacteria was 6.9 (Log CFU/ml) and the number of crude oil degradaing bacteria was 1.49 Log (CFU/ml)

3.4.2. Effect of different nitrogen sources on crude oil biodegradation in contaminated sea water

To test the effect of nitrogen sources, the ammonium nitrate which was the sole nitrogen source in the last experiment (3.4.1) was replaced by local, ammonium sulfate , sodium nitrate , local nitrogen fertilizer (calcium ammonium nitrate),local urea, urea 46% (Egyptian commercial fertilizer) on equal nitrogen basis. Manure was also used as nitrogen source at a concentration of 5% (w/v). Samples were taken each week for determination of the residual crude oil and the bacterial density.

The results presented in Table 30 and Fig. 31 revealed that using commercial fertilizers as nitrogen sources stimulated crude oil degradation in comparison with chemical nitrogen sources. It was found that the lowest degradation values in the first week were obtained with the pure chemical nitrogen sources (ammonium sulfate and sodium nitrate) except in case of bioaugmentation with *Rhodococcus* sp. S and consortium M with ammonium sulfate. Increasing the time increased the degradation of crude oil. After five weeks, the results show that the maximum biodegradation was obtained with local nitrogen fertilizer (95.5, 94.5 and 92.5 for *Rhodococcus* sp. S, consortium M and *Pseudomonas* sp. S respectively) with the exception for biostimulation in which the maximum biodegradation (83%) was obtained with local urea. On the other hand, after six weeks local urea gave the highest degradation with consortium M and *Pseudomonas* sp. S. It was observed that the addition of manure stimulated the biodegradation of crude oil.

The highest cumulative percent was obtained in bioaugmentation by *Rhodococcus* sp.S with manure as nitrogen source (79.5%), while the lowest cumulative percent was recorded in case of biostimulation with ammonium sulfate (51.42%).

The number of total aerobic heterotrophic bacteria and crude oil degrading bacteria is demonstrated in Table 31 and Fig.32 . The results showed that the bacterial density increased in time with fluctuations in some cases. The heterotrophic bacterial density at zero time was low (6.9 Log CFU/ml), and it increased with increasing time to reach 8.3-9.47 Log CFU/ml after six weeks. The lowest bacterial densities were obtained in the presence of chemical nitrogen sources. The highest densities were observed in crude oil contaminated sea water treated by consortium M in presence of urea 46 % and local urea as nitrogen sources (9.47 and 9. 44 Log CFU/ml respectively). This was followed by *Rhodococcus* sp.S with the urea 46%, local urea and local nitrogen fertilizer nitrogen (9.3; 9.35 and 9. 36 Log CFU/ml respectively). The lowest density was obtained in biostimulation treatment with sodium nitrate as nitrogen source (8.3%).

The results also revealed that the increase in the total heterotrophic bacterial density is accompanied with an increase in the number of crude oil degrading bacteria. It was found that the number of crude oil degrading bacteria at zero time was 1.49 Log CFU/ml and reached its maximum (5.35 Log CFU/ml) in the microcosm treated with the consortium M and urea 46% as nitrogen source after six weeks. On the other hand, the lowest number of crude oil degrading bacteria was obtained in the biostimulated microcosm using ammonium phosphate as nitrogen source.

From the results demonstrated in Fig. 31 and 32, it was observed that the biodegradation of crude oil more or less correlated with the number of crude oil degrading bacteria. Local urea was selected to complete this part of the present research, due to its availability and inexpensive cost.

Table 30: Effect of some nitrogen sources on crude oil degradation in contaminated sea water

Time (Week) / Treatment	week 1	week2	week3	week4	week5	week6	Cumulative %
	% Degradation						
Rhodococcus sp. S+ ammonium sulfate	43.5	67.5	73.5	71.5	83	85.5	70.75
Rhodococcus sp. S+ sodium nitrate	34	62.5	75	85.5	81.5	85.5	70.67
Rhodococcus sp. S+ urea 46%	55.5	63.5	85.5	71.5	90.5	94	76.75
Rhodococcus sp. S+ local urea	46.5	53.5	85	73.5	89.5	92	73.33
Rhodococcus sp. S+ N fertilizer	40.5	59.5	73.5	93.5	95.5	93.5	74.83
Rhodococcus sp. S+ manure	60	77.5	63.5	89	95.5	91.5	79.5
Consortium M+ ammonium sulfate	41.5	57	75.5	78.5	84.5	90.5	71.25
Consortium M+ sodium nitrate	40.5	48.5	73.5	65.5	87.5	92.5	68.00
Consortium M+ urea 46%	47.5	55.5	78.5	87.5	95	93.5	76.25
Consortium M+ local urea	37.5	55.5	77.5	93.5	86	94	74.00
Consortium M+ N fertilizer	55.5	71	68.5	85	94.5	92.5	77.83
Consortium M + manure	50	63.5	75	70.5	90.5	90	73.25
Pseudomonas sp. S+ ammonium sulfate	25.5	45.5	65.5	60	78.5	67.5	57.08
Pseudomonas sp. S+ sodium nitrate	40.5	35.5	61	53.5	79.5	77.5	57.92
Pseudomonas sp. S+urea 46%	63	43.5	67.5	55	78	84.4	65.23
Pseudomonas sp. S+ local urea	50.5	47.5	57	88	74.5	91.5	68.17
Pseudomonas sp. S+ N fertilizer	47.5	68	74.5	88.5	92.5	87.5	76.42
Pseudomonas sp. S + manure	53.5	65	87	85.5	91	88.5	78.42
Biostimulation+ ammonium sulfate	35.5	43.5	61.5	45.5	55.5	67	51.42
Biostimulation + sodium nitrate	25.5	45.5	51.5	48	71.5	83.5	54.25
Biostimulation + urea 46%	52.5	63	47.5	59.5	70	78.5	61.83
Biostimulation + local urea	50	46.5	55	68.5	83	78.5	63.58
Biostimulation + local N fertilizer	51	62.5	75	78.5	67	81	69.17
Biostimulation + manure	56.5	70.5	81.5	68	74	75.5	71

pH: 7.4- 7.6
Temperature: 19- 22°C
* Cumulative % = ∑ % of degradation / time

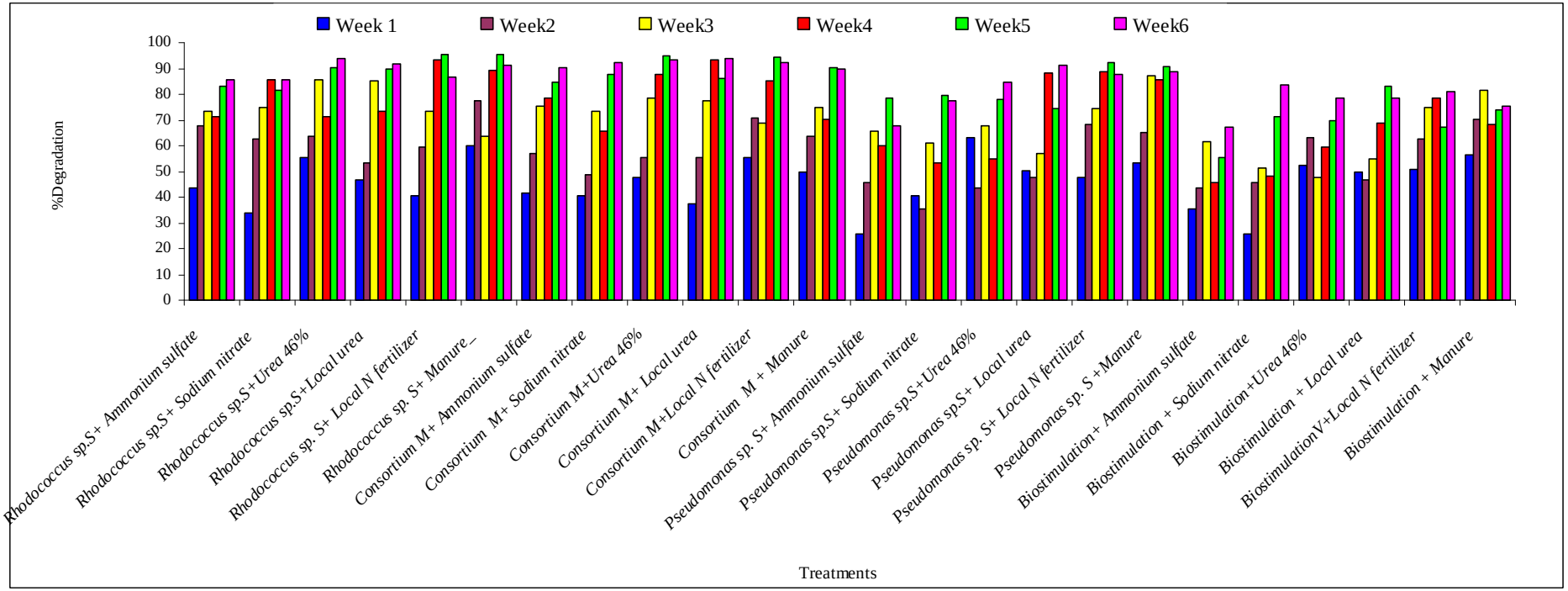


Fig.31. Effect of different nitrogen sources on crude oil degradation in contaminated sea water

Table 31:Effect of different nitrogen sources on the number of heterotrophic bacteria and crude oil degrading bacteria

Time (Week) / Treatment	Week1		Week2		Week3		Week4		Week5		Week6	
	H	C	H	C	H	C	H	C	H	C	H	C
Rhodococcus sp. S+ Ammonium sulfate	7.81	3.51	8.06	4.1	8.3	4.3	8.6	4.45	8.88	4.55	9.07	4.88
Rhodococcus sp. S+ Sodium nitrate	8.35	3.35	8.55	3.5	8.74	3.48	9	4.01	8.85	4.22	9.01	4.7
Rhodococcus sp. S+ Urea 46%	8.75	4.35	8.6	4.25	8.95	4.6	9.11	4.7	9.17	4.85	9.3	4.75
Rhodococcus sp. S+ Local urea	8.88	4.41	8.9	4.55	9.01	4.65	9.07	4.88	9.18	5.1	9.35	5.15
Rhodococcus sp. S+Local N fer tilizer	8.2	3.57	8.35	3.85	8.65	4.3	9.03	4.25	9.19	4.6	9.36	5.11
Rhodococcus sp. S+ Manure	8.5	4.1	8.75	4.38	9.05	4.45	8.94	5.15	9.11	5.06	9.26	5.02
Consortium M+ Ammonium sulfate	8	3.22	8.08	3.54	8.22	4.01	8.55	4.02	8.77	4.3	9.06	4.44
Consortium M+ Sodium nitrate	7.87	3.3	8.29	4.15	8.3	4.49	8.88	4.66	8.71	4.96	9.03	4.64
Consortium M+ Urea 46%	8.67	4.28	8.81	4.49	9.23	4.6	9.22	4.83	9.17	5.06	9.47	5.35
Consortium M+ Local urea	8.77	4.36	8.85	4.48	9.14	4.6	9.35	4.44	9.03	5.22	9.44	5.15
Consortium M+ Local N fertilizer	8.71	4.28	8.88	4.48	9.02	4.72	8.88	4.81	9.03	4.93	9.23	5
Consortium M + Manure	8.47	4.41	8.6	4.48	9.11	4.6	9.35	5.06	9.26	5.11	9.11	5.04
Pseudomonas sp. S+	7.89	3.14	7.9	3.2	8.04	3.32	8.18	3.38	8.47	4.07	8.91	5.1

Ammonium sulfate												
Pseudomonas sp. S+ Sodium nitrate	7.96	3.17	8.06	3.34	8	3.27	8.55	4.32	8.3	4.3	8.94	4.38
Pseudomonas sp. S+Urea 46%	8.43	3.97	8.39	4.13	8.55	4.11	8.95	4.48	9.11	4.73	9.22	4.94
Pseudomonas sp. S+ Local urea	8.24	3.85	8.54	4.05	8.8	4.25	9,03	4.68	9.17	4.83	9.07	5.18
Pseudomonas sp. S+ Local N fertilizer	8.13	3.96	8.6	3.99	8.88	4.36	8.98	4.67	8.88	4.58	9.23	4.48
Pseudomonas sp. S + Manure	8.34	3.9	8.22	4.06	8.69	4.38	8.9	4.72	9.01	4.87	9.22	4.7
Biostimulation+ Ammonium sulfate	7.79	3.07	7.92	3.32	8.13	3.18	7.99	3.58	8.3	3.51	8.43	4.15
Biostimulation + Sodium nitrate	8	3.06	8.47	3.62	8.39	4.07	8.47	4.06	8.55	4.32	8.3	4.22
Biostimulation + Urea 46%	8.39	3.5	8.43	3.22	8.59	4.14	8.71	4.38	8.88	4.22	8.79	4.75
Biostimulation + Local urea	8.07	3.2	8.39	3.48	8.59	4.08	8.55	4.3	8.6	4.1	8.88	4.85
Biostimulation + Local N fertilizer	7.81	3.16	8.3	3.39	8.47	3.62	8.71	4.49	8.69	4.45	8.95	4.7
Biostimulation + Manure	8.3	3.48	8.28	3.35	8.77	4.32	8.75	4.61	9	4.7	9.01	4.88

H: The number of aerobic heterotrophic bacteria (Log CFU/ml)
C: The number of crude oil degraing bacteria (Log CFU/ml)
At zero time the number of heterotrophic bacteria was 6.9 and the number of crude oil degradaing bacteria was 1.49

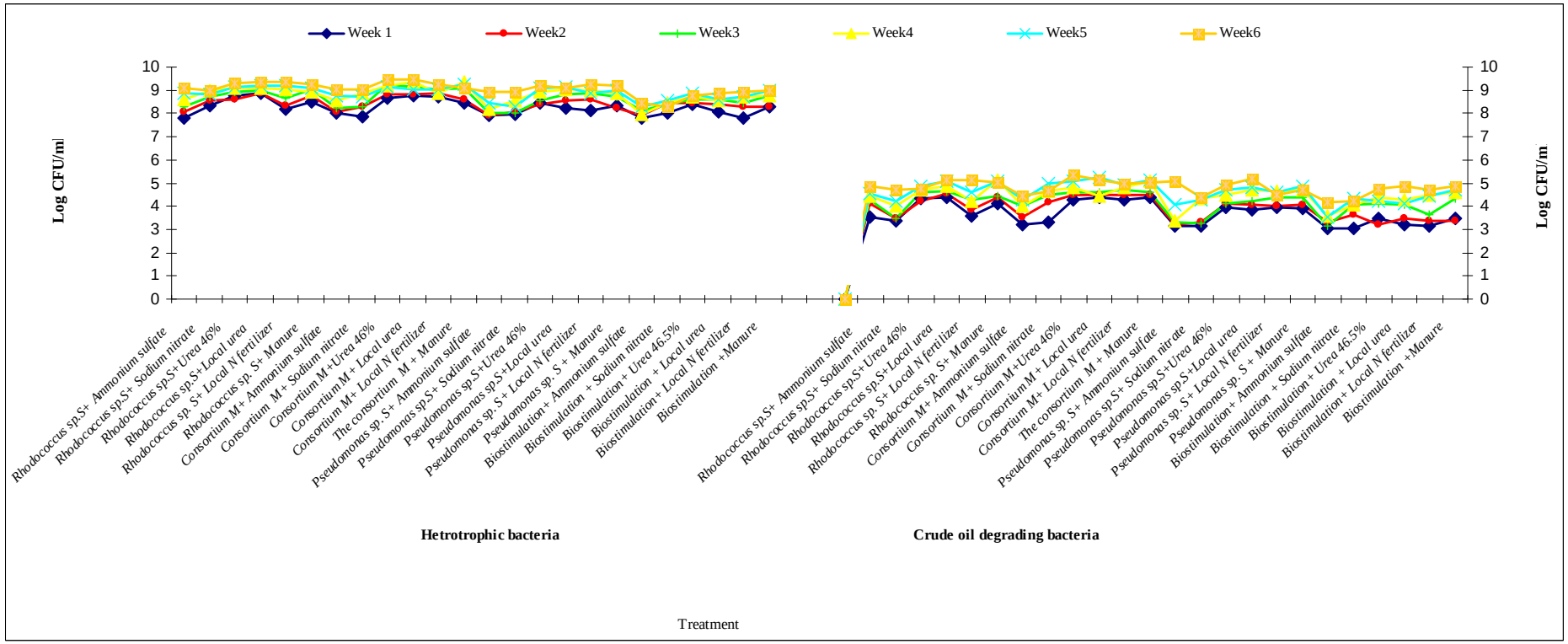


Fig.32: Effect of different nitrogen sources on the number of heterotrophic bacteria and crude oil degrading bacteria
At zero time the number of heterotrophic bacteria was 6.9 (Log CFU/ml) and the number of crude oil degradaing bacteria was 1.49 (Log CFU/ml).

3.4.3. Effect of different phosphorus sources on crude oil biodegradation in contaminated sea water

The disodium hydrogen phosphate and sodium dihydrogen phosphate used as phosphorus sources in the last experiment (3.4.2.) were substituted, on equal phosphorus basis, with super phosphate 15.5% (commercial source), ammonium phosphate and a local phosphorus fertilizer. Local urea was used as nitrogen source. Samples were taken each week for determination, the residual crude oil and the bacterial density.

The results are illustrated in Table 32 and Fig. 33. It was found that using super phosphate 15.5% and the local phosphorus fertilizer as phosphorus sources increased the rate of crude oil degradation in comparison with ammonium phosphate. After one week, the crude oil degradation was ranged between 40-57% in treatments with phosphorus fertilizers, except in bioaugmentation with *Pseudomonas* sp. S and super phosphate 15.5%. Maximum value (57%) was obtained with *Rhodococcus* sp. S in presence of super phosphate 15.5% after the same period. The rate of crude oil degradation increased with increasing the time to reach 95% in the microcosm bioaugmented with consortium M using super phosphate 15.5% as phosphorus source after 5 weeks. On the other hand, using local phosphorus fertilizer maximum degradation (92%) was obtained with consortium M, followed by *Rhodococcus* sp.S (90.5%), then biostimulation treatment (84.5%) and finally by *Pseudomonas* sp. S (71.5%) after the same time.

The cumulative percent of degradation 75.92% was recorded in contaminated sea water treated with consortium M using super phosphate 15.5% as phosphorus source. The lowest cumulative percent (56.67%) of degradation was obtained in contaminated sea water biostimulated with ammonium sulfate as phosphorus source.

The variation in the number of heterotrophic bacteria and crude oil degrading bacteria is presented in Table 33 and Fig.34. The density of heterotrophic bacteria increased with time. The results revealed that the number of heterotrophic bacteria at zero time was 6.7 and ranged between 8-8.94 Log CFU/ml after the first week.

The results showed that the development in the number of heterotrophic bacteria was high in treatments with phosphorus fertilizer in comparison with ammonium phosphate. It reached 9.03 Log CFU/ml after two weeks in the treatments with *Rhodococcus* sp.S and consortium M using the local phosphorus fertilizer. The highest value of heterotrophic bacteria was obtained in the treatment with consortium M and *Rhodococcus* sp.S using super phosphate 15.5% and local phosphorus fertilizer respectively (9.6 Log CFU/ml).

On the other hand, it was found that the increase in the number of heterotrophic bacteria was correlated with an increase in crude oil degrading bacteria for each treatment. Increasing time had a stimulating effect on the number of crude oil degrading bacteria. The results show that the highest bacterial densities were obtained with the consortium M in presence of super phosphate 15.15% (5.18 Log CFU/ml), followed by *Rhodococcus* sp.S (5.11 Log CFU/ ml) in presence of the same phosphorus source.

From the results demonstrated in Fig. 33 and 34, it was observed that the biodegradation of crude oil correlated with the number of crude oil degrading bacteria. The increase in the number of crude oil degrading bacteria was accompanied with an increase in the degradation rate with some fluctuations.. Local phosphorus fertilizer was selected to complete this part of the present research, due to its availability and inexpensive cost.

Table 32: Effect of different phosphorus sources on crude oil degradation in contaminated sea water

Time (week) / Treatment	1	2	3	4	5	6	Cumulative % of degradation*
	% Degradation						
Rhodococcus sp.S+ Super phosphate 15.5%	57	67	63.5	78.5	89.5	93	74.75
Rhodococcus sp.S+Local phosphorus fertilizer	43	58	70.5	86.5	90.5	88.5	72.83
Rhodococcus sp. S+Ammonium phosphate	37.5	50	41.5	64.5	82.5	87	60.5
Consortium M+ Super phosphate 15.5%	45	60.5	71.5	89.5	95	94	75.92
Consortium M+ Local phosphorus fertilizer	50.5	58.5	68	77.5	92	90	72.75
Consortium M+ Ammonium phosphate	41.5	69.5	75.5	77	67	88	69.75
Pseudomonas sp. S+ Super phosphate 15.5%	38.5	50	67	85.5	91.5	87	69.92
Pseudomonas sp.S+ Local phosphorus fertilizer	48.5	46.5	66.5	83	71.5	89.5	67.58
Pseudomonas sp.S+ Ammonium phosphate	38.5	53.5	47.5	75.5	66	79.5	60.08
Biostimulation+ Super phosphate 15.5%	45.5	40	65.5	78	84	80	65.5
Biostimulation + Phosphorus fertilizer P_2O_5	40	40.5	58.5	69.5	84.5	87	63.33
Biostimulation+ Ammonium phosphate	37.5	32.5	54.5	60	78	77.5	56.67

pH: 7.5-7.8
Temperature: 23-25°C
* Cumulative % = ∑ % of degradation / time

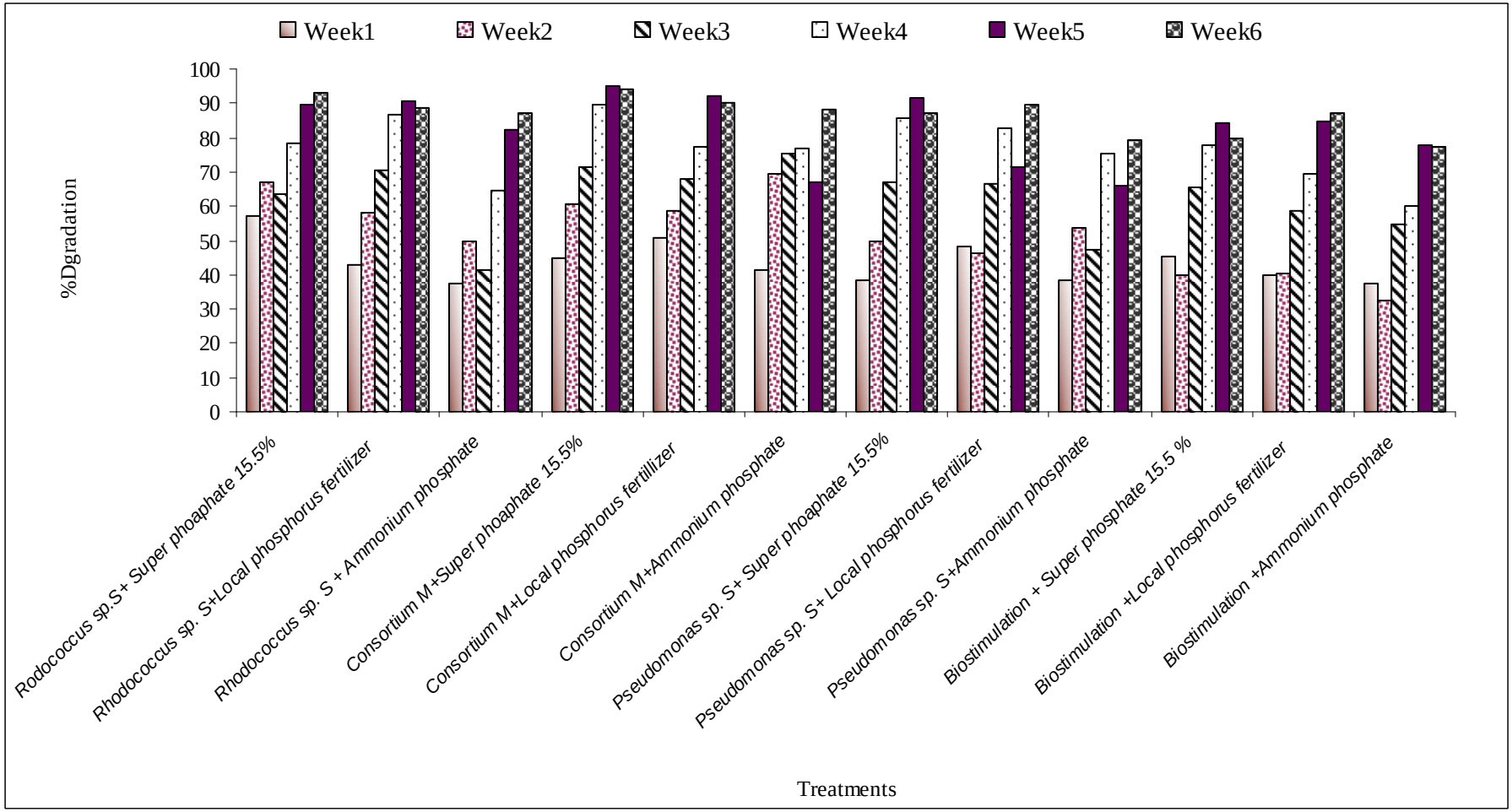


Fig.33: Effect of different phosphorus sources on crude oil degradation in contaminated sea water

Table 33: Effect of different phosphorus sources on the number of heterotrophic bacteria and crude oil degrading bacteria

Time (Week) / Treatment	Week1		Week2		Week3		Week4		Week5		Week6	
	H	C	H	C	H	C	H	C	H	C	H	C
Rhodococcus sp.S+ Super phosphate 15.5%	8.34	3.96	8.71	4.1	8.88	4.25	9.19	4.65	9.44	5.1	9.32	5.11
Rhodococcus sp.S+Local phosphorus fertilizer	8.74	3.85	9.03	4.49	9.11	4.6	9.44	4.83	9.26	5.04	9.6	5.06
Rhodococcus sp. S+Ammonium phosphate	8.06	3.54	8.32	3.71	8.55	3.97	8.75	4.62	8.69	4.85	9.01	4.9
Consortium M+ Super phosphate 15.5%	8.94	4.25	8.67	4.55	9.07	4.7	9.36	4.85	9.47	5.15	9.6	5.18
Consortium M+ Local phosphorus fertilizer	8.67	4.15	9.03	4.36	9.19	4.81	9.22	4.93	9.35	5.04	9.41	5.06
Consortium M+ Ammonium phosphate	8.55	4.13	8.71	3.97	8.88	4.72	8.69	4.66	9	4.83	8.94	5.02
Pseudomonas sp. S+ Super phosphate 15.5%	8.26	3.95	8.55	4.28	8.43	4.49	8.26	4.85	8.8	4.75	9.09	4.9
Pseudomonas sp.S+ Local phosphorus fertilizer	8.24	4.2	8.69	4.3	8.88	4.65	9.17	4.45	8.94	5	9.14	5.1
Pseudomonas sp.S+ Ammonium phosphate	8.32	3.85	8.47	4.01	8.55	4.3	8.97	4.55	9.14	4.7	9.19	4.88
Biostimulation+ Super phosphate 15.5%	8.11	3.32	8.34	4.06	8.28	4.38	9.03	4.25	9.09	4.38	8.94	4.75
Biostimulation + Phosphorus fertilizer P_2O_5	8.13	3.22	8.43	3.95	8.5	4.35	8.6	4.47	8.9	4.72	9	4.75
Biostimulation+ Ammonium phosphate	8	3.14	8	4.2	8.3	3.85	8.55	4.35	8.6	4.45	8.8	4.25

H: The number of aerobic heterotrophic bacteria (Log CFU/ml)

C: The number of crude oil degraing bacteria (Log CFU/ml)

At zero time the number of heterotrophic bacteria was 6.7 and the number of crude oil degradaing bacteria was 1.3

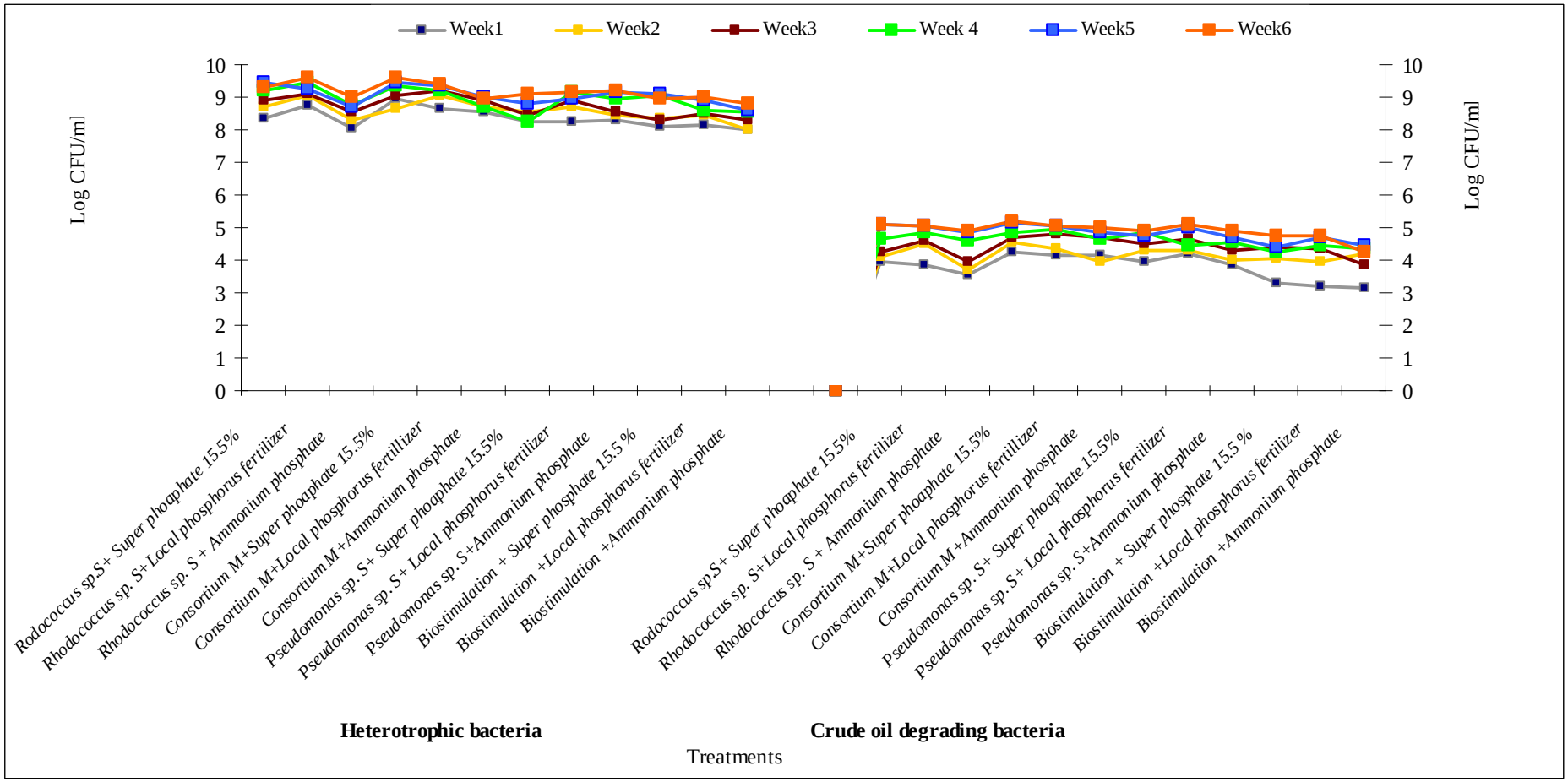


Fig. 34: Effect of different phosphorus sources on the number of heterotrophic bacteria and crude oil degrading bacteria
At zero time the number of heterotrophic bacteria was 6.7 (Log CFU/ml) and the number of crude oil degradaing bacteria was 1.3(Log CFU/ml).

3.4.4. Crude oil biodegradation in contaminated sea water using free and immobilized cells at the temperature range 18-20ºC

The effect of free and wheat straw immobilized cells on crude oil degradation in contaminated sea water was tested (Fig.35). The results are demonstrated in Table 34 and Fig. 36. It was found that the immobilized cells stimulated crude oil degradation than free cells in all treatments. The results after four weeks show that the immobilized cells degraded crude oil faster than the free cells by about 20, 13 and 16% for *Rhodococcus* sp. S, consortium M and *Pseudomonas* sp. S respectively and decreased with increasing time to reach about 9, 11 and 15% in the same order. The data of cumulative percent of degradation also revealed that immobilized cells enhanced the degradation of oil by about 18, 13 and 21% than the free cells. It was also noted that the time needed for crude oil degradation was reduced from six weeks to four weeks in case of immobilized cells, which ranged from 88to 92%. Fig. 37 shows the chromatographic analysis of crude oil after treatment with free and immobilized cells of the tested organisms.

Dehydrogenase activity in sea water during the bioremediation process has been used to monitor the microbial activity. Table 35 and Fig. 38 show the results obtained for dehydrogenase activity measured as increase in optical density (OD) at 546 nm. It was found that the activity depends on the incubation time, bioremediation treatments and the state of the cells (free or immobilized).

Increasing the incubation time increased the value of dehydrogenase (increase in OD) in both treatments (free and immobilized) with a slight fluctuation in some cases. Maximum OD (0.305) was obtained with immobilized cells of consortium M after five weeks. On the other hand, minimum value (0.055) was obtained in bioaugmentation treatment with *Pseudomonas* sp. S after one week. Generally, the dehydrogenase activity was higher in treatment with immobilized cells than in free cells.

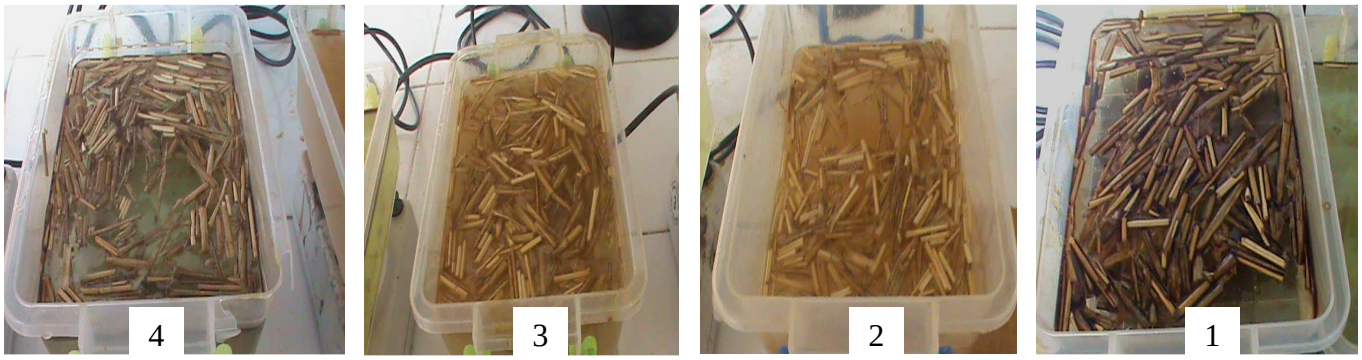


Fig. 35. Biodegradation of crude oil using immobilized cells on wheat straw after six weeks
1) Control; 2) *Rhodococcus* sp. S,3) Consortium M; 4) *Pseudomonas* sp.S

Table 34: Biodegradation of crude oil in sea water using free and immobilized cells at temperature range 18-20ºC

Time (weeks) / Organism	Free cells							Immobilized cells on wheat straw						
	1	2	3	4	5	6	Cumulative % of degra-dation	1	2	3	4	5	6	Cumulative % of degra-dation
	% Degradation							% Degradation						
Rhodococcus sp. S	32	44.5	65	71	68.75	80	60.22	41.5	65	71	88	88	87	73.42
Consortium M	40.25	56.25	70.25	81	85	82.75	69.25	47.25	68.5	82	92.75	92	92	79.08
Pseudomonas sp. S	25.5	37	56	72.75	70	77	56.38	38	45	77.25	86.5	91.25	90.5	71.42

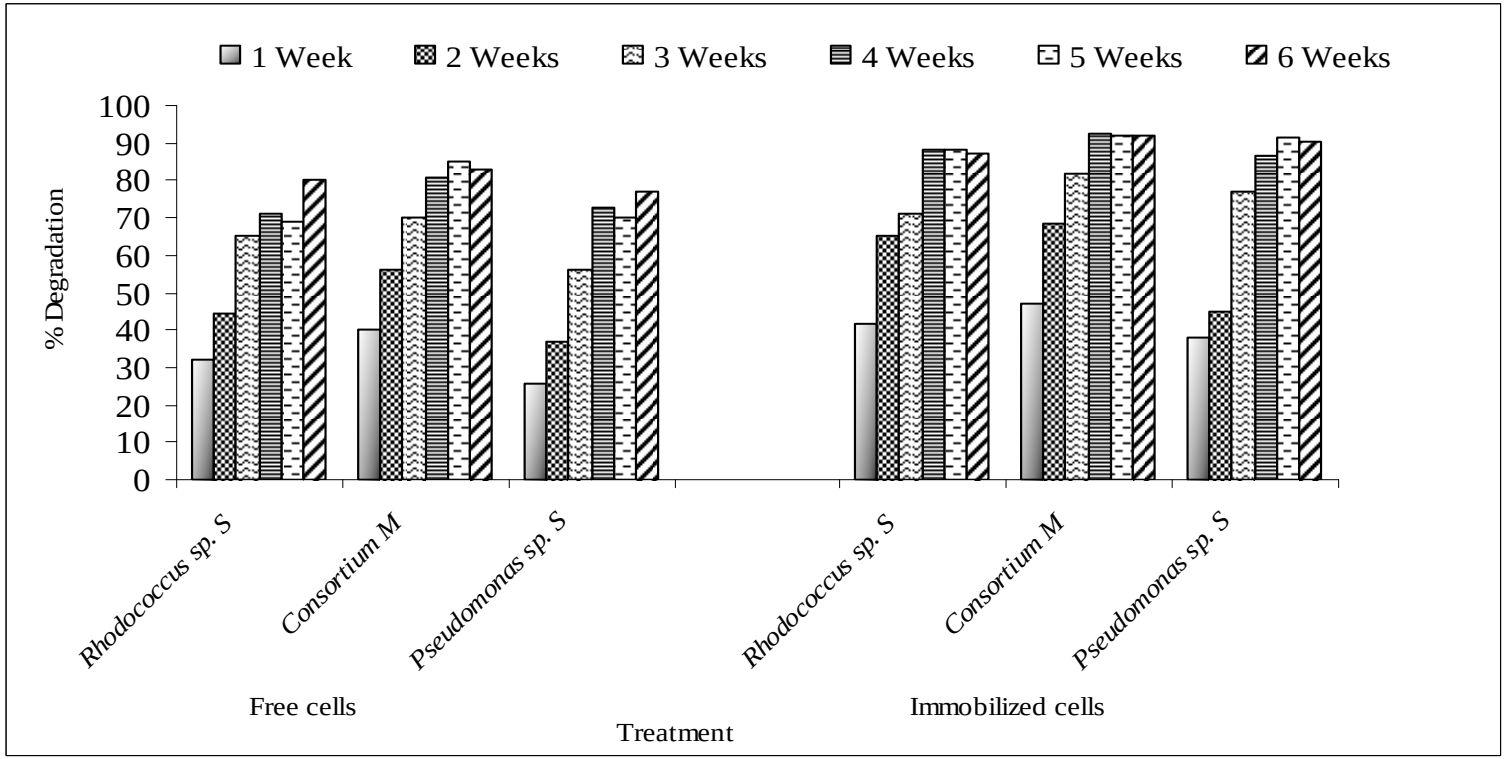


Fig.36: Biodegradation of crude oil in sea water using free and immobilized cells at temperature range 18-20◦C

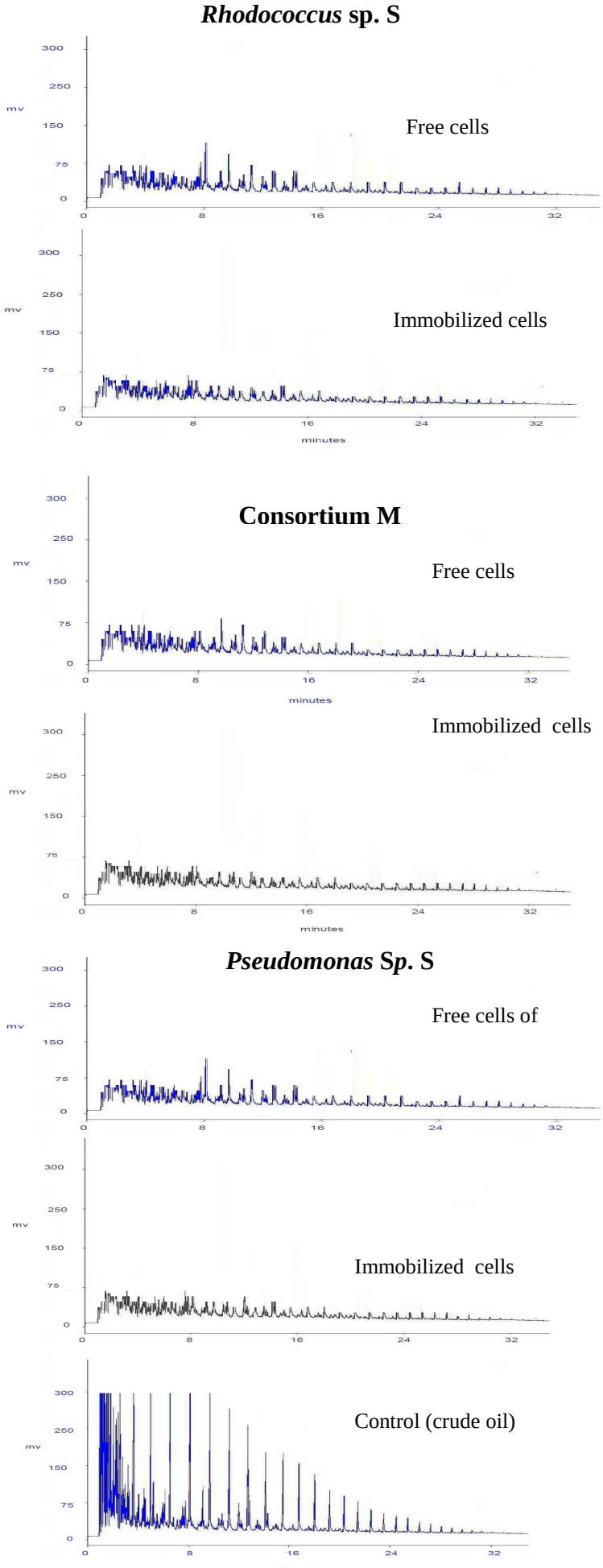


Fig. 37: Gas chromatogram of crude oil degradation in contaminated sea water using free and immobilized cells after four weeks.

Table 35 : Dehydrogenase activity in contaminated sea water treated by free and immobilized cells as measured by the increase in optical density at 546 nm at temperature range 18-20ºC

Time (Days) / Organism	Free cells						Immobilized cells					
	1	2	3	4	5	6	1	2	3	4	5	6
	OD at 546 nm						OD at 546 nm					
*Rhodococcus s*p. DM	0.06	0.141	0.135	0.213	0.189	0.176	0.11	0.237	0.304	0.257	0,245	0.287
Consortium M	0.08	0.152	0.167	0.278	0.301	0.294	0.12	0.226	0.275	0.303	0.305	0.284
Pseudomonas sp. S	0.055	0.111	0;123	0.118	0.121	0.117	0.098	0.23	0.202	0.212	0.235	0.228

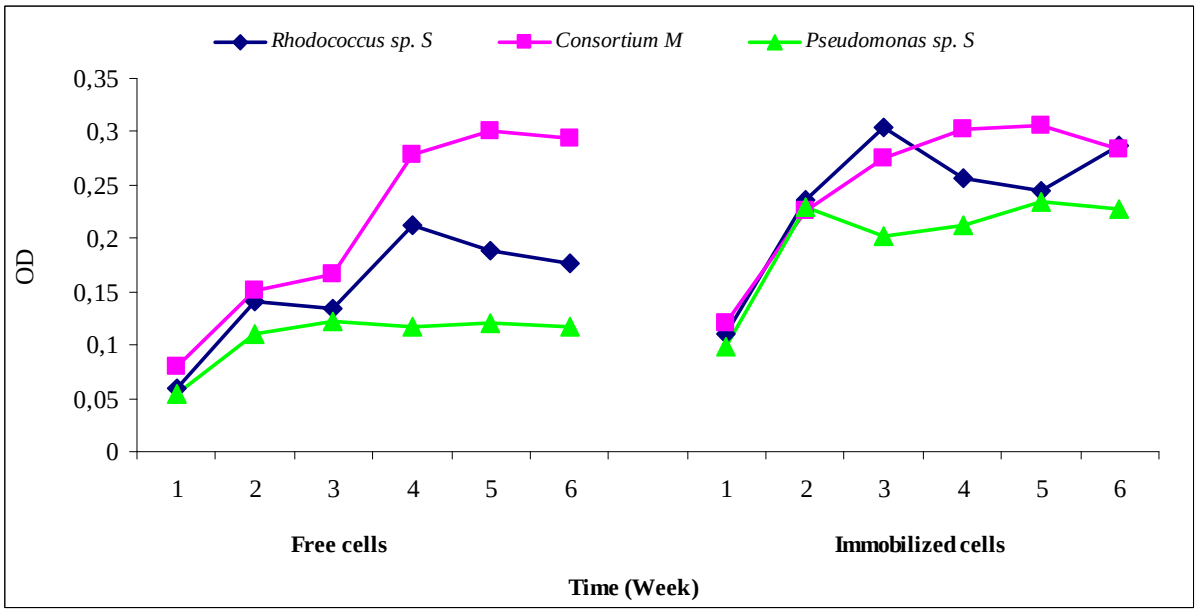


Fig.38. Dehydrogenase activity in contaminated sea water treated by free and immobilized cells as measured by the increase in optical density at 546 nm.

3.4.5. Effect of temperature on crude oil degradation by free cells

The treatment of oil contaminated sea water using local urea and local phosphorus fertilizer as nitrogen and phosphorus sources was performed at two different temperature ranges namely 18-20°C and 22-26°C. The results are demonstrated in Table 36 and Fig. 39. The results revealed that the degradation of crude oil was enhanced by increasing the temperatures range from 18 - 20°C to 22 - 26°C in all treatments with some fluctuations, especially in biostimulation treatment.

It was also observed that after six weeks the degradation of crude oil was ranged from 68-82.75 at the temperature range 18-20°C whereas at the temperature range 22-26C it was ranged between 70.25-92.5%. The highest cumulative percent (76.04) was obtained in bioaugmentation with consortium M at the temperature range 22-26°C whereas the lowest one (54.17) was obtained in the biostimulation treatment at the temperature range 18- 20°C.

The percentage of crude oil degrading bacteria in contaminated sea water treated by free cells at the two different temperature range (18-20°C) and (22-26°C), is presented in Table 37 and Fig.40. High temperature slightly increased the percentage of crude oil degrading bacteria comparing with low temperature. At the temperature range 18-20°C the percentage of crude oil degrading bacteria was ranged from about 41-52% after six weeks, while it ranged from about 44-55% at temperature range 22-26°C after the same time.

Table 36: Effect of temperature on crude oil degradation in sea water at different temperature ranges using free cells

Time (weeks) / Organism	18-20°C							22-26°C						
	1	2	3	4	5	6	Cumu-lative% of degra-dation	1	2	3	4	5	6	Cumu-lative% of degra-dation
	% Degradation							% Degradation						
Rhodococcus sp. S	32	44.5	65	71	68.75	80	60.21	42.25	40	65	75.75	83.25	85	65.22
Consortium	40.25	56.25	70.25	81	85	82.75	69.25	51.5	61.25	84.25	78.75	88	92.5	76.04
Pseudomonas sp. S	25.5	37	56	72.75	70	77	56.38	37.25	54.5	60.5	83	77.25	85	66.25
Biostimulation	30.5	49.5	41.25	65.5	70.25	68	54.17	35.5	51.5	50.5	68	75.75	70.25	58.58

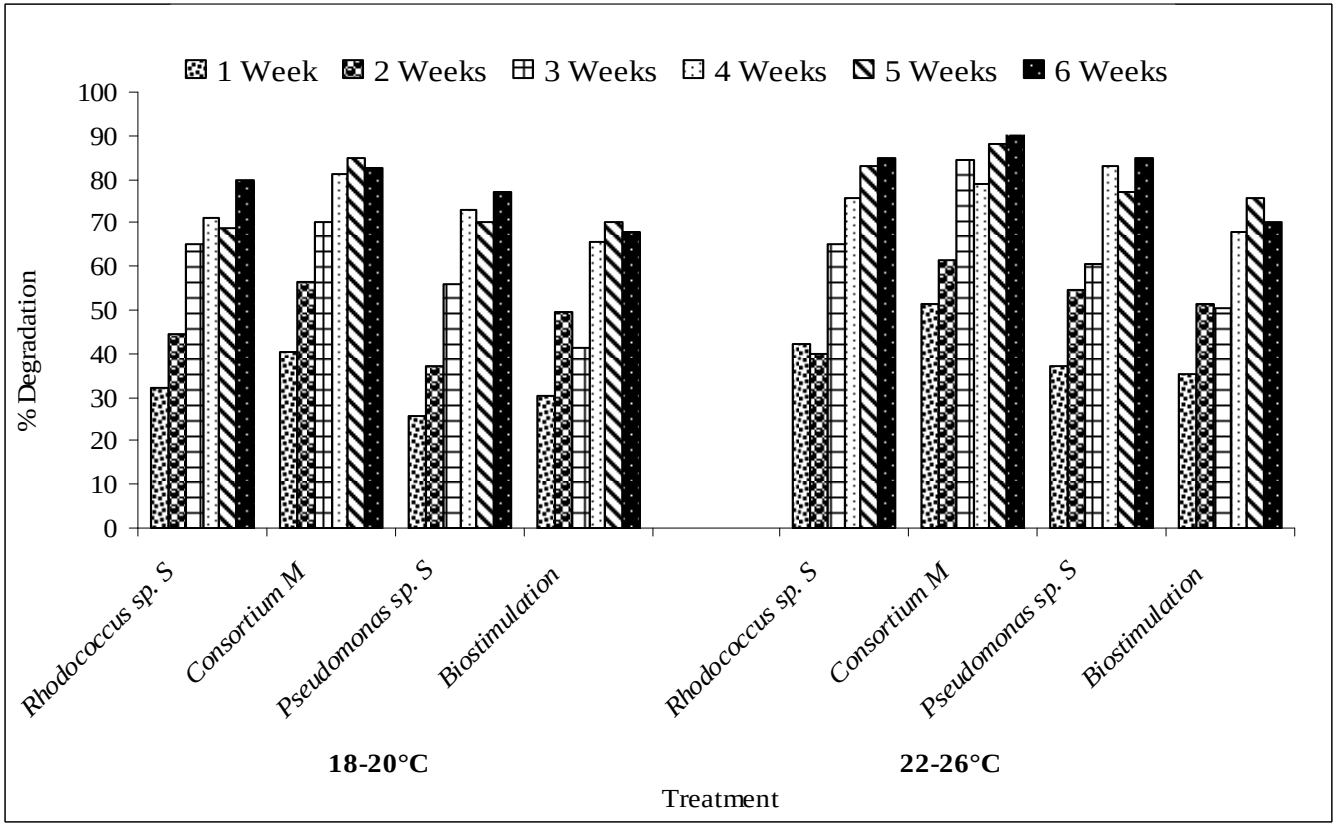


Fig.39. Effect of temperature on crude oil degradation in sea water at different temperature ranges using free cells

Table 37: Effect of temperature on the percentage of crude oil degrading bacteria (using free cells)

Time (weeks) / Organism	18-20°C						22-26°C					
	1	2	3	4	5	6	1	2	3	4	5	6
	% of crude oil degrading bacteria						% of crude oil degrading bacteria					
Rhodococcus sp. DM	40.24	38.04	47.56	52.64	51.96	48.39	48.56	46.03	50.07	54.88	53.6	52
Consortium M	45.5	41.23	48.55	52.81	49.89	51.84	52.43	50.94	52.39	53.63	54.84	55.31
Pseudomonas sp. S	38.07	43.81	40.44	48.42	46.58	47.03	47.45	49.75	47.57	49.04	51.2	52.34
Biostimulation	32.42	37.83	36.54	38.12	40.67	40.9	40.53	42.76	40.56	43.07	42.88	44.39

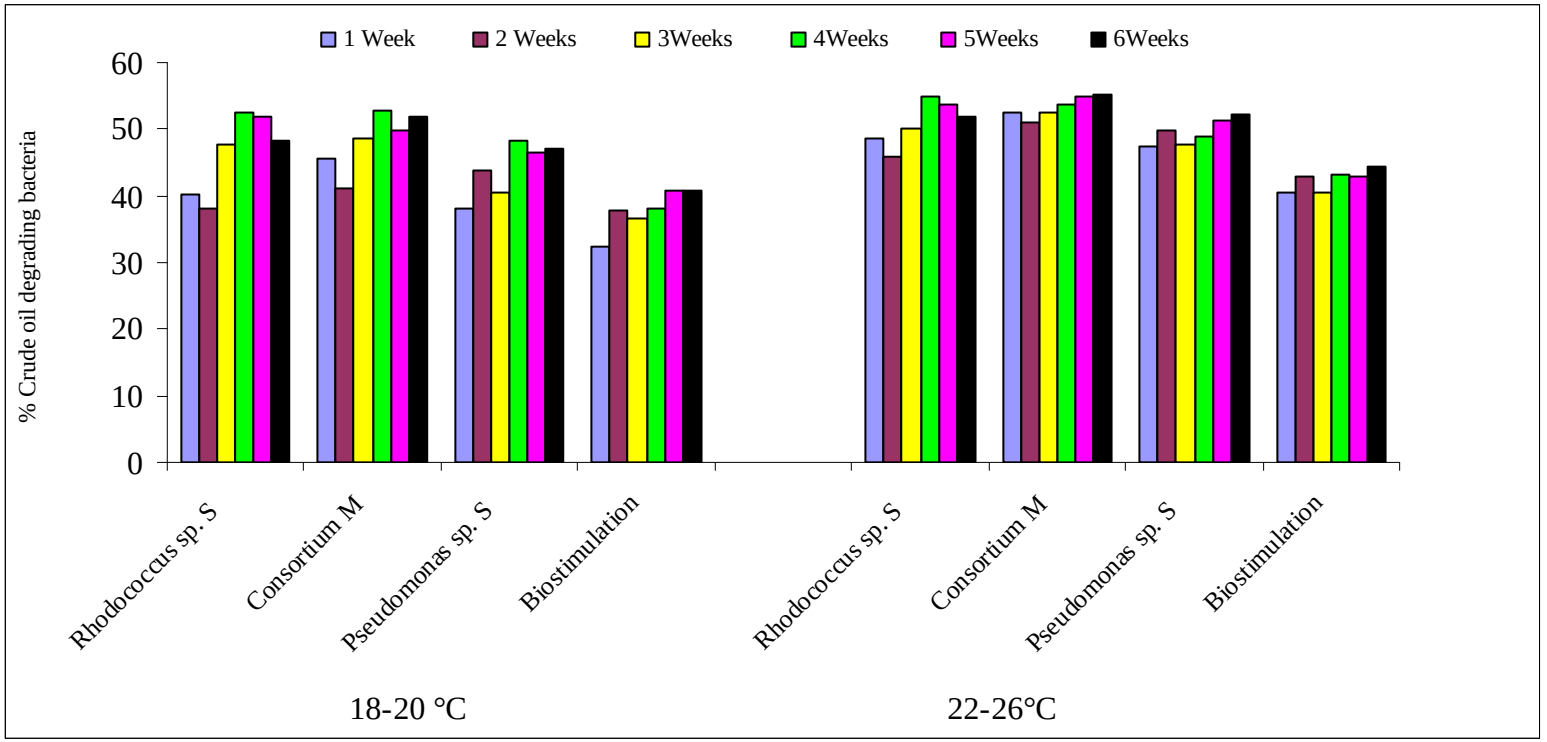


Fig.40: Effect of temperature on the percentage of crude oil degrading bacteria (using free cells)

3.4.6. Effect of temperature on crude oil degradation by immobilized cells

The experiment was performed with wheat straw immobilized cells at two different temperature ranges namely 18-20°C and 22-26°C. The results are demonstrated in Table 38 and Fig.41. The temperature range, 22-26°C had a better effect on crude oil degradation compared with 18-20°C. After three weeks, the degradation was increased at the temperature range (22-26°C) by about 9, 8 and 7% for *Rhodococcus* sp. S, the consortium M and *Pseudomonas* sp. S, respectively in comparison with the degradation at temperature range (18-22°C).

The highest value of cumulative percent at temperature (18-22°C) was 79.08% using the consortium M, while it increases to reach 82.17% at temperature range (22-26°C) by the same treatment.

Table 38: Effect of temperature on crude oil degradation using immobilized cells

Time (weeks) / Organism	18-20°C							22-26°C						
	1	2	3	4	5	6	Cumulative% of degradation	1	2	3	4	5	6	Cumulative% of degradation
	% degradation							% Degradation						
Rhodococcus sp. S	41.5	65	71	88	88	87	73.42	42.25	67.5	77.75	91.5	92.25	90.5	76.96
Consortium M	47.25	68.5	82	92.75	92	92	79.08	50.5	73.25	88.75	94	92.5	94	82.17
Pseudomonas sp. S	38	45	77.25	86.5	91.25	90.5	71.42	46.75	67.25	8.3	89.25	88.25	93.25	77.96

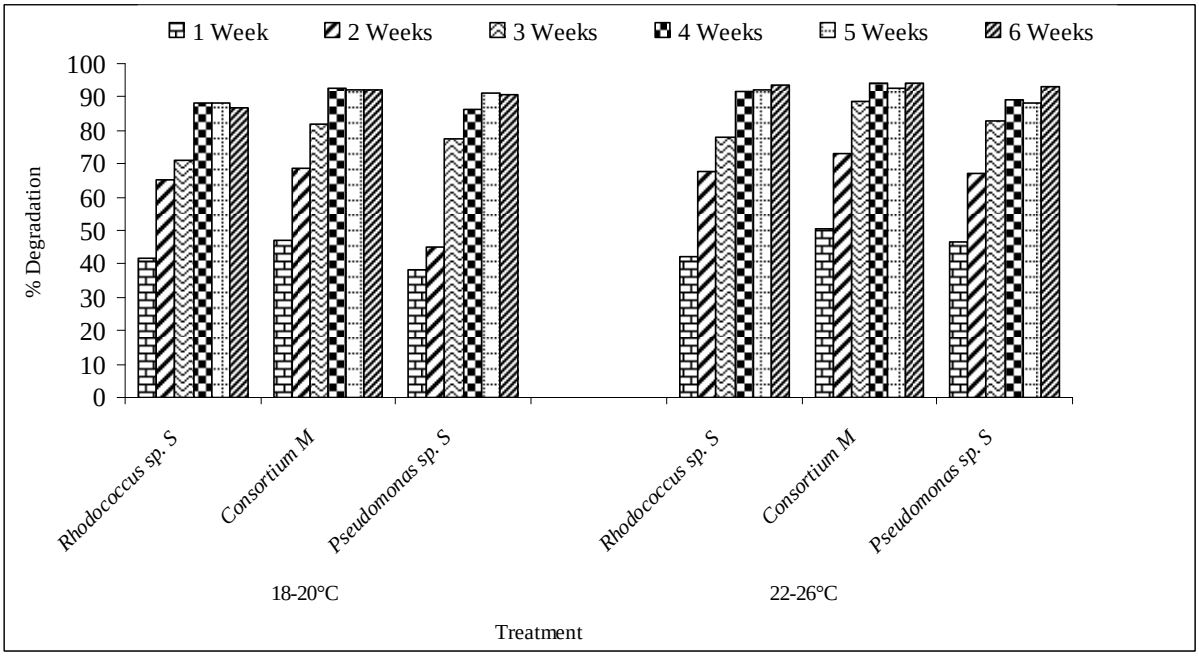


Fig. 41. Effect of temperature on crude oil degradation using immobilized cells

3-4-7.Effect of crude oil concentrations on the degradation using immobilized cells:

In this experiment wheat straw immobilized cells of *Rhodococcus* sp.S, the consortium M and *Pseudomonas* sp. S were used. Crude oil concentration was elevated from 1 to 6%.The experiment was performed without surfactants and in presence of 1% of Triton X-100. Local urea and local phosphorus fertilizer were used as nitrogen and phosphorus sources respectively. N/P ratio was adjusted at 10/1. The temperature range was 25-31°C. Samples were taken after 4 weeks to determine the residual crude oil and the bacterial density.

The results mentioned in Table 39 and Fig 42 revealed that the addition of 1% Triton X-100 at lower crude oil concentration had no significant effect on degradation of oil in sea water bioaugmented by the tested organisms, while its addition at higher oil concentration had stimulating effect on degradation, which decreased by increasing oil concentration. The results show that at 3% oil concentration, the addition of surfactant had no significant effect on degradation by *Rhodococcus* sp. S and consortium M, while it enhanced the degradation by *Pseudomonas* sp. S by about 20%. It was also found that the addition of surfactant at 4% oil concentration enhanced the degradation by about 16, 15 and 17% by *Rhodococcus* sp. S, consortium M and *Pseudomonas* sp. S respectively. On the other hand, the addition of surfactant at oil concentration 5 and 6% had a negative effect on degradation by *Rhodococcus* sp. S, consortium M in comparison with its absence, while slightly enhanced the degradation by *Pseudomonas* sp. S at the same oil concentration (5 and 6%).

Generally, the results revealed that the addition of surfactant had no significant effect on oil degradation by *Rhodococcus* sp. S, consortium M and slightly enhanced the degradation by *Pseudomonas* sp. S. Therefore, *Rhodococcus* sp. S, consortium M was selected to scale-up the Lab microcosms without surfactant.

Table 39: Effect of crude oil concentration on the degradation using wheat straw immobilized cells in presence and absence of 1%TritonX-100

Crude oil concentration / Organism	With 1% of Triton X-100						Without Triton X-100					
	1	2	3	4	5	6	1	2	3	4	5	6
	% Degradation						% Degradation					
Rhodococcus sp. S	90.5	92.5	88	77.5	62	59	92	90.5	89.5	65	65.5	50.5
Consortium	95	94	91.5	87.5	61	65.5	94	92.5	90.5	74.5	75.5	70.5
Pseudomonas sp. S	92	90	90	73	54	50	91.5	88.75	72	60.5	45.5	40

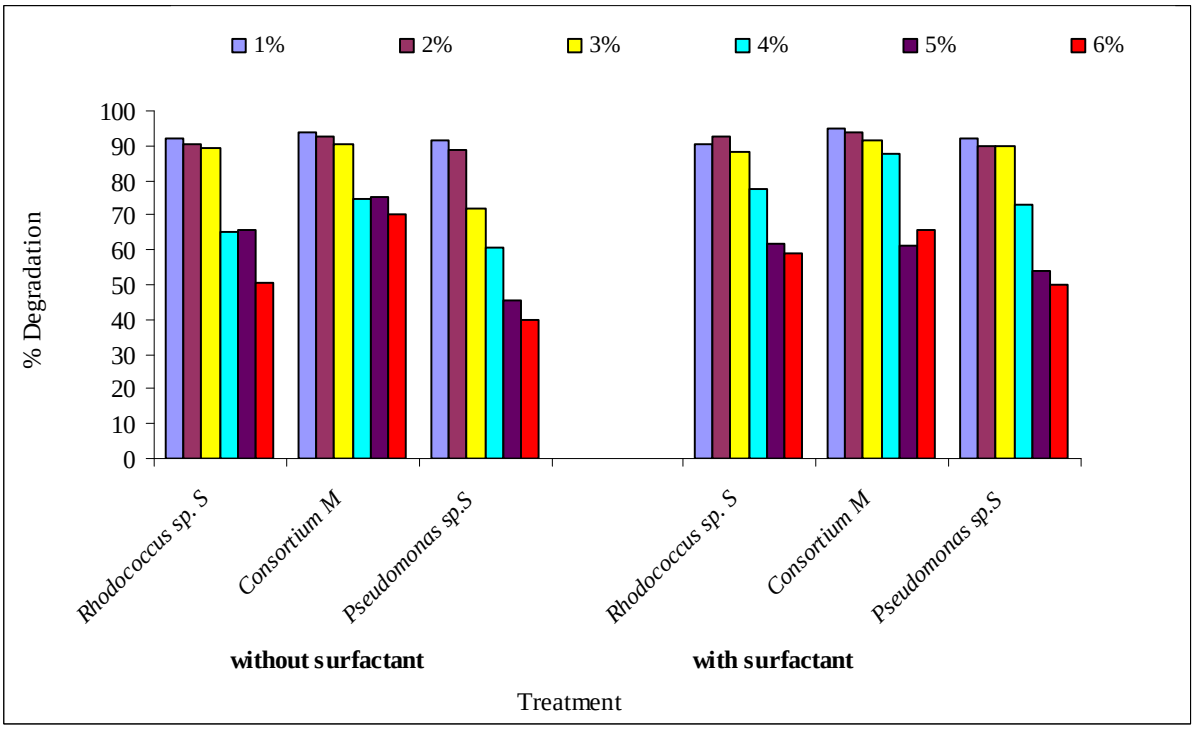


Fig.42. Effect of crude oil concentration on the degradation using wheat straw immobilized cells in presence and absence of 1%TritonX-100

3.5. Scale-up of Lab microcosms

The scale-up of Lab microcosms was performed by four treatments; bioaugmentation by wheat straw immobilized cells of: *Rhodococcus* sp. S and consortium M; biostimulation and attenuation. A control microcosm was also prepared by sterilizing the contaminated sea water (Fig.43). The results are presented in Table 40 and demonstrated in Fig. 44. The results show that the degradation began after the first week by about 41.5, 45, 40 and 33.5 *Rhodococcus* sp. S, consortium M, biostimulation and attenuation respectively. Increasing the time increased the biodegradation in all treatments to reach the maximum values after five weeks. On the other hand, the maximum degradation in attenuation was observed after three weeks, and then begins to decrease to reach the minimum value (55.5%) after five weeks. It was also observed that the loss in crude oil concentration in control was begin with 16% after the first week and reached its maximum (22.5%). The results also revealed that there was no significant differences in cumulative percent of degradation between *Rhodococcus* sp. S, consortium M and biostimulation. Fig. 45 shows the chromatographic analysis of crude oil after treatment.

Dehydrogenase activity was measured as increase in OD at 546 nm (Table 41 and Fig.46). Low dehydrogenase activity was observed after the first week in all treatments. Increasing the time increased the dehydrogenase activity with some fluctuations. Maximum value of dehydrogenase activity was obtained in case of consortium M after five weeks (0.149). Dehydrogenase activity in the control pilot was about zero.

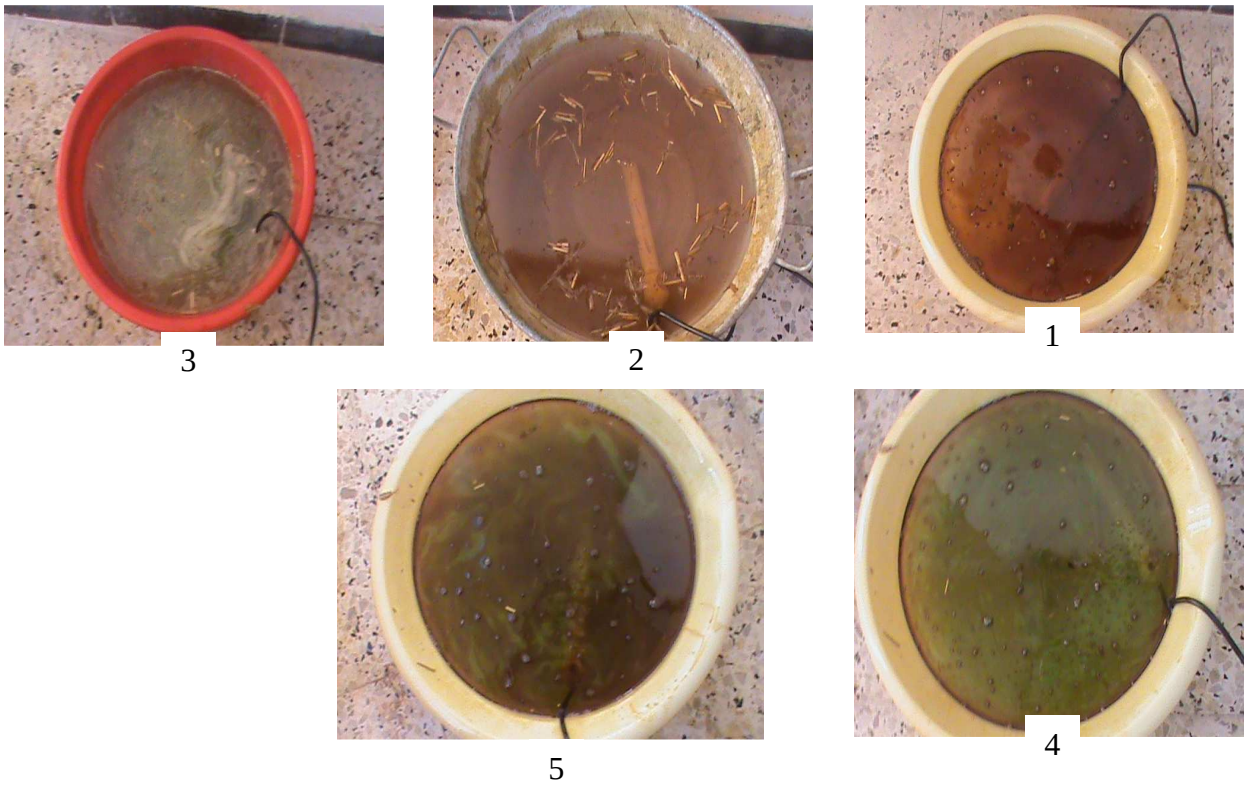


Fig.43. Bioremediation of crude oil contaminated sea water in scaled up- lab microcosms

1)Control; 2) Bioaugmentation by immobilized cells of *Rhodococcu*s sp. S; 3) Bioaugmentation by immobilized cells of consortium M; 4) Biostimulation; 5) Attenuation

Table 40: Scale-up process of lab microcosms

Time (weeks) / Treatment	1	2	3	4	5	Cumulative % of degra-dation
	%Degradation					
Rhodococcus sp.S (Immobilized cells)	41.5	51	68.25	75.25	90.5	65.3
Consortium M (Immobilized cells)	45	51,5	65.75	88.75	92.25	68.65
Biostimulation	40	57.5	66.5	73	78.75	63.15
Attenuation	33.5	41.25	64.75	50.5	55.5	49.1
Control	16	10.5	14.75	20	22.5	16.75

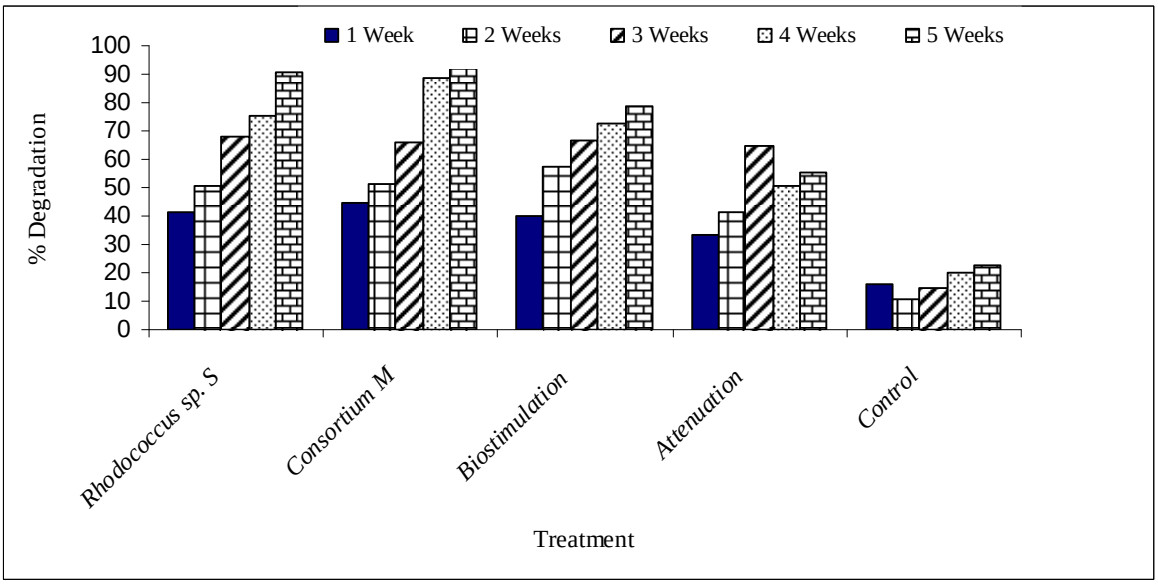
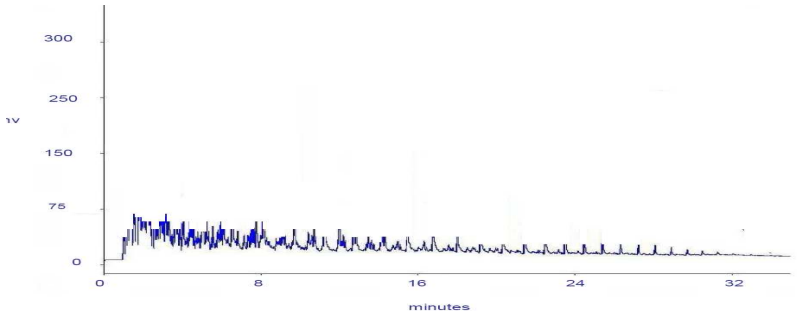
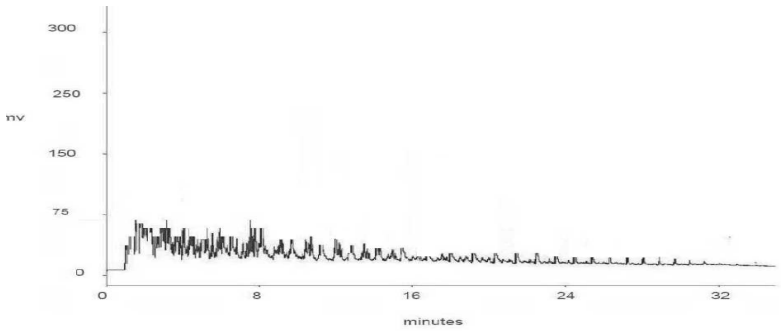


Fig.44. Scale-up process of lab microcosms

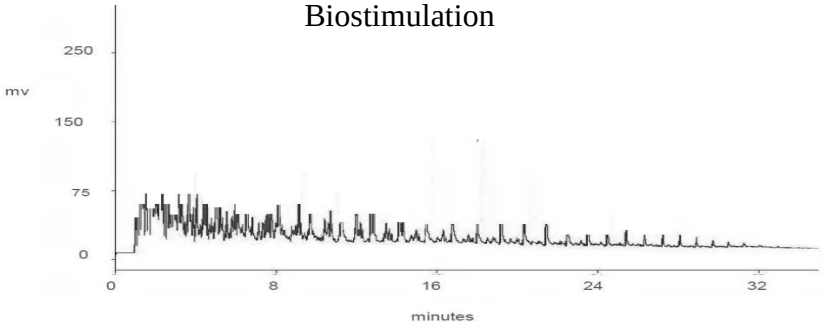
Rhodococcus sp. S



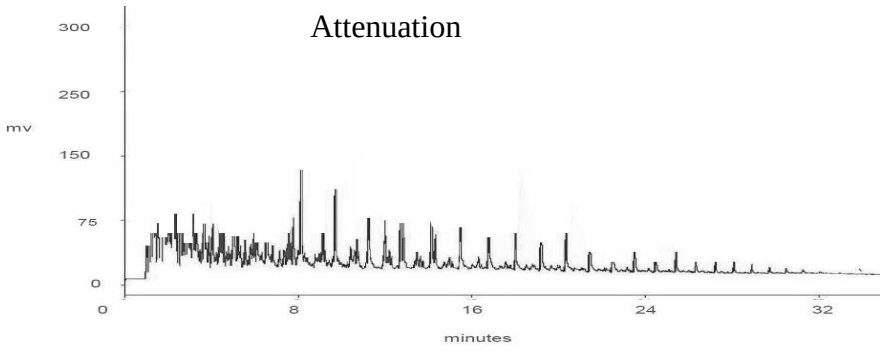
Consortium M



Biostimulation



Attenuation



Control

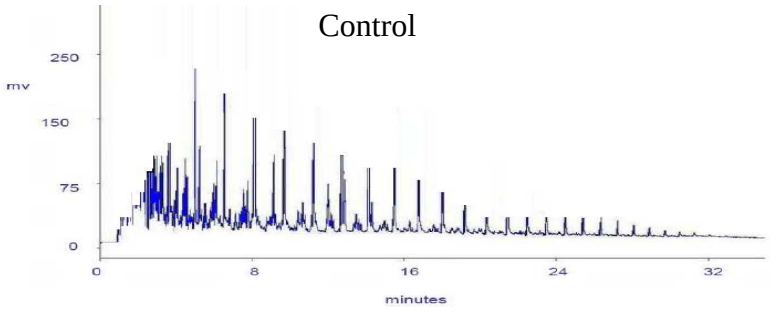


Fig.45. Gas chromatogram of the scale-up process (after 5 weeks) of lab microcosms

Table 41 : Dehydrogenase activity in scale-up process of lab microcosms measured as increase in OD at 546 nm.

ime (weeks) / Treatment	1	2	3	4	5
	OD				
Rhodococcus sp.S (Immobilized cells)	0.05	0.045	0.067	0.071	0.122
Consortium M (Immobilized cells)	0.068	0.121	0.075	0.134	0.149
Biostimulation	0.021	0.034	0.122	0.097	0.107
Attenuation	0.03	0.04	0.052	0.11	0.096
Control	0.01	0.01	0.002	0.012	0.005

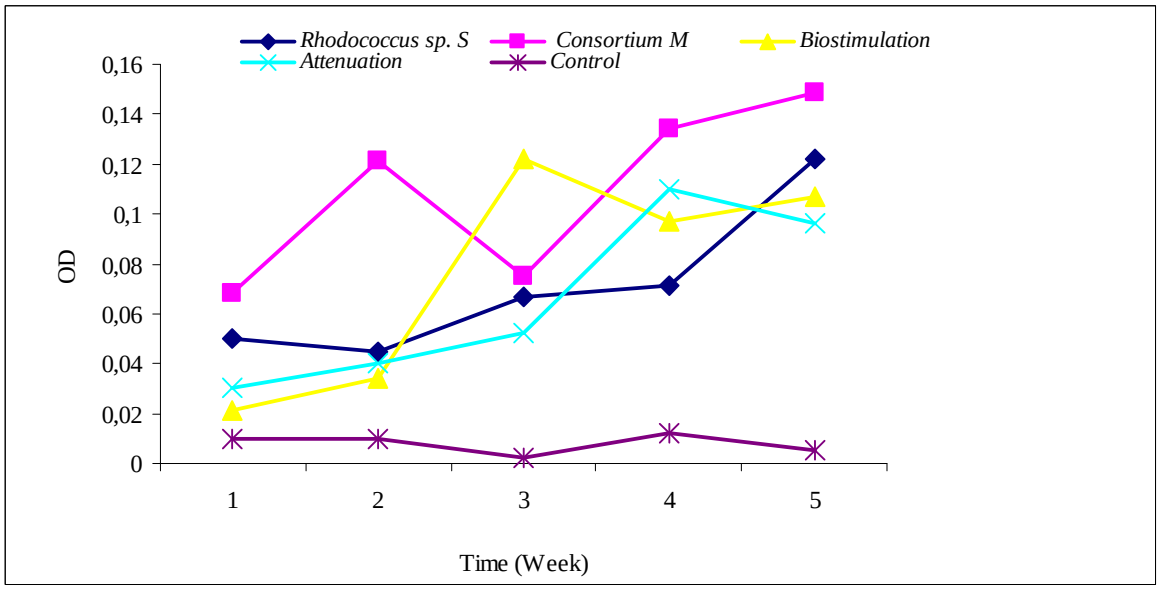


Fig.46. Dehydrogenase activity in scale-up process of lab microcosms measured as increase in O.D. at 546 nm.

3.6. Growth of Algae in crude oil contaminated and bioremediated sea Water

An experiment was designed to grow an algae obtained from Ben Mhidi beach in the treated sea water (after five weeks) to evaluate the bioremediation process of crude oil contaminated sea water (Fig.47). The results are shown in Table 42. The growth was monitored for seven days . The highest percentage of algae growth was recorded in the control pot (86.91%). The percentage of growth in the bioaugmented pots by consortium M sp. S and *Rhodococcus* sp. S reached 83.81 % and 83.44% respectively followed by 76.74% in the biostimulated pot . A moderate growth was obtained in the attenuated pot (67.74%). The untreated seawater had an inhibitory effect on the growth of the algae, the percentage of growth was (-39.60%).

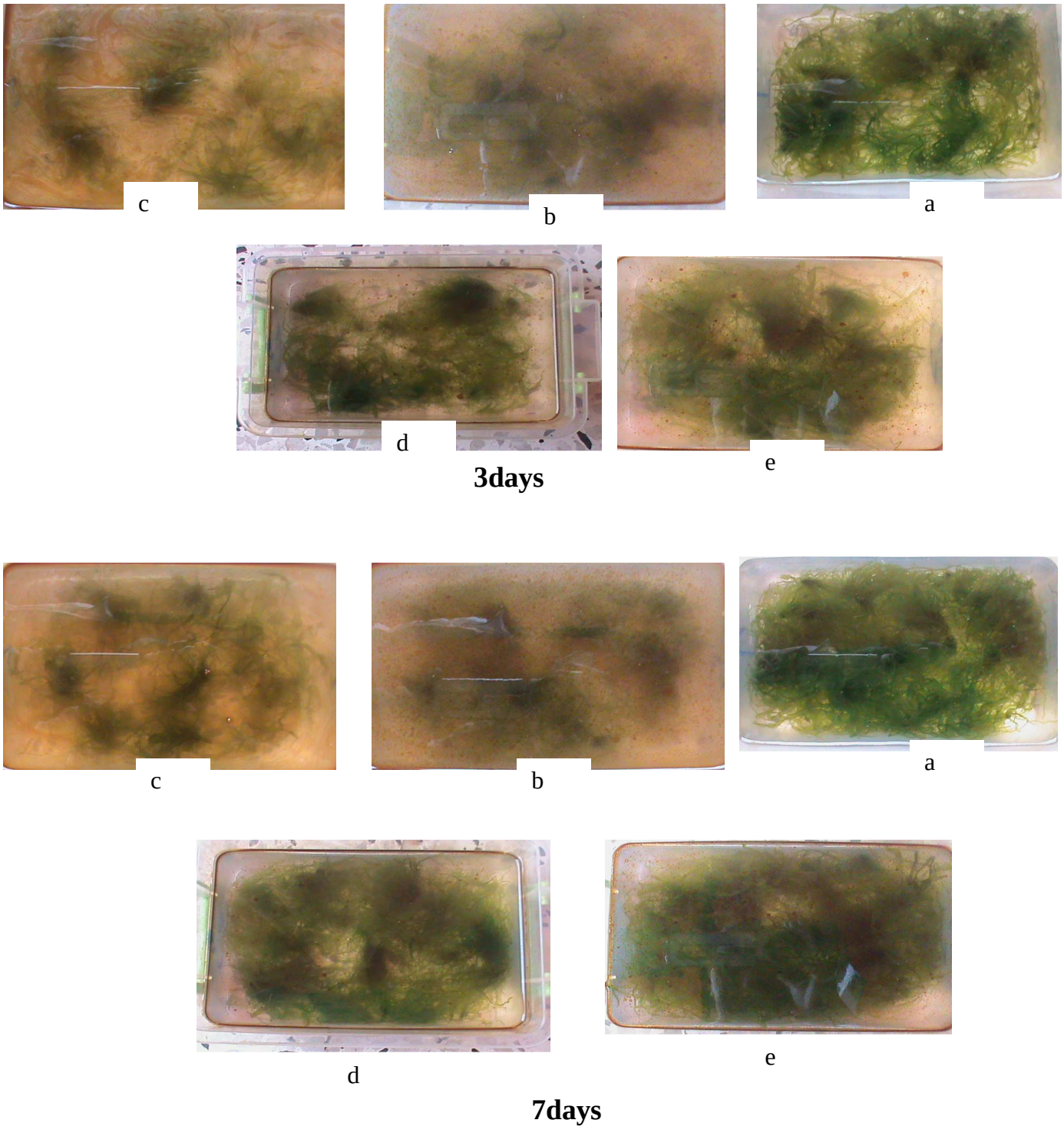


Fig.47. Growth of algae in bioremediated sea water

a)Control; b) Attenuation; c) Biostimulation; d) Bioaugmentation by immobilized cells of *Rhodococcus* sp. S ; e) Bioaugmentation by immobilized cells of Consortium M

Table 42: Percentage of algae growth in the bioremediated sea water after seven days

Treatment / Growth(%)	Control	*Rhodococcus* sp.S	Consortium M	Biostimulation	Attenuation	Untreated sea water
	86.91	83.44	83.81	76.74	67.74	-39.60

IV.
DISCUSSION

IV Discussion

Petroleum-based products are the major source of energy for industry and daily life. Petroleum is also the raw material for many chemical products, such as plastics, paints and cosmetics. The transport of petroleum across the world is frequent and the amounts of petroleum stocks in developed countries are enormous. Consequently, the potential for oil spills is significant. The volume of spills usually exceeds the inherent remediation for any given environment, resulting in a significant ecological impact (Chekroud, 2006).

The elimination of hydrocarbons from marine environments needs the intervention of many biotic and abiotic factors. Among these factors the use of microorganisms and in particular the bacteria in biodegradation. Bacteria are considered as the most important natural process in the depollution of marine environments. Accordingly, the mechanisms of oil hydrocarbons degradation by bacteria and the parameters, which could influence the degradation, have been largely studied (Soltani, 2004).

Skikda, which is a port city, is doted of a petrochemical industrial platform, which constitutes the principal source of hydrocarbons pollution (Bourdjiba et al., 2009). Sea water in Skikda is under great pressure because of industrial development (Boudelaa and Medjram, 2011) and the release of hydrocarbon pollutants in the sea, which has affected the quality of life in sea water.

This work simulates a possible crude oil spill in sea water of Skikda and the possibility to bioremediate the crude oil contaminated sea water using bacterial strains. From the 30 isolates three bacteria (O, R and V) were able to grow in the presence of crude oil, in addition to the consortium (M) and the bacterial strain (VA) isolated from Alexandria (Egypt). The microorganisms O, M and V were selected to complete the research. The bacterial strain VA was identified by physiological and biochemical methods as *Pseudomonas* sp. CK (DSMZ, Braunschweig, Germany). The selected isolates (O and V) were identified using gram staining and API 20 kits as *Rhodococcus* sp. S and *Pseudomonas* sp. S. The consortium is composed of *Bacillus sp. S, Acinetobacter sp. S and Aerobacter sp. S.* The results of flask experiments in the present work revealed that *Rhodococcus sp*. S could degrade 86.75% of crude oil after 15 days of incubation, the consortium M degraded 90% of crude oil and *Pseudomonas* sp. S 81% of crude oil after the same period. The capacity of bacteria, especially *P. aeruginosa*, to metabolize aerobically heavy oil or aliphatic hydrocarbons is well known since a long time (Chaerun et al., 2004). Nine non-sporulating Actinomycetes belonging to *Rhodococcus, Noccardia, Gordonia* and *Dietzia* genera were investigated for their hydrocarbon-degrading abilities. The studied strains showed affinity with kerosene, pentadecane, hexadecane, 2,6,10,14-tetramethyl pentadecane (peristane), phenyldecane and gas-oil (Alvarez, 2003). Gentili et al. (2006) reported

that Rhodococcus *corynebacterioides* could degrade hydrocarbons in sea water. The removal of n- alkanes or branched alkanes in sea water by *Rhodococcus erythropolis* was also tested by Liu et al. (2009). Gargouri et al. (2011) isolated a strain of *Rhodococcus* sp. able to degrade hydrocarbons in contaminated water

The chromatographic analysis of crude oil under test before and after degradation shows that n-alkane fractions are easily degraded than aromatic fractions. It was also observed that the lighter part of crude oil was degraded much faster than the heavier one. These results are in agreement with the results obtained with many authors (Diaz et al., 2002; Gouda et al., 2007a; Nocentini et al., 2000; Wang et al., 1998). The fraction C4- C16 was rapidly degraded by all tested organisms. Above C16 the degradation pattern differs according to the organisms under test. Xu (2010) demonstrated that the fraction C12-C29 of oil was more degradable in some treatments, whereas there was a greater extent of consumption of C19-C23 in other treatments.

The bioremediation effectiveness mainly depends on some limitations (Si-Zhong et al., 2009). Most microbes that degrade hydrocarbons require oxygen, water, suitable pH and nutrients such as nitrogen and phosphorus (Xu and Obbard, 2003; Baszczyc-Maleszak et al., 2006). In order to enhance the biodegradation of crude oil in marine medium, different cultural conditions were tested in the present work.

To test the effect of co-substrates on crude oil degradation by the three tested organisms, glucose, molasses, peptone, wheat bran and yeast extract were added at 0.5% as co-substrate. Table 5and Fig. 10 demonstrate that glucose had an inhibitory effect on the tested organisms with a maximum inhibition in case of *Rhodococcus* sp. S. On the other hand, the supplementation of yeast extract had no significant effect on all organisms. It was also found that wheat bran stimulated crude oil degradation by all microorganisms under test. Molasses slightly inhibited the biodegradation by *Rhodococcus* sp. S and inhibit the biodegradation by consortium (M) and *Pseudomonas* sp. S. Peptone slightly stimulated the effect of *Pseudomonas* sp. S and inhibited the effect of *Rhodococcus* sp. S and consortium M.

These results are in partial agreement with the results described by Chekroud (2006), who found that the addition of glucose had an inhibitory effect on kerosene degradation by *Pseudomonas* sp. CK, *Pseudomonas* sp. AP and *Gordonia* sp. DM while the supplementation of molasses, yeast extract and peptone had no significant effect on kerosene degradation by the same organism. Chekroud (2006) also reported that wheat bran stimulated kerosene degradation by *Pseudomonas* strains only. On the other hand, the results of the present study are contradictory to that obtained by Kim et al. (2003) who reported that the degradation of phenanthrene was enhanced 1.5 times in presence of glucose, yeast extract or peptone as co-substrates. Beahm and Perry (1973) also found that the relative abundance of cycloalkanes

utilizers and the capacity for oxidation of cycloalkanes to homologous alkanine strongly suggest that co-metabolism does play a role in the degradation of these compounds in nature. The capacity of *Rhodococcus erythropolis* to degrade hexane in sea water was 1100ppmv in presence of nutrient broth and 850ppmv without nutrient broth after 140 days of incubation. Kageyama et al. (2005) isolated two organisms that degrade trichloroethylene (TCE) without addition of co-substrate; the two species were classified as *Ralstonia* sp. Some other organisms belonging to the genera *Pseudomonas* and *Burkholdria* can degrade TCE without co-substrate (Leahy et al., 1996; McClay et al., 1995).

The nutrient status of a sea water directly impacts microbial activity and biodegradation. A group of nutrient elements or organic compounds is required as a source of carbon or electron donor/acceptor. Inorganic nutrients including exchangeable cations, nitrates, and phosphates are important for bioremediation (Si-Zhong et al., 2009). In this study, the effect of different nitrogen and phosphorus sources and some commercial fertilizers on the degradation capacity of the tested organisms was evaluated. The results obtained in Table 8and Fig. 12 demonstrated that ammonium sulfate had an inhibitory effect on crude oil biodegradation by all tested microorganisms in comparison with ammonium nitrate used in the basal medium. Urea 46% slightly enhanced oil degradation by *Rhodococcus* sp. S and *Pseudomonas* sp. S and it had no significant effect on consortium M. Local urea slightly stimulated oil biodegradation by consortium M and *Pseudomonas* sp. S and it had no significant effect on *Rhodococcus* sp.S. The nitrogen fertilizer had a negative effect on *Pseudomonas* sp.S and *Rhodococcus* sp. S while it had a stimulating effect on oil biodegradation by the consortium. Although inorganic salts ($NaNO_3$ and KNO_3) were used in some laboratory and field experiments with conflicting results (Wrenn et al., 1994; Mearns et al., 1995), the addition of reduced nitrogen was more successful and usually supplied in commercial fertilizers as ammonium salts (i.e NH_4NO_3 or NH_4Cl) or as urea $(NH_2)_2CO$ (Wrabel and Peckol, 2000) . The availability of added nutrients and their stimulating effects seem to be linked to their chemical speciation and their ability to stay at the remediation site (Pelletier et al., 2004). Some organic fertilizers containing fishbone meal were also used in experimental plots with limited success (Lee et al., 1995). The authors observed an increase of the toxicity following periodic additions of the fish compost which was attributed to anoxia and also to the formation of toxic metabolites from a too rapid degradation of the fertilizer itself instead of the treated oil. In contrast, Santas et al. (2001) reported a successful bioremediation of crude oil by a fish compost in mesocosm assays simulating Mediterranean winter conditions with 70% alkane degradation in 30 days. In the present work, the use of manure as nitrogen source gave the maximum degradation of crude oil (94.25-97%) for the tested organisms, but it was not selected to complete this work due to hygiene purposes. Local

urea was selected to complete the work due to its low cost and availability as commercial fertilizer.

The results presented in Table 11 and Fig.14 show that the use of super phosphate 15.5% and local urea increased the oil biodegradation by *Rhodococcus* sp. S and consortium M while it had no significant effect on *Pseudomonas* sp.S. The local phosphorus fertiliser slightly inhibited oil biodegradation by the consortium, while the ammonium phosphate increased the activity of *Rhodococcus* sp. S., and slightly inhibited the effects of *Pseudomonas* sp. S and consortium.

Sharma and Pant (2000) isolated a species of *Rhodococcus*, which degraded up to 50% of the aliphatic fraction of Assam crude oil in seawater supplemented with 35 mM nitrogen as urea and 0.1 mM phosphorus as dipotassium hydrogen orthophosphate after 72 h at 30°C. Nawar (1997) in his study on the biodegradation of hydrocarbons reported that using NH_4Cl as nitrogen source and Na_2HPO_4 and KH_2PO_4 as phosphorus sources enhanced solar biodegradation in comparison with local fertilizers (urea and Abu Tartour phosphate mixture) which decreased degradation by about 14.19% to 71.26%.

For economic reasons commercial fertilizers (local urea and local phosphorus fertilizer) were used as nitrogen and phosphorus sources in the present study.

The effect of different nitrogen phosphorus ratios (1/1, 3/1, 7/1, 10/1 and 20/1) on oil biodegradation was also tested. Local urea and local phosphorus fertiliser were used as nitrogen and phosphorus sources respectively. The ratios 1/1 ,3/1 and 20/1 decreased the activity of all the bacterial isolates under test, While the ratio 7/1 decreased crude oil degradation by *Rhodococcus* sp. S and *Pseudomonas* sp. S. The ratio10/1 stimulated oil biodegradation by *Rhodococcus* sp. S and consortium and it had it had no significant effect on the activity of *Pseudomonas* sp. S. It was reported that the ratios carbon/ nitrogen/ phosphorus varied to some extent in the different literatures (Gouda et al., 2007a). Some ratios reported in the literatures are 100 :15 :3, 33 :5 :1 (Zitrides, 1983; Riser-Roberts, 1998). Gouda and co-workers (2007) observed that the N/P ratio had no effect on kerosene biodegradation. Filler et al. (2006) reported that when nutrients are not limited, the desired ratio of C, N, P, is 100:15:1.

The effect of pH on oil biodegradation was also tested in this study. It was observed that the best results of oil degradation were obtained at pH 6, 7and 8 , while the pH 4 and 9 inhibited oil degradation by all tested microorganisms. Kim et al. (2003) found that maximum degradation was observed at pH 7 and was better under weak acidic conditions (pH 5-7) than under alkaline conditions. The results obtained in this study were in partial agreement with that reported by Baszczyk-Maleszak et al. (2006), who found that 60-70% reduction in oil content was observed at pH 5 and 80 - 90% at pH 7 and 9 after 21 days of cultivation.

According to Munro (1970) many microbes have an optimum pH for the growth around 7 and most prefer the pH range 5-8, although there are many exceptions to these trends. Shin et al. (2004) reported that the pH did not have a dramatic effect on cells growing in the presence of phenanthrene as carbon source. Arafa (2003) in his study on the biodegradation of some aromatic hydrocarbons, benzene, toluene, ethylbenzene and xylene, (BTEXs) isolated a consortium of bacteria from a polluted site in Saudi Arabia, which was affected by the pH. He found that the biodegradation rate was superior at pH 7 than at pH 6 and pH 8.

As reported by Gouda et al. (2007a), the use of chemical surfactants may accelerate the degradation rate. The biodegradation proceeds more rapidly when the oil is emulsified into small droplets (Munn, 2004). In the present study, five chemical surfactants (Tergitol, Triton X-100, Igepal, Tween 20 and Tween 80) were tested. Using these surfactants, crude oil degradation reached (90.5-96%) with the tested organisms except in case of *Rhodococcus* sp. S with Tergitol (88.5%). These results are in agreement with that obtained by Allen et al. (1999) who reported that Triton X-100 increased the biotransformation of naphthalene and phenanthrene by two oxygenase expressing bacteria *Pseudomonas* sp. strain 9816/11 and *Sphingomonas yanoikyae* B8/39. Triton X-100 was selected to complete this study, due to its low price compared to the other surfactants. Chemical surfactants have the ability to emulsify or pseudomobilize water-soluble compounds thus potentially improving their accessibility to microorganisms. Willumsen and co-workers (1998) found that Tween 80 had no toxic effect on biodegradation of fluranthene by two strains of *Mycobacterium* and two strains of *Sphingomonas.* Boonchan et al. (1998) in their examination of surfactant-amended biodegradation of higher molecular weight PAHs by *Stenotrophomonas maltophlia* VUN 10,010 reported that anionic and cationic surfactants were seen to be highly toxic to this strain, while Tween series of surfactants were used as growth substrate. Igepal CA-630 inhibited pyrene degradation and microbial growth. The addition of Tergitol NP-10 to VUN 10,010 cultures substantially improved degradation of PAHs individually as in mixtures (Boonchan et al., 1998). Tween 20 is relatively non-toxic surfactant, it appeared least inhibitory to the microbial mineralization of phenanthrene (Bramwell and Laha, 2000). Lee et al. (2006) tested the effect of a mycolic acid surfactant on the biodegradation of diesel oil by a newly identified *Rhodococcus baikonurensis* EN3. They found that the synthetic mycolic acid has potential for the remediation of petroleum-contaminated sites from both an economic and applied perspective as it can stimulate biodegradation at low concentrations. The concentration of surfactants used is an important factor affecting the biodegradation of hydrocarbons.

The effect of oil concentrations was investigated in this work. The results revealed that the microorganisms under test were able to degrade 87.5-90.5% of 2% of crude oil in comparison

with 94-95% in presence of 1% of oil. The surfactants increase the contact between the microorganism and the contaminant (Yerushalmi et al., 2003). The results are in agreement with those found by Okuda and co-workers (2007) who reported that the use of Triton X- 100 enhanced the dodecane degradation. According to Gouda and co-workers (2007a) the addition of Triton X-100 increased the kerosene biodegradation by *Pseudomonas* sp. AP, *Pseudomonas* sp. CK and *Gordonia* sp. DM.

The time needed for oil biodegradation was decreased from 15 days to 12 days when immobilised cells on wheat straw were used and it reduced to 9 days in presence of immobilised cells by entrapment in alginate. The immobilised cells on wheat straw had the capacity to degrade 88-90% of oil after 12 days, whereas the immobilised cells by entrapment in alginate could degrade 87.75-92% from 2% of oil after 9 days. These results are in agreement with those found by Diaz and co-workers (2007) who reported that the bacterial consortium shows good stability in immobilised systems. Apparently the increased stability of intracellular activities can be attributed to the protective effect of the biofilm against physicochemical stress. The use of alginate as a carrier accelerated the degradation of oil in comparison with the wheat straw; this might be due to the high immobilisation efficiency of the cells on the immobilisation material and the high affinity between the hydrophobic immobilisation material and the substrate (Quek et al., 2005), which makes the substrate more available for the bacterial cells. The findings obtained in this study indicate that immobilised cells on showed faster and better oil degradation than free cells. This is due to immobilisation material that protects the bacterial cells from the contaminants (Gouda et al., 2007b) On the other hand, the oil –absorbing capacity of wheat straw (8-10%) can be used to prevent migration of floating petroleum products from an oil to spill to beaches and shorelines.

In all previously mentioned experiments, the growth of the tested organisms was calculated in terms of intracellular proteins and as increase in OD at 600nm. It was observed in most cases that there was a correlation between the degradation percent and the total amount of intracellular proteins which indicates that the bacterial strains utilized crude petroleum oil as sole source of carbon and energy which was evident from the increase in cells density and protein content as reported by Das and Mukherjee, (2007) .

However flask experiments do not reflect field conditions, the selected organisms were tested for their ability to bioremediate crude oil contaminated sea water in lab-scale microcosms. In biological treatments, it is necessary to perform laboratory feasibility tests to determine the microbial potential to degrade the pollutants and whether nutrients are required to increase the degradation rate (Bomlen and Kossan, 1995). In this thesis the effect of bioaugmentation of the tested organisms (alone or in consortium), biostimulation of indigenous sea water flora or its

attenuation on bioremediation of crude oil contaminated sea water. The bioremediation was done in pots (23 x 17 x 12 cm) containing 1L of crude oil contaminated sea water.

At the end of the experiment (after 6 weeks) crude oil degradation was about 70.5-95% whereas it was only 55.5% in attenuation treatment. After the first week, the highest degradation (55%) was observed upon bioaugmentation of sea water with consortium M*;* this was followed by the *Rhodococcus* sp.S (48%). After the same time (one week) the lowest degradation (25.5%) was obtained in attenuated sea water. Moderate degradation (38 and 35%) was observed upon bioaugmentation of sea water with consortium (C) and *Pseudomonas sp. S* respectively. Laboratory microcosms were conducted by Chaerun et al. (2004), they studied the role of hydrocarbon degrading bacteria in bioremediation processing of heavy oil since 1997 until 2001. According to some authors bioaugmentation is more effective when the environment is not nutrient deficient, but the indigenous microbial population lacks the required activity or metabolic capability (Yerushalmi et al., 2003) or when contaminants have a toxic effect on the indigenous microorganisms (Margesin and Schinner, 1997a).

Biostimulation of sea water microorganisms upon the addition of nutrients (basal medium) resulted in 40.5% % degradation after the first week with gradual increase until the end of the experiment, which reached 70.5%. Many research studies have demonstrated the feasibility and efficacy of fertilization with nitrogen and phosphorus to combat oil spills in marine environments (Nikolopoulou and Kalogerakis, 2008). The addition of inorganic nutrients increased significantly hydrocarbon removal, confirming previous laboratory and field experiments, which stated that nutrient availability is a key limiting factor for the efficient removal of hydrocarbons by microbes in contaminated sediments (Swannell et al., 1996; Röling et al., 2004, Xu et al., 2005).

It was also observed that the greatest cumulative percent of degradation was obtained in bioaugmented sea water with consortium M followed by *Rhodococcus* sp. S. The results presented in Table 28 and Fig. 28 revealed that the bioaugmentation of sea water with consortium (C) of the tested organisms decreased the cumulative percent of degradation to 62.42% .

Natural attenuation of the indigenous microorganisms of sea water gave the lowest degradation (55.5%) and the lowest cumulative percent (43.5%) by the end of the experiment (6 weeks). These results are better than that obtained by Beolchini et al. (2010), who reported that hydrocarbon removal due to natural attenuation processes was very low since only <5% of the HMW aliphatic and aromatic compounds were removed after five weeks of incubations. These results are contradictory to that observed by Bento et al. (2005), who reported that natural attenuation resulted in the highest degradation of the light fraction of total petroleum

hydrocarbons (TPH). Sarkar et al. (2005) also reported that 93.8% reduction in TPH was observed in natural attenuation after 8 weeks. Greer et al. (2003) reported that natural attenuation pilots did not show any significant hexadecane mineralization indicating that biostimulation had a significant influence on the alkane mineralization, which was in agreement with the results obtained in this study. In the present study, the low degradation capacity upon natural attenuation could be due to the nutrient deficiency in the tested sea water hence microorganisms need nutrients to grow (Chekroud, 2006), or due to the low density of crude oil degrading microorganisms (1.3- 1.49 Log CFU/ml). Forsyth et al. (1995) reported that biodegradation would not occur at a significant rate if population of indigenous microorganisms is less than 10^5 CFU/g.

The number of heterotrophic bacteria in sea water without any addition was 6.9 Log CFU/ml/. This number increased to (7.2-8.79 Log CFU/ml) after one week by addition of nutrients (biostimulation) and microorganisms (bioaugmentation). After six weeks, it ranged between 8.01-9.4 Log CFU/ml (Table 29 & Fig. 30). The number of crude oil degrading bacteria was 1.49 Log CFU/ml, it increased after one week to the range 2.5-3.73 Log CFU/ml while it reached the values (3.85-5.18 Log CFU/ml) after six weeks. The increase in heterotrophic bacterial number was correlated with an increase of crude oil degrading bacteria. Pellitier et al. (2004) reported that the number of hydrocarbon degrading bacteria found in hydrocarbon polluted interdial sediments before treatment did not exceed 1% of the total saprophytic abundance but it reached to 45–50% of the total between 177-208 days. Chaerun et al. (2004) during his study on bioremediation of a oil spill polluted sea in Japan found that the number of crude oil degrading bacteria was about 10^5 - 10^6 at the beginning of the experiments and reached 10^4 - 10^6 cells/ml after five years of bioremediation. The results proved that there were no great differences in the number of crude oil degrading bacteria between the beginning and the end of the experiment.

Generally, it was observed that there was a correlation between the CFU/ml and the percentage of degradation, crude oil degrading bacteria used carbon from crude oil to proliferate (Pellitier et al., 2004). On the other hand, Chaîneau et al. (2005) reported that the biodegradation of crude oil was not concurrent with the heterotrophic bacterial population. Other authors experimenting with nutrient-supplemented hydrocarbon contaminated soils (Ting et al., 1999; Vasudevan and Rajaram, 2001) have reported dramatic changes in microbial populations. The microbial population in the nutrient system studied by Ting et al. (1999) decreased after the increase and, then increased again. These changes may be due to interactions between various microbial populations and the resulting changes to the environment (Sarkar et al., 2005). It could be also due to the byproducts of crude oil degradation, which may induce or inhibit the indigenous flora.

The acceleration of natural hydrocarbon biodegradation processes through the addition of nitrogen and phosphorus containing fertilizers has been tested in both marine and terrestrial ecosystems during the last two decades (Gentili et al.,2006).Thus, when bioremediation is conducted suitable nitrogen and phosphorus are usually applied to the contaminated environment to stimulate biodegradation (Prince, 1993). Various nitrogen sources such as inorganic fertilizers, urea, saw dust, compost, manure and biosolids have been used in biostimulation (Cho et al., 1997; Namkoong et al., 2002; Rosenberg et al., 1992; Walworth and Reynolds, 1995; Williams et al., 1999). In this study, the effect of nitrogen and phosphorus sources on bioremediation of crude oil contaminated sea water.

In the present study, the bioremediation of crude oil contaminated sea water was also evaluated in Lab-microcosm. Urea 46%, local urea, sodium nitrate, ammonium sulfate and local nitrogen fertilizer were used as nitrogen sources, in addition to the organic source (manure). The results obtained in Table 30 demonstrated that the degradation of crude oil was dependent on the type of treatment and the nitrogen source used. It was about 67.5-94% after 6 weeks of treatment. It was also found that in presence of local urea as nitrogen source, the degradation of crude oil reached more than 90% in all treatments except in case of biostimulation (78.5%). The results also proved that the addition of manure stimulated the biodegradation of crude oil in biostimulation or bioaugmentation with *Rhodococcus* sp. S and *Pseudomonas* s. S treatments in comparison with local urea as nitrogen source. Adesodun and Mbagwu (2008) added organic wastes from animal droppings as bioremediation alternative for soils spiked with waste-lubricating oil (spent oil). The total hydrocarbon contents (THC) with time of sampling were markedly reduced with addition of cow dung (CD), poultry manure (PM) and pig wastes (PW). The general trend in the first year indicated that PW stimulated the highest net percentage loss in THC for soils polluted with 5000 mg/ kg (0.5%SP) and 50,000 mg /kg (5% SP) oil levels. Poultry manure induced the highest reduction in soils polluted with medium, i.e. 2.5%SP (25,000 mg kg_1) oil concentration.

The use of urea with different phosphorus sources(ammonium phosphate, super phosphate 15.5% and local phosphorus fertilizer) resulted in a degradation percentage between 77.5- 94% (Table 32). When local phosphorus fertilizer was used as phosphorus source crude oil degradation was 87-90% compared with 77.5-88% when we used ammonium phosphate. It was concluded that different organisms have different requirements for N and P and provision of these nutrients at different concentrations will differentiate that the organisms most able to utilize the nutrients at levels provided in the oiled habitat (Chekroud, 2006). Mryyan and Battikhi (2005) reported that addition of $NaNO_3$ had beneficial effects on hydrocarbon degradation, which is in agreement with some treatments in this study (like bioaugmentation with consortium

M*;* 92.5% after six weeks). Rojas-Avelizapa et al. (2007) used urea and K_2HPO_4 as nitrogen and phosphorous sources in order to achieve a C/N/P ratio of 100/3/0.5 for the bioremediation of drilling mud polluted site. McCarty et al. (2004) used urea 46% as nitrogen source and mono-ammonium phosphate as phosphorus source to stimulate biodegradation of hydrocarbons in a hydrocarbon contaminated site. The addition of these fertilizers enhanced biodegradation even at low temperatures. An oleophilic fertilizer Inipol EAP 22 which is a microemulsion containing urea as a nitrogen source, lauryl phosphate(as phosphorus source) was applied by Nikolopoulou and Kalogerakis (2008) to enhance bioremediation of crude oil. Nitroammophosqua (potassium, nitrogen and phosphorus content is 16% each) is a commercial fertilizer used by Murygina et al. (2000) for the bioremediation of diesel oil polluted aquatic systems and soils. Ruiz et al.(2006) used NH_4NO_3 and K_2HPO_4 in concentrations to keep a C:N:P ratio of 100:10:1 for the bioremediation of sea water polluted with weathered crude oil by a salt tolerant consortium.

Adding nutrients enhanced the number of heterotrophic and crude oil degrading bacteria in contaminated sea water (Fig.32 & 34) in all treatments, indicating that sea water had high microbial population natively and updated hydrocarbon degrading population which are stimulated by nutrients supplementation. Chaîneau et al. (2005) and Sarkar et al. (2005) obtained similar results.

To evaluate the effect of immobilized cells on crude oil degradation in sea water, the tested organisms were immobilized on rice straw. The results mentioned in Table 34 indicated that immobilized cells showed faster and better crude oil degradation than free cells. The time needed for crude oil removal was reduced from six weeks in case of bioaugmentation by free cells to four weeks when contaminated sea water was bioaugmented by immobilized cells. The cumulative percent of degradation in the treatments with immobilized cells was higher than free cells (56.38-69.25% in comparison with 71.42-79.08% respectively) . This could be explained that immobilization protect the cells from the contaminants. There have been several studies reporting similar results (Diaz et al., 2002; Na et al., 2000; Manohar et al., 2001). The better and faster degradation rate observed was most likely due to the high immobilization efficiency of the cells onto the immobilization material. Diaz et al. (2002) used fibers made from 100% polypropylene material with oleophilic and hydrophobic properties to immobilize bacterial consortium. They found that when the salinity of the medium exceeded 20 g/l, hydrocarbon removal by immobilized cells was higher than free cells but at low salinity (0-20 g/l) there was no difference between the effect of free and immobilized cells. For the treatment of oil field wastewater, Zhao et al. (2006) used immobilized cells on a carrier called FPUFS and found that the immobilized cells are effective in treating field wastewater that is high in salinity, lacks nitrogen and phosphorus and contains PAHs. On the other hand, Quek et al. (2005) reported that

the degradation extent of total n- alkanes in Arabian light crude (ALC) by *Rhodococcus* sp. *F 92* immobilized on D14 polyuthrene foam (D14 PUF) was similar to those of free cells. Tay et al. (2005) to overcome the inhibitory difficulties associated with high strength phenolic wastewaters used aerobic acetate granules, which are self immobilized aggregates of microorganisms and organic and inorganic matter held together by a matrix of extracellular polymers. Gentili et al.(2006) used a strain of *R. corynebacteriorides* immobilised on chitin and chitosan flakes to bioremediate a crude oil polluted sea water. They found that 60% of hydrocarbons in the hexanic extract were removed compared with 30% in case of cells without carrier.

The petroleum adsorbed products may be then degraded in-situ or ex-situ (Quek et al.2005). The wheat straw immobilised cells could be easily collected from marine waters and can keep their capacity to degrade oil for a certain period. This finding can be applied to the polluted sea waters of the industrial zone of skikda, Algeria, where the Mediterranean waters suffer from the petroleum refinery activities.

Dehydrogenase activity has been correlated with hydrocarbon degradation (Margesin et al., 2000; Marin et al., 2005). Increasing the incubation time increased the value of dehydrogenase (increase in O.D.) in both treatments (free and immobilized) with some fluctuation in some cases. Maximum O.D. (0.305) was obtained with immobilized cells of the consortium after five weeks. Lee et al. (2008) reported that dehydrogenase acitivity increased after 15 days of treatment, then decreased to background levels and remained unchanged. Generally, the dehydrogenase activity was higher in treatment with immobilized cells than in free cells. Dehydrogenase activity can be considered as an indicator for aerobic biodegradation (Chekroud,2006). It was found that dehydrogenase activity correlated with the degradation rate of crude oil. These results are in agreement with those found by Lee et al. (2008). They reported that dehydrogenase activity was significantly negatively correlated with total extracted matter (TEM) concentrations. This suggests that there was an increase in microbial growth with a consequent increase in enzyme activity (Reddy and Fazza, 1989; Perucci, 1993)

=It was also reported that the ambient temperatures influence the physical nature and chemical composition of oil, rate of hydrocarbon degradation, and composition of microbial communities (Atlas, 1981; Hoff, 1993). The effect of temperature on the rate of crude oil biodegradation in sea water under test was evaluated by free and immobilized cells of the tested organisms at different temperature ranges. In case of free cells, the cumulative percent of degradation was better by about 5-10% at temperature range 22–26°C than at 18–20°C. Biostimulation of the indigenous flora at temperature range 22–26°C increased the degradation of crude oil in sea water by about 4% in comparison with the temperature range 18–20°C (Table 36). It was also found that temperature affects the crude oil degradation by immobilized cells

(Table 38). An increase of 3–6.5% in the cumulative percent of degradation was observed by immobilized cells at temperature 22-26°C than at 18-20°C. As the results indicate, temperature has a marked effect on the rate of crude oil degradation in sea water. At low temperatures, the biodegradation of crude oil was reduced. It is suggested to carry out remediation of crude oil contaminated sea water at temperature ≥ 30°C. Therefore, in situ remediation of sea water will be more efficient in warm seasons. The results are in agreement with the results of Seklemova et al. (2001), who reported that when the bioremediation operations commence during the warm season, the biodegradation process will be faster. Iwashita et al. (2004) isolated a *Bacillus licheniformis* strain, which was able to degrade the metal lubricant at 37°C and faster at 42°C. The biotransformation and mineralization of two (PAHs), anthracene and pyrene, by an enrichment culture were investigated at two temperatures, 10°C and 25°C (Sartoros et al., 2005). At 25°C, the overall mineralization of anthracene was 48% and that of pyrene was 66.1%, while at 10°C, there was a decrease in the mineralization of anthracene and pyrene (18.5 and 61.5% respectively).

The effect of temperature on bioremediation is dependent on the type of microorganisms involved in bioremediation. The percentage of crude oil degrading bacteria in contaminated sea water slightly increased at the temperature range 22–26°C in comparison with the range 18-20°C. Leahy and Colwell (1990) and Olivera et al. (1997) reported that the percentage of degradation decreased with decreasing temperature, which seems to be primarily related to the decrease in enzymatic activity, whereas higher temperatures increased the rate of hydrocarbon metabolism to the maximum.

The effect of bioaugmentation by immobilized cells on the degradation of elevated concentrations of crude oil was testes in lab-microcosms. The experiment was performed in presence and in absence of Triton X-100. It was found that at 3% of crude oil , bioaugmentation by *Rhodococcus* sp. S and the consortium was not affected by the absence of Triton X-100. This is may be due to the high hydrophobicity of the cells since cells exhibiting the highest hydrophobicities were among the fastest hydrocarbon degraders (Zhang and Miller, 1994). Therefore, isolates with high hydrophobicity are likely to be more efficient degraders (Cameotra and Singh, 2008). Cell hydrophobicity is also an indication of biosurfactant production (Pruthi and Cameotra, 1997).

Many microbes have hydrophobic surfaces and adhere to small droplets of oil and many also produce extracellular compounds which disperse the oil (Munn, 2004). Bonilla et al. (2005) isolated a strain of *Pseudomonas putida* ML2 that was able to produce a bioemulsifier polysaccharide.

In this part of the present study four treatments were prepared for scale-up of lab microcosms (40 cm of diameter); attenuation of natural flora, biostimulation and bioaugmentation with wheat straw immobilized cells of *Rhodococcus sp S* and consortium M. A control without any treatment was performed. The results in Table 40 demonstrated that the best treatment was in bioaugmentation with consortium M (cumulative percent of degradation 68.65%) in comparison with the other treatments. Ojo (2006) used the bacterial consortium isolated from wastewater contaminated with crude oil as seed onto oil-impacted environment, which is a more environmentally friendly approach to bioremediation rather than the use of exotic bacterial strains. In the present study, biostimulation of the cude oil contaminated sea water resulted in 78.5% degradation after five weeks while only 55.5% was obtained in attenuation of the natural flora after the same time. These results are in agreement with Röling et al. (2004) who reported that the treatment of plots contaminated with buried oil by adding nutrients was faster than the attenuated plots. In the control seawater, the degradation of crude oil due to abiotic effect (evaporation) was low (22.5% after five weeks) in comparison with the biological treatments. In this study, the degradation of crude oil due to abiotic effect in the scale-up process was higher than that in lab microcosms (it was almost zero in lab microcosms), this may be due to the climatic factors (like temperature and air) of the scale-up microcosms. Röling et al. (2004) found differences between laboratory and field experiments. These differences indicate that great care should be taken when results of laboratory experiments are extrapolated to field situations (Swannell et al., 1995).

Dehydrogenase activity in the scale up experiment was increased with time in biological treatments (Table 40 and Fig. 43, this increase may be due to the microbial activity. The low values of dehydrogenase activity in control sea water (OD was 0.005 after five weeks) indicates that the loss in crude oil concentration was due to abiotic effects.

Algae are often used in toxicity testing (Staveley and Smrchek, 2005). To test toxicity of crude oil contaminated sea water, an algae native to Ben Mhidi beach was grown in contaminated sea water before and after treatment (five weeks). The growth of the algae was better in treated sea water by immobilized cells of *Rhodococcus* sp. S and Consortium M. The untreated sea water inhibited the algal growth. This means that treated sea water showed less toxicity in comparison with untreated one. Bruno and Eklund (2003) applied an inhibition test , with two filamentous algae *Ceramium stricum* and *Ceramium tenuicorne* in phenol contaminated sea water. Han and Choi (2005) also used the green macro algae *Ulva pertusa* as a test toxicity of heavy metals in sea water.

V. CONCLUSION

V.Conclusion

This work was done to understand the role of microorganisms in the bioremediation of crude oil polluted sea in Skikda, since Skikda is one of the most oil spills polluted towns.We concluded that

- Bioremediation using bacterial strains is an effective technique for the decontamination of oil polluted marine media,
- Local fertilisers (local phosphorus fertiliser and local urea) may be used as phosphorus and nitrogen sources,
- The use of wheat straw immobilised cells is a cost effective technique, it accelerates the rate of oil biodegradation and could be employed as a practical technology for oil biodegradation in oil polluted sea ,
-The use of bacterial strains able to produce biosurfactants instead of adding chemical surfactants is cost effective and helps in accelerating the rate of degradation,
- The lab microcosm, can be scaled up successfully with small differences and
-It is recommended to perform the bioremediation process in warm seasons to accelerate the biodegradation of crude oil.

VI.

REFERENCES

Adesodun J.K. and Mbagwu J.S.C. (2008). Biodegradation of waste-lubricating petroleum oil in a tropical alfisol as mediated by animal droppings.
Biores. Tech. **99** :5659–5665

Admaski M., Manko T., Sebastian I., Lukasik W., Plaza G., Ulfig K., **Wolsky P., Worsztynowicz A. and Zachaz K**. **(2000).** Bioremediation. In: Bioremediation of Petroleum Hydrocarbon-contaminated soils. Comprehemsive Report .WSRC-RP-2000-00975 (U).

Alef K. and Nannipieri P. (1995). Methods in Applied Soil Microbiology and Biochemistry. Academic Press, New York. 576 P.

Allen C. C. R., Boyd D. R., Hempenstall F., Larik M. J. and Sharma N. D. (1999). Contrasting effects of a nonionic surfactant on the biotransformation of polycyclic aromatic hydrocarbons to cis-dihydrodiols by soil bacteria. Appl.Environ.Microbiol.**65**:1335-1339.

Alvarez H. M. (2003). Relation between β-oxidation pathway and the hydrocarbon-degrading profile in actinomycetes bacteria. Int. Biodeter. Biodeg. **52**: 35-42.

American Society for Testing and Materials (D 3921-96). (1996). Standard Test Methods for Oil and Grease Petroleum Hydrocarbons in Water. American Society for Testing and Materials. 100 Barr Harbor DR.,West Conshohoken, PA 19428.

Arafa M. A. (2003). Biodegradation of some aromatic hydrocarbons (BTEXs) by a bacterial consortium isolated from polluted site in Saudi Arabia. Pakistian J. Biol. Sci. **6**:1482-1486.

Atlas R. M. (1977). Stimulated petroleum biodegradation. CRC Crit. Rev. Microbiol. **5**: 371-386.

Atlas R. M. (1981). Microbial degradation of petroleum hydrocarbons: an environment perspective. Microbiol. Rev. **45**: 180-209.

Atlas R. M. (1991).Microbial hydrocarbon degradation-bioremediation of oil spills. J.Chem. Technol. Biotechnol. **52**:149-156.

Atlas R.M. and Bartha R. (1973). Fate and effects of pollution in the marine environment. Residue Rev.**49**: 49-85.

Atlas R. M and Unterman R. (1999). Bioremadiation. In: A. Demain, J. Davies and R. M. Atlas (Ed). Manual of industrial microbiology and biotechnology. Vol.2. Societies and Associations. PP. 666-681.

Baker K. H. and Herson D. S. (1994). Microbiology and biodegradation. In: K. H. Baker and D.S. Herson (Ed). Bioremediation. McGraw- Hill, Inc. New York. PP. 9-60.

Bartha R. and Atlas R. M. (1977). The microbiology of aquatic oil spills. In: D. Pearlman (Eds). Advances in applied microbiology. Vol. 2. Academic learn New York, San Fransisko. PP. 225-266.

Baszczyk-Maleszak H., Zabost A., Wolicka D. and Kacieszczenke J. (2006). Effectiveness of biodegradation of petroleum products by mixed bacterial population in liquid medium at different pH values. Polish J. Microbiol. **55**: 69-73

Beahm H. W. and Perry J. J. (1973). Co-metabolism as a factor in microbial degradation of cycloparaffinic hydrocarbons. Arch. Mikrobiol. **91**:87-90

Beolchini F., Rocchetti L., Regoli F. and Dell'Anno A. (2010). Bioremediation of marine sediments contaminated by hydrocarbons:Experimental analysis and kinetic modeling. Journal of Hazardous Materials **182**:403–40

Bento F.m., Amargo F.A.O.,Okeke B.C.and Frankenberger W.T.(2005). Comparative bioremediation of soils contaminated with diesel oil by natural attenuation, biostimulation and bioaugmentation,.Biors. Technol. 96:1049-1055.

Bomlen G. F. and Kossan D. S. (1995). In situ process for bioremediation of BTEX and petroleum fuel products. In: L. Y Yong and C.F. Cerniglia (Eds). Microbial transformation and degradation of toxic organic chemicals. Wiley Liss, New York.

Bonilla M., Corona M., Alvarez A. and Soubes M. (2005). Production and characterization of a new bioemulsifier from *Pseudomonas putida* ML2. Appl. Microbiol. 98: 456-463.

Boonchan S., Britz M. L. and Stanley G. A. (1998).Surfactant-enhanced biodegradation of high molecular weight polycyclic aromatic hydrocarbons by *Stenotrophomonas maltophilia*. Biotechnol. Bioeng. **59**: 482-494.

Bosma T. N. P., Middeldor P., Schraa G. and Zender A. J. B., (1997).Mass transfer limitation of biotransformation: quantifying bio-availability. Environ. Sci. Technol. **31**: 248-252

Bossert I. and Bartha R. (1984).The fate of petroleum in soil ecosystem. In: R. M. Atlas (Ed.). Petroleum microbiology. Macmillan, New York. PP. 435-476.

Boudelaa S. and Medjram M.S. (2011). Assessment of Chemical and Petrochemical Effluent: - Case ofHydrocarbon Pole of Skikda, Algeria.European Journal of Scientific Research. **ISSN 1450-216X :63 (4**):563-584

Bourdjiba O., Bekhouche F., Hassaine A. and Djenidi R. (**2009)**. Impact de la pollution par les hydrocarbures sur la qualité des eaux usées dans la région de Skikda (Nord- Est Algérien). European Journal of Scientific Research **26(1)**: 87-97.

Braddock J. F., Ruth M. L., Catterall P. H., Walworth J. L. and McCarthy K. A. (1997). Enhancement and inhibition of microbial activity in hydrocarbon-contaminated arctic soils: implications for nutrient amended bioremediation.*Environ. Sci. Technol.* **31**: 2 078–2 084.

Bragg J. R., Prince R. C., Harner E. J. and Atlas R. M. (1994). Effectiveness of bioremediation for the *Exxon Valdez* oilspill. *Nature*. **368**: 413–418.

Bramwell D.-A. P. and Laha S. (2000). Effects of surfactant addition on the biomineralization and microbial toxicity of phenanthrene. Biodeg. **11**:263-277.

Brennan P. J. and Nikaido H. (1995). The envelope of mycobacteria. Annu. Rev. Biochem. **64**: 29-63.

Bressler D. C. and Gray M. R. (2003). Transport and reaction processes in bioremediation of organic contaminants. 1. Review of bacterial degradation and transport. *Int. J. Chem. React. Eng*. **1**: R3.

Britto R., Scherrard J. H. and Truax D. (1994). Kinetics of pseudo-continuous flow bioreactor treatment of diesel contaminated soils. Water, Air and Soil Pollut. **86**: 125-136.

Bruno B. and Eklund B. (2003).Two new growth inhibition tests with the filamentous algae Ceramium strictum and C. tenuicorne (Rhodophyta). Environ Pollut. **125(2):**287-93.

Cairney T. (1993). Contaminated Land, Blackie, London. P.4

Cameotra S. S. and Singh P. (2008). Bioremediation of oil sludge using crude biosurfactants. International Biodet. & Biodeg. **62** : 274–280

Champion J. T., Gilkey J. C., Lamparske H., Retterer J. - and Miller R. M. (1995). Electron Microscopy of Rhamnolipid (Biosurfactant) Morphology: Effects of pH, Cadmium, and Octadeane. Colloid and Interface Sci. **170**: 569-574.

Chaerun S.K., Tazaki K.., Asada R.and Kogure K. (2004). Bioremediation of coastal areas 5 years after the Nakhodka oil spill in the Sea of Japan: isolation and characterization of hydrocarbon-degrading bacteria. Environ. Int. 30 :911 – 922.

Chaîneau C. H., Rougeux G. C., Yéprémian C. and Oudot J. (2005).Effects of nutrient concentration on the biodegradation of crude oil associated microbial populations in the soil. Soil Biol. Biochem. **37**: 1490-1497.

Chekroud Z. (2006). Bioremediation of kerosene contaminated clay. M.Sc Thesis, Botany Depart. Faculty of Science, Alexandria University, Egypt.

Cho B.-H., Chino H., Tsuji H., Kunito T., Nagoaka K., Otuka S., Yamashita K., Matsumoto S. and Oyaiz, H. (1997). Laboratory scale bioremediation of oil contaminated sites of Kuwait with soil amendment materials. Chemosphere. **35**: 1599-1611.

Cote R. P. (1976). The effects of petroleum refinery liquid wastes on aquatic life with special emphasis on the Canadian environment. Reaserch Council of Canada NRC Association Committee for Environmental Quality. Ottawa Ontario, Canada KIA 0R6, publication number 15021:77.

Cunningham J. A., Hopkins G. D., Lebron C. A., and Reinhard M. (2000). Enhanced anaerobic bioremediation of groundwater contaminated by fuel hydrocarbons at Seal Beach, California. Biodeg. **11**: 159-170.

Das K., Mukherjee A.K. (2007). Crude petroleum-oil biodegradation eYciency of *Bacillus subtilis* and *Pseudomonas aeruginosa* strains isolated from a petroleum-oil contaminated soil from North-East. India.Biores.Tech. **98**: 1339–1345

Diaz M. P., Kenneth G. B. and Grison S. J. W. (2002). Biodegradation of crude oil across a wide range of salinities by an extremely halotolerant bacterial consortium M.P.D-M, immobilized onto polypropylene fibers.
Biotechnol. Bioeng.**79**:145-153.

Dibble J. E and Bartha R. (1979). Leaching aspects of oil sludge biodegradation in soil. Science. **127**: 365-370.

Dineen D., Slater J. P., Jicks P. and Holland I. (1990). In situ biological remediation of petroleum hydrocarbons in unsaturated soils. In: P. T. Kostecki and E .J. Calabrese (Eds). Hydrocarbon Contaminated Soils and Ground Water: Analysis, Fate, Environmental and Public Health Effects, Remediation. Vol.1. Lewis Publishers, Chelsea, Mich. PP. 177-187.

Dionex(2004). Accelerated solvent extraction (ASE) of hydrocarbon contaminants (BTEX,Diesel, and TPH) in soils.Application note 324, Dionex corp, USA. 4**.**

Einsele A. (1983). Biomass from higher n-alkanes. In: H.-J. Rehm and G. Reed (Ed). H. Dellweg (Vol. Ed.). Biotechnology. Vol.3. Verlag Chemie, Weinheim, Deerfield. PP. 44-81.

Einsele A. and Fiechter A. (1971). Liquid and solid hydrocarbons. Biochem. Eng. **1**: 169-194.

Filler D. M., Reynolds C. M., Snape I., Daugulis A. J., Barnes D. L. and Williams P. J. (2006). Advances in engineeredremediation for use in the Arctic and Antarctica. *Polar Rec*. **42**: 111–120.

Forsyth J. V., Tsao Y. M. and Bleam R. D. (1995). Bioremediation: when is bioaugmentation needed? In: R. E. Hinchee, J. Frederickson and B. C. Alleman (Eds). Bioaugmentation for site remediation. Battele Press, Columbus, OH. PP. 1-14

Fukui S. and Tanaka A. (1981). Metabolism of alkanes by yeasts. In: A. Fiechter (Ed). Advances in biochemical engineering. Reactors and Reactions. Springer-Verlag, Berlin, Heidelberg, New York. PP.217-235.

Gallegot J. L. R., Loredo J., Liamas J. F., Vasquez F. and Sanchez j. (2001). Bioremediation of diesel contaminated soils: evaluation of potential in situ techniques by study of bacterial degradation. Biodeg. **12**: 325-335.

Gargouri B., Karray F., Mhiri N., Aloui F.and Sayadi S.(2011). Application of a continuously stirred tank bioreactor (CSTR) for bioremediation of hydrocarbon-rich industrial wastewater effluents.Journal of Hazardous Materials **189** : 427–434.

Gaudy A. F. Jr. and Gaudy E. T. (1980). Microbiology for Environmental Scientists and Engineers. McGraw-Hill Book Company. New York.

Gentili A.R., Cubitto M.A., Ferrero M.and Rodrigue´z M.S. (2006). Bioremediation of crude oil polluted seawater by a hydrocarbon degrading bacterial strain immobilized on chitin and chitosan flakes.Int. biodeter. Biodegr. **57** :222–228.

Gianfreda L. and Rao M. A. (2004). Potential of extra cellular enzymes in remediation of polluted soils: a review. Enzyme Microbiol. Technol. **35**: 339–354.

Gouda M.K., Omar S.H., Chekroud Z.A., Nour Eldin H.M. (2007a) Bioremediation of kerosene I: A case study in liquid media. Chemosphere **69**: 1807-1814.

Gouda M.K., Omar S.H., Nour Eldin H.M. and Chekroud Z.A. (2007b). Bioremediation of kerosene II: A case study in contaminated clay (laboratory and field : scale microcosms). World J. Microbial. Biotechnol. DOI 10. 1007/s11274-007-9634-x

Greer C. W., Fortin N., Roy R., Whyte L. G. and Lee K. (2003). Indigenous sediment microbial activity in response to nutrient enrichment and plant growth following a controlled oil spill on a fresh water wetland. Bioremed. j. **7:** 69-80.

Gruiz K. and Kriston E. (1995). In situ bioremediation of hydrocarbon in soil. Soil Contamination. **4**:163-173.

Haines J. R. and Alexander M. (1974). Microbiol degradation of high-molecular weight alkanes. Appl. Microbiol. **28**: 1084-1085.

Hamman S. (2004). Bioremediation capabilities of white rot fungi. BI570-review article spring 2004.

Han T. and Choi G.W. (2005). A novel marine algal toxicity bioassay based on sporulation inhibition in the green macroalga *Ulva pertusa* (Chlorophyta). Aquatic Toxicol. **75** :202–212

Harms H. and Bosma T. N. P. (1997). Mass transfer limitation of microbial growth and pollutant degradation. Indust. Microbiol. **18**: 97-105.

Hoff R. Z. (1993). Bioremediation: an overview of its development and use for oil spill clean up. Mar. Pollut. Bull. **26**: 476-481.

Huesemann M. H and Moore K. O. (1993). Compositional changes during land farming of weathered Michigan crude oil- contaminated soil. Soil Contamination. **2**: 245-264.

Hunt G. R. (1979). Near-infrared (1.3-1.4 mm) spectra of alteration minerals-potential for use in remote sensing. Geophysics.**42**: 501-513.

Irwin W. H. (1965). Fifty-seven species of fish in oil refinery waste biomassay, North American Wildlife and Natural Resources Conference Transcripts. **30**: 89-99.

Iwashita S., Callahan T. P. and Haydu J. (2004). Mesophilic aerobic degradation of metal lubricant by a biological consortium. Appl. Microbiol. Biotechnol. **65**: 620-626.

Jackson W. A. and Pardue J. H. (1998). Potential of enhancement of biodegradation of crude oil in Louisiana salt marshes using nutrient amendments. Water, Air, and Soil Pollut. **190**: 343-355.

Johnsen A. R., Wick L. Y. and Harms. H. (2005). Principals of microbial PAH degradation in soil. Environ. pollut. **133**: 71-84.

Jones J. G., Knight M. and Byrom J. A. (1970). Effect of gross pollution by kerosene hydrocarbons on the microflora of a Moorland soil. Nature (London). **227(5263):** 1166

Jones III V. D., Matthew M. D. and Richers D. M. (1999). Light hydrocarbons for petroleum and gas prospecting. In: M. Hale (Ed). Handbook of exploration of geochemistry. Vol.7. Elsievier Sience B.V.

Kageyama C., Ohta T., Hiraoka K., Suzuki M., Okamoto T. and Ohishi K. (2005). Chlorinated aliphatic hydrocarbon-induced degradation of trichloroethylene in *Wautersia numadzuensis* sp. nov. Arch. Microbiol. **183**: 56-65.

Kanaly R. A and Harayama S. (2000). Biodegradation of high- molecular weight aromatic hydrocarbons by bacteria. Bacteriol. **82**: 2059-2067.

Kim T. J., Lee E. Y., Kim Y. J., Cho K.-S. and Ryu H. W. (2003). Degradation of polyaromatic hydrocarbons by *Burkholderia cepacia* 2A-12. Microbiol. Biotechnol. **19**: 411-417.

Kim Y.-H., Freeman J. P., Moody J. D., Engesser K. H. and Cerniglia C. E. (2005). Effects of pH on the degradation of phenanthrene and pyrene by *Mycobacterium vanbalenii* pyr-1. Appl. Microbiol. Biotechnol. **67**: 275-285.

Klug M. J. and Markovetz A. J. (1971). Utilization of aliphatic hydrocarbons by microorganisms. Adv. Microbiol. Physiol. **5**: 1-43.

Korda A., Santas P., Tenente A., Santas R. (1997). Petroleum hydrocarbon bioremediation: sampling and analytical techniques, in situ treatments and commercial microorganisms currently used. Appl. Microbiol. Biotechnol. **48**: 677-686.

Leahy J. G. and Colwel R. R. (1990). Microbial degradation of hydrocarbons in the environment. Microbiol. Rev. **54**: 305-315.

Leahy J. G., Byrn A. M. and Olsen R. H. (1996). Comparison of factors influencing trichloroethylene degradation by toluene-oxidizing bacteria. Appl. Envioron. Microbiol. **62**:825-833.

Lee K., Siron R., and Tremblay G. H. (1995). Effectiveness of bioremediation in reducing toxicity in oiled intertidal sediments. In R. E. Hinche, F. J. Brockman, & C. M. Vogel (Eds.), Microbial processes for Bioremediation. Columbus: Battelle Press. pp. 117–127

Lee M., Kim M. K., Singleton I., Goodfellow M. and Lee T. (2006). Enhanced biodegradation of diesel oil by a newly identified *Rhodococcus baikonurensis* EN3 in the presence of mycolic acid. Appl. Microbiol. **100**: 325-333.

Lee S.H. Bang-Il O.and Jeong-gyu K. (2008). Effect of various amendments on heavy mineral oil bioremediation and soil microbial activity. Biores. Technol.**99:** 2578–2587.

Li K. Y., Zhang Y. and Xu T. (1995). Bioremadietion of oil contaminated soil-aerate model. Waste Manag. **15**: 335-338.

Li Z., Obika H., Kamishima H., Fukuoka S., Kakita H., Kobayashi Y. and Higashihara T. (1994). Biodegradation of floating oil by a bio-system co-immobilizing marine oil-degrading microorganism and nutrients. Seibutsu-Kogaku.**72**: 363-370.

Li Z., Obika H., Kamishima H., Fukuoka S., Kakita H., Kobayashi Y. and Higashihara T. (1995). Improvement of immobilization conditions for biodegradation of floating oil by a bio-system co-immobilizing marine oil-degrading yeast *Candida sp*. and nutrients. Seibutsu-Kogaku. **73**: 295-299.

Lieberg E. W. and Cutright T. J. (1999).The investigation of enhanced bioremediation through the addition of macro and micro nutrients in PAH contaminated soil. Int. Biodeter. Biodeg. .**44**: 5-64.

Liu C.W.,. Chang W.N and Liu H.S. (2009). Bioremediation of *n*-alkanes and the formation of bioflocculesby *Rhodococcus erythropolis* NTU-1 under various saline conditions and sea water. Biochemical Engineering Journal. **45**: 69–75

Lode A. (1986). Changes in bacterial community after application of oily sludge to soil. Appl. Microbiol. **25**:295-299.

Loynachan T. E. (1978). Low temperature mineralization of crude oil in soil. Environ.Qual.**7**: 494-500.

Lowry O. H., Rosenbrough N. J., Farr A.L and Rundall R. L. (1951). Protein measurement with folIin reagent. J. Biol. Chem. **193**: 265-275.

Manohar S., Kim C. K. and Karegoudar T. K. (2001). Enhanced degradation of naphthalene by immobilization of *Pseudomonas sp.* strain NGK1 in polyurethane foam. Appl. Microbiol. Biotechnol. **55**: 311-316.

Margesin R. and Schinner F. (1997a). Bioremediation of diesel-oil contaminated alpine soils at low temperatures.Appl. Microbiol. Biotechnol. **47**:462-468.

Margesin R. and Schinner F. (1997b). Laboratory bioremediation experiments with soil from a diesel-oil contaminated site- significant role of cold-adapted microorganisms and fertilizers. Chem. Biotechnol. **70**: 92-98.

Margesin R., Zimmerbauer A. and Schinner F. (2000). Monitoring of bioremediation by biological activities. Chemosphere **40**: 339–346.

Marin J.A. Hernandez T. and Garcia C. (2005) Bioremdiation of oil refinery sludge by landfarming in semiarid conditions: Influence on soil microbial activity. Environ. Res. **98**: 185–195.

Mearns A., Doe K., Fisher W., Hoff R., Lee K., Siron R., Mueller C. and Venosa A. (1995). Toxicity trends ruding an oil spill bioremediation experiment on a sandy shoreline in Delaware, USA. Proceed.18th AMOP Tech. Seminar, Vol. 2, pp. 1133–1145, Edmonton, Canada.

McClay K., Streger S. H. and Steffan R. J. (1995). Induction of toluene oxidation activity in *Pseudomonas mendocina* KR1 and *Pseudomonas sp. strain* ENVPC5 by chlorinated solvents and alkanes.Appl. Enviorn. Microbiol. **61**: 3479-3481.

McCarty K., Walker L., Vigoren L. and Bartel J. (2004). Remediation of spilled petroleum hydrocarbons by in situ land farming at an arctic site. Cold Region Sci. Technol. **40**: 31-39.

Miyoshi M. (1895). Die durchbohrung von membranen durch Pilzbaden. Jahrb. Wiss. Bot. **28**:269-289.

Moran A.C., Olivera N., Commendatore M., Esteves J. and Sifieriz F.(2000). Enhancement of hydrocarbon waste biodegradation by addition of a biosurfactant from *Bacillus subtilus* O9. Biodeg. **11**: 65-71.

Morgan P. and Watkinson R. J. (1989). Hydrocarbon degradation and methods for soil biotreatment. CRC Crit. Rev. Biotechnol. **8**: 305-333.

Mryyan B. and Battikhi M. N. (2005). Biodegradation of total organic carbons (TOC) in Jordanian petroleum sludge. J. Haz. Mat. **120**: 127-134.

Mueller J. G, Chapman P. J. and Pritchard P. H. (1989). Action of a fluoranthene-utilizing bacteria community on polycyclic aromatic hydrocarbon components of creosote. Appl. Environ. Microbiol. **55**: 3085-3090.

Munn C. B. (2004). Marine Microbes and Human Society. In: C.B. Munn (Ed). Marine microbiology. Ecology and applications. Bios Scientific Publishers. PP. 255-271.

Munro A. L. S. 1970.Measurement and control of pH values. In: J.R Norris and D.W. Ribbons (Eds). Methods in microbiology. Vol.2. Academic Press, London. PP.39-89.

Murakami A., Omi S., Kozwa H. and Yamane A. (1985). Enhancement of biodegradation of spilled oil in the sea by nutrients released from microcapsules. Hakkokogaku. **63**: 145-151

Murygina V., Arinbasarov M. and Kalyuzhnyi S. (2000). Bioremediation of oil polluted aquatic systems and soils with novel preparation "Rhoder". Biodeg. **11**: 385-389.

Na K., Lee Y., Lee W., Huh Y., Lee J., Lee J., Kubo M. and Chung S. (2000). Characterization of PCB-degrading bacteria immobilized in polyurethane foam. J. Biosci. Bioeng. **90**: 368-373.

Namkoong W., Hwang E., Park J. and Choi J. (2002). Biormediation of diesel contaminated soil with composting. Environ. Pollut. **119**: 23-31.

National Academy of Sciences. (1975)."Petroleum in the marine environments" NAS, Washington D.C.

Nawar H.F.F. (1997). Bioremediation of petroleum hydrocarbons. M.Sc Thesis, Botany Depart. Faculty of Science, Alexandria University, Egypt.

Nelson M. J., Compeau G. C., Mariarz T. and Mahaffey W. R. (1994). Laboratory treatability testing for assessement of field applicability. In: P. E. Flathman, D.E. Jerger and J. H. Exner (Eds). Bioremediation: field experience. Lewis Publishers. London. PP: 59-78.

Nikolopoulou M. and Kalogerakis N. (2008). Enhanced bioremediation of crude oil utilizing lipophilic fertilizers combinedwith biosurfactants and molasses. Marine pollut. Bul. **56:** 1855–1861.

Nobert-King T. J. and Mount D. I. (1986). Validity of effluent and ambient toxicity tests for predicting biological impact. Skeleton Creek, Emid, Oklahoma.U.S. Environmental Protection Agency Report. Oklahoma, U.S.A. p.35.

Nocentini M., Penilli D. and Fava F. (2000). Bioremediation of soil contaminated by hydrocarbon mixtures: the residual concentration problem. Chemosphere. **41**: 1115-1123

Ogram A.V., Jessup R. E., Ou L. T. and Rao P. S. C. (1985). Effect of sorption on biological degradation rates of (2, 4-Dichlorophenoxy) acetic acid in soils. Appl. Environ. Microbiol. **49**: 582-587.

Oh Y.-S., Maeng J. and Kim S. J. (2000). Use of microorganism immmobilized polyurethane foams to absorb and degrade oil on water surface. Appl. Microbiol. Biotechnol. **54**:418-423.

Ojo O. A. (2006). Petroleum-hydrocarbon utilization by native bacterial population from a waste water canal Southwest Nigeria. African J. Biotechnol. **5**: 333-337.

Okuda T., Alcantara-Garduno M.E., Suzuki M., Matsui C., Kose T. et al. (2007) Enhancement of biodegradation of oil adsorbed on fine soils in a bioslury reactor. Chemosphere, doi: 10.1016/j chemosphere. 2006.12.081.

Olivera N. L., Esteves J. L. and Commendatore N. G. (1997). Alkane biodegradation by a microbial community from contaminated sediments in Patarogia, Argentina. Int. Biodeter. Biodeg. **40**: 75-79.

Omar S. H. and Rehm H.-J. (1980). Physiology and metabolism of two alkane oxidizing and citric acid producing strains of *Candida parapsilosis*.I. Activity of tricarboxilic and glyoxylate cycle enzyme. Appl. Microbiol. Biotechnol. **11**: 35-41.

Omar S. H. and Rehm H.-J. (1988). Degradation of n-alkanes by *Candida parapsilosis* and *Penicillium frequentans* immobilized on granular clay and aquifer sand. Appl. Microbiol**.** Biotechnol**. 28:** 103-108**.**

Omar S. H., Bϋdecker U. and Rehm H.-J. (1990). Degradation of oily sludge from a floating unit by free and immobilized microorganisms. Appl. Microbiol. Biotechnol. **34**: 259-263.

Pankrantz T. M. (2001).Environmental Engineering Dictionary and Directory.CRC Press. Boca Raton, FL.

Pelletier E., Delille D.and Delille B. (2004). Crude oil bioremediation in sub-Antarctic intertidal sediments: chemistry and toxicityof oiled residues.Marine Envron. Res. **57** :311–327

Perucci P.(1993). Enzyme activity and microbial biomass in a field soil amended with municipal refuse. Biol. Fert. Soils. **24:** 429–434.

Perrier A. (1913). Ann. Sci. Agron. Fr. Etrang. **30**: 321. (Cited in Biotechnology, 1983. H.-.J. Rehm and G. Reed (Ed). H. Dellweg (Vol. Ed). Verlag Chemie Weinheim Deerfield.).

Pfaender F. K. and Buckley E. N. (1984). Effects of petroleum microbial communities. In: R.M. Atlas (Ed). Petroleum microbiology. Macmillan, New York. P.507.

Pothuluri J. V. and Cerniglia C. E. (1994). Microbial metabolism of polyaromatic hydrocarbons. In: G.R. Chaudhry (Ed). Biological degradation and bioremediation of toxic chemicals. Kluwer, Dordrecht. PP. 92-124.

Prescott L. M., Harley J. P., and Klein D. A. (2002). Industrial microbiology and biotechnology. In: L. M. Prescott, J. P. Harley, and D. A. Klein. (Eds). Microbiology. 5th ed. McGraw Hill. PP: 941-1022.

Prince R. (1993). Petroleum spill bioremediation in marine environments. Microbiol. **19**: 217-242.

Pruthi V., Cameotra S.S. (1997). Rapid identification of biosurfactant producing bacterial strains using a cell surface hydrophobicity technique. Biotechnol. Tech. **11**: 671–674.

Quek E., Ting Y.-P. and Tang H. T. (2005). *Rhodococcus sp*.F92 immobilized on polyurethane foam shows ability to degrade various petroleum procucts. Biores. Technol. Article in Press.

Raymond R. L., Hudson J. O. and Jamison V. W. (1976). Oil degradation in soil. Appl. Environ. Microbiol. **31**: 522-535.

Reddy G.B. and Fazza A. (1989). Dehydrogenase activity in sludge amended soil. Soil Biol. Biochem. **21**: 327–332.

Resnick J. A. (1998). Degradation of petroleum hydrocarbons with organisms encapsulated in wax. US patent US5807724.

Rijnaarts H. H. M., Bachmann A., Jumelet J. C. and Zehnder A. J. B. (1990). Effect of desorption and intraparticular mass-transfer on the aerobic biomineralization of alpha-hexachlorocyclohexane in contaminated calcareous soil. Environ. Microbiol. **49**: 582-587.

Riser-Roberts E. (1998) Remediation of petroleum contaminated soil: biological, physical and chemical processes,CRC Press LLC, Bocca Raton, FL**.**

Rittmann B. E and McCarty P. L. (2001). Bioremediation. In: B. E Rittmann and P. L. McCarty. Environmental biotechnology: Principles and applications. McGraw Hill. PP.595-628.

Rodier J. (1996).L'analyse de l'eau, eaux naturelles, eaux résiduaires, eau de mer. 8 eme édition Dunod, Paris, France, 1384P.

Rojas-Avelizapa N.G., Rolda´n-Carrillo T., Zegarra-Martı´nez H., Mun˜oz-Colunga A.M. and Ferna´ndez-Linares L.C. (2007). A field trial for an ex-situ bioremediation of a drilling mud-polluted site.Chemosphere **66** :1595–1600.

Röling W.F.M., Milner M.G., Jones D.M., Fratepietro F., Swannell R.P.J., Daniel F. and Head I.M. (2004). Bacterial community dynamics and hydrocarbon degradation duringa field-scale evaluation of bioremediation on a mudflat beach contaminated with buried oil. Appl.Environ. Microbiol. **70**: 2603–2613.

Rosenberg E., Legmann R., Kushmaro A., Taube R., Aldere E. and Ron E. Z. (1992). Petroleum bioremediation a multiphase problem. Biodeg. **3**: 337-350.

Ruiz M., Pasadakis N. , Kalogerakis N. (2006). Bioremediation and toxicity determination of natural seawater polluted with weathered crude oil by salt-tolerant consortia in a SBR. Mar.polut. Bull. **52** : 1490–1493

Saha M. K. and Konar S. K. (1985). Chronic effects of petroleum refinery effluent on aquatic ecosystem. Environ. Ecol. **3**: 17-20.

Santas E., Korda A. Tenente A., Buchholz K. and Santas P. H. (2001). Mesocosm assays of oil spill bioremediation with oleophilic fertilizers: Inipol, F1 or both? Marine Pollut. Bul. **38**: 44–58.

Sarkar D., Ferguson M., Datta R. and Burnbaum S. (2005).Bioremediation of petroleum hydrocarbons in contaminated soils: comparison of biosolids addition, carbon supplementation and monitored natural attenuation. Environ. pollut. **136**: 187-195.

Sartoros C., Yerushalmi L., Beron P. and Guiot S. R. (2005). Effects of surfactant and temperature on biotransformation kinetics of anthracene and pyrene. Chemosphere. **61**: 1042-1050.

Schwarzenbach R. P., Gschwend P. M. and Imboden D. M. (1993). Environmental Organic Chemistry. Wiley Interscience,New York. 681 P.

Seklemova E., Pavlova A. and Kovacheva K. (2001). Biostimulation-based bioremediation of diesel fuel: field demonstration. Biodeg. **12**: 311-316.

Sextone A. J. and Atlas R. M. (1977a). Persistence of oil in tundra soils. Dev. Ind. Microbiol. **18**: 673-684.

Sextone A. J. and Atlas R. M. (1977b). Response of microbial populations in Arctic tundra soils to crude oil. Can. J. Microbiol. **23**: 1327-1333.

Sharma S. L. and Pant A. (2000). Biodegradation and conversion of alkanes and crude oil by a marine *Rhodococcus* sp. Biodeg. **11**:289-294.

Shin K.-H., Kim K.-W. and Seargen E. A. (2004). Combined effects of pH and biosurfactant addition on solubilization and biodegradation of phenanthrene. Appl. Microbiol. Biotechnol. **65**: 336-343.

Si-Zhong Y. Hui-Jun J., Zhi1 W., Rui-Xia H., Yan-Jun J., Xiu-Mei L.and Shao-Peng Y. (2009). Bioremediation of Oil Spills in Cold Environments: A Review. *Pedosphere* **19**(3): 371–381.

Soltani M.(2004). Distribution lipidique et voies métaboliques chez quatre bactéries Gram négatif hydrocarbonoclastes. Variation en fonction de la source de carbone,Thèse de PHD, Université de Paris6.

Staveley J.and Smrchek J.(2005). Algal Toxicity Test**.** Small-scale Freshwater Toxicity Investigations181-202, DOI: 10.1007/1-4020-3120-3_5

Steel R.G. and Torrie J. H. (1980). Principles and procedures of statistics. 2nd ed. Mc Graw Hill, New York, USA.

Sutherland J. B., Rfii F., Khan A. A., and Cerniglia C. E. (1995). Mechanisms of polycyclic aromatic hydrocarbons degradation .In: L.Y. Yong and C.E. Cerniglia (Ed). Microbial transformation and degradation of toxic chemicals. Wiley-Liss New York. PP.269 -306.

Swannell R. P. J., Croft B. C., Grant A. L. and Lee K. (1995). Evaluation of bioremediation agents in beach microcosms. Spill Sci. Technol. Bull. **2**: 151-159.

Swannell R.P.J., Lee K.and McDonagh M. (1996). Field evaluations of marine oil spill bioremediation, Microbiol. Rev. **60** :342–365.

Tay S. T.-L., Benjamin Y.-P. M., Maszenan A. M. and Tay J.-H. (2005). Comparing activated sludge and aerobic granules as microbial inocula for phenol biodegradation. Appl. Microbiol. Biotechnol. **67**: 708-713.

Ting Y. P., Hu H. L. and Tan H. M. (1999). Bioremediation of petroleum hydrocarbons in soil microsoms. Res. Environ. Biotechnol. **2**:197-218.

US. Environmental Protection Agency (1985). Hand Book of Remedial Action at Waste Disposal Sites (Revised). EPA Report No. EPA/625/6-85/006.

Van der Meer J. R., Devos W. M., Harayama S. and Zehnder A. J. B. (1992). Molecular mechanisms of genetic adaptation to xenobiotic compounds. Microbiol. Rev. **56**: 677-694.

Vandevivere P. and Verstraete W. 2001. Environmental applications**.** In: C. Ratledge and B. Kristiansen (Ed). Basic Biotechnology. Vol.2. Cambridge University. PP. 531-557.

Van Hamme J. D., Singh A. and Owen P. W. (2003). Recent advances in petroleum microbiology. Microbiol. Mol. Biol. Rev. **67**: 503-549.

Vasudevan N. and Rajaram P. (2001). Bioremediation of oil sludge contaminated soil. Environ. Int. **26**: 409-411.

Verstraete W. and Devliegher W. (1996). Formation of non-available organic residues in soil: Perspectives for site remediation. Biodeg. **7**: 471-485.

Vidali M. (2001). Bioremediation. An overview. Pure Appl. Chem.**73**:1163-1172.

Volkering F., Breure A. M., Sterkemburg A. and Van Angel J. G. (1992). Microbial degradation of polycyclic aromatic hydrocarbons: effect of substrate availability on bacterial growth kinetics. Appl. Microbiol. Biotechnol. **36** : 548-552.

Wake H. (2005). Oil refineries: a review of their ecological impacts on the aquatic environment. Estewarine Coastal Shelf Sci. **6**: 131-140.

Walworth J. L. and Reynolds C. M. (1995). Bioremediation of petroleum-contaminated cryic soil: effects of phosphorus, nitrogen and temperature. Soil Contamination. **4**: 1599-1611.

Wang Z., Fingas M., Blenkinsopp S., Sergy G., Landriault M., Sigouin L., Foght J., Semple K. and Westlake D.W.S. (1998). Comparison of oil composition changes due to biodegradation and physical weathering in different oils.J. Chromatog. A. **809**: 89-107.

Westlake D. W. S., Jobsoo A. M. and Cook F. D. (1978). In situ degradation of oil in a soil of the boreal region of the Northwest Territories. Can. J. Microbiol. **24**: 254-260.

Williams C. M., Grimes J. L. and Mikkelsen R. L. (1999). The use of poultry litter as co-substrate and source of inorganic nutrients and microorganisms for the ex-situ biodegradation of petroleum compounds. Poultry Sci. **78**: 956-964.

Willumsen P. A., Karlson U. and Pritchard P.H. (1998). Response of fluoranthene-degrading bacteria to surfactants. Appl. Microbiol. Biotechnol. **50**: 475-483.

Wrabel,M. L. and Peckol, P. (2000). Effects of bioremediation on toxicity and chemical composition of no. 2 fuel oil: growth responses of the brown alga Fucus vesiculosus. Marine Pollut. Bul. **40**, 135–139

Wrenn B. A., Haines J. R., Venosa, A. D., Kadhodayan, M., and Suidan M. T. (1994). Effects of nitrogen source on crude oil biodegradation. J. Indust. Microbiol.. **13**: 279–286.

Wright A. L. Weaver R. W. and Webb J. W. (1997). Oil bioremediation in salt marsh mesocosms as influenced by N and P fertilization, flooding, and season.*Water Ai.r Soil Pollut.* **95**(1–4): 179–191.

Xu R. and Obbard J. P. (2003). Effect of nutrient amendments on indigenous hydrocarbon biodegradation in oil-contaminated beach sediments. J. Environ. Qual. **32**: 1234-1243.

Xu R., Yong L.C., Lim Y.G., Obbard J.P (2005). Use of slow-release fertilizer and biopolymer for stimulating hydrocarbon biodegradation in oil-contaminated beach sediments, Mar. Pollut. Bull. **51**: 1101–1110.

Xu Y., Lu M.(2010). Bioremediation of crude oil contaminated-soil: comparison of different biostimulation and bioaugmentation treatments.Journal of hazardous materials. 183: 395-401.

Yerushalmi L., Rochleau S., Cimpoi R., Sarrazin M., Sunarah G., Peisajovich A., Leclair G. and Guiot S. (2003). Enhanced biodegradation of petroleum hydrocarbons in contaminated soil. Bioremed. J. **7**: 37-51.

Zajic J. E. (1964). Biochemical reactions in hydrocarbon metabolism. Dev. Indust. Microbiol. **6**: 16-27.

Zhang Y. and Miller R.M. (1994). Effect of a Pseudomonas rhamnolipid biosurfactant on cell hydrophobicity and biodegradation of octadecane. Applied and Environmental Microbiol. **60**: 2101–2106.

Zhanovich A. V., Gridin O. M and Gridin A. O. (1995). Hydrophobic organomineral sorbents for elimination of oil spills. In: Proceeding of the All-Russian Conference on Ecological Problems. Moscow. PP.3-6

Zhao X., Wang Y. and Ye Z. (2006). Oil field wastewater treatment in biological aerated filter by immobilized microorganisms. Process in Biochem. **41**: 1475-1483.

Zhou E. and Crawford R. (1995). Effects of oxygen, nitrogen and temperature on gasoline biodegradation in soil. Biodeg. **6:** 127-140.

Zitrides T. (1983). Biodecantation of spill site**.** pollut. Eng. **15:** 25-27.

Zobell C. E. (1946). Action of microorganism on hydrocarbons. Bacteriol. Rev. **10**: 1-49.

Zobell C. E.(1973). Bacterial degradation of mineral oils at low temperature. In: D.G. Ahearn and S.P. Meyers (Ed). The microbial degradation of oil pollutants. Louisiana State University. Baton Rowge. PP. 153-161.