Phytoremediation of oil-polluted desert soil in Kuwait using native plant species

Sarah Salah Al-Ateeqi

This Thesis has been submitted in fulfilment of the requirements for the Degree of Doctor of Philosophy.

School of Life Science
College of Medical, Veterinary and Life Sciences
University of Glasgow

November 2014

Abstract

As a result of damage caused during the First Gulf War in 1990-1991, the Kuwaiti environment suffered from drastic pollution caused by massive petroleum hydrocarbon contamination resulting from the destruction and burning of 700 oil wells across Kuwait. A range of types of polluted soils, including fresh oil lakes, dry oil lakes, and tarcretes, damaged desert wildlife. The idea of phytoremediaton using native plants was introduced and concluded that the native species *Haloxylon salicornicum* (Amaranthaceae) had potential as a phytoremediator.

In the initial phase of this study a follow-up survey of clean and polluted sites in 7 areas in Kuwait was undertaken: Bahra, Sabah Alahmad protected area, Burgan oil field, Um Alaish oil field, Um Alrros Military Base, Sabriya oil field, and Um Ghadaier oil field where 41 plant species were found to be present. TWINSPAN classification of the dataset identified four assemblages of plant species, occurring in four ecologically-distinguishable habitat types (represented by 7 sample-groups produced by the classification procedure); one of them is mostly north of Kuwait where Sabah Alahmad protected area is and Bahra and Um Alaish oil fields and tends to be more in the oil damaged areas and characterized by the presence of the *Haloxylon salicornicum*; the other one is in both the north (Um Alaish oil field) and south of Kuwait (Burgan oil fields) and is characterized by the presence of both *Cyperus conglomeratus* and *Rhanterium epapposum* while the third and fourth assemblages can be mostly considered variant and characterized by the presence of *Pennisitum divisum*. These native species (former 3) were hence selected as the focus for subsequent investigation.

The survival of *Haloxylon salicornicum* plants in weathered oil-polluted soils was experimentally investigated under greenhouse conditions, using a random block design with 4 replicates and 5 treatments: pots containing 100%, 75%, 50%, and 25% polluted soil, mixed with clean soil, and clean soil only as a control. The results indicated that the plants could grow successfully even in 100% oil-polluted soil. The experimental results also provided evidence that water applied to the surface (simulating rainfall) could reach the root system in all of the treatments (even for the 100% oil contamination treatment).

Following on from the greenhouse study, a field trial was undertaken to examine the survival and growth of *Haloxylon* plants introduced into clean and oil-polluted soils (in and adjacent to a weathered dry oil lake) under field conditions. Three replicate two-year old

(nursery-grown) *Haloxylon* plants were planted at each randomly-chosen location in the dry oil lake soil, and the design was repeated at 10 random locations in clean soil close to the lake boundary. The experiment was set up in two different locations (Bahra area in the north; and in Burgan oil field in the south of Kuwait). there was no significant difference in growth rates between plants in clean and polluted soil, in either area. The biomass data showed a significant difference in fresh weight between plants from clean and polluted soils, with those growing in clean soil having higher moisture content (possibly less woody than those from the polluted sites). However there was no significant difference in either fresh or dry biomass between the two experimental areas in the north and south. Data produced by analysis of amount of TPH in the polluted soils in both experimental areas showed some variability, but overall there was no significant difference between the two polluted areas, in terms of their weathered petroleum hydrocarbon content.

Successful phytoremediation usually, if not always, is a function not only of phytoremediator plant physiology, but also the activity of the phytoremediator species associated rhizosphere microflora. In order to gain some insight into the hitherto unknown rhizosphere microflora of *Haloxylon salicornicum* plants, bacterial and fungal isolation procedures were carried out on samples taken from the roots of wild *Haloxylon salicornicum* plants, and from cultured plants growing in oil-contaminated soils in the greenhouse experiment, using media enriched with petroleum hydrocarbons to encourage the culture to survive in such conditions. Bacteria organisms found to be associated with the rhizosphere of wild *Haloxylon* are *Streptomyces* spp. and *Inquilinus* sp., while in 100% oil contaminated soil *Rhodococcus manshanensis, Agrobacterium tumefaciens, Nocardia cyriacigeorgica, Gordonia lacunae / Gordonia terrae* and *Lysobacter* spp., occurred. In the 50% oil contamination treatment soil, around the roots of *Haloxylon* plants contained *Gordonia lacunae / Gordonia terrae* and *Agrobacterium tumefaciens* and finally the clean soil *Sphingopyxis* spp. was present. Fungi found in the *Haloxylon* rhizosphere included organisms known to be associated with petroleum hydrocarbon degradation, including *Penicillium* spp. in wild *Haloxylon* (and also 50%, 100% and clean soil from the greenhouse trial), as well as *Trichoderma asperellum* in the clean soil.

The conclusion is that *Haloxylon salicornicum*, together with its rhizosphere microflora it contain, offers high potential for use in phytoremediation operations designed to assist in the clean-up of oil polluted desert soils in Kuwait.

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Acknowledgments

To my Supervisor Dr. Kevin Murphy for the most amazing years of my life, he gave me the confidence, space, time and most importantly his patience and I really hope that I was as good a student for him as he was a supervisor for me. I wish him the best health and happiness Ensha'allah in his life for everything he's done for me and for others. I couldn't ask for a better supervisor, and I want to thank him from my heart...God bless him.

To my dearest friend and Co-Supervisor Dr. Layla Al-Musawi, I don't think that I could have passed the whole thing that smoothly especially the field work without her help/guidance for the last four years; I will never forget the way she helped me for all of it.

Thank you to Dr. Redha Al-hasan from Kuwait University (KU) for helping me immensely, and his technician Ms. Majda Khanafer. Thanks to my colleagues Mohammad Al-Khrafi (PAAF), Andy Hafferty (PAAC), Memo Porcelli (KISR) and Ali Al-Azmi (PAAF), Meshal Abdullah (PAAC), Shayma Hasan (PAAF), Divya Saju, and Tequila (KU). I would love to thank Dr. Mohamed Al-Ahmad (KEPS) for the great help in the last field work and for the base maps he provided me and to Mr. Ismail (EPA) for being there for me. Thank you to the Al-Shimmery family for their help, as well as Mr. Abdurrahman Al-Farsi for helping me finds an assistant, and his moral support.

I want to thank Public Authority of Agriculture (PAAF) and Offset program and Kuwait Foundation for the Advancement of Sciences (KFAS) for the financial support, thank you to everyone that helped the thesis come alive...God Bless You All.

Finally to my parents and my sisters and brothers, to my beloved grandmothers Mama Fatma Al-Dakhan and Mama Taiba Al-Ateeqi thank you very much for believing in me and for supporting me in every way possible, and for being there for me when I am in need, God bless you, I really hope that they are proud of me as their daughter for everything I've done through the years. To my best friend Nouf Al-Hashash, thank you for your support I couldn't have asked for a better friend, you helped me in this thesis and in my life, this work is dedicated to all of you.

Despite all the kind and generous help I received while doing this work, the fault of any mistake in this thesis would be mine and mine alone.

Author's declaration

I declare that, except where explicit reference is made to the contribution of others, that this dissertation is the result of my own work and has not been submitted for any other degree at the University of Glasgow or any other institution.

Signature _____
Printed name _____

Abbreviations

Code Key	Explanation
01BA0013	Quadrate # in specific area, Bahra Oil Field, total Quadrate # and a Key code for oil damaged soil.
01SA0311	Quadrate # in specific area, Sabah Alahmad Protected area, total Quadrate # and a Key code for oil damaged soil.
01BU0763	Quadrate # in specific area, Burgan oil field, total Quadrate # and a Key code for oil damaged soil.
01UM1063	Quadrate # in specific area, Um Al-Aish oil field, total Quadrate # and a Key code for oil damaged soil.
01UA1261	Quadrate # in specific area, Um Al-Rros Military base, total Quadrate # and a Key code for oil damaged soil.
01SB1363	Quadrate # in specific area, Sabriya oil field, total Quadrate # and a Key code for oil damaged soil.
01UG1563	Quadrate # in specific area, Um-Ghadair oil field, total Quadrate # and a Key code for oil damaged soil.

Abbreviations	Explanation
PAAF	Public Authority for Agriculture and FisheriesAffairs
KISR	Kuwait institute for Scientific reserch
KOC	Kuwait Oil Company
Max GR ftBO	Maximum Growth rate at field trips to Bahra Oil plots
Max GR ftBC	Maximum Growth rate at field trips to Bahra Clean plots
Max GR ftBuO	Maximum Growth rate at field trips to Burgan Oil plots
Max GR ftBuC	Maximum Growth rate at field trips to Burgan Clean plots
TPH	Total Petroleum Hydrocarbon

1 Chemicals	2 Abbreviations
3 Polycyclic Aromatic Hydrocarbon	4 PAH
5 Acenaphthylene	6 (ANAY)
7 Anthracene	8 (ANTH)
9 Benzo(a)anthracene	10 (B[a]ANTH)
11 Benzo(a)pyrene	12 (B[a]P)
13 Benzo(b)fluoranthene	14 (B[b]FLAN)
15 Benzo(g,h,i)perylene	16 (B[ghi]PERY)
17 Benzo(k)fluoranthene	18 (B[k]FLAN)
19 Chrysene	20 (CH)
21 Dibenz(a,h)anthracene	22 (D[a, h]AN)
23 Fluoranthene	24 (FLAN)
25 Indeno(1,2,3-cd) pyrene	26 (I[123-cd]PY)
27 Naphthalene	28 (NA)
29 Phenanthrene	30 (PH)
31 Pyrene	32 (PY)
33 Acenaphthene	34 (ANA)

Species Names	Abbreviations
Cyperus conglomeratus	Cyco
Rhanterium epapposum	Rhep
Calligonum polygonoides	Capo
Arnebia decumbens	Arde
Stipa capensis	Stca
Senecio glaucus	Segl
Salsola imprecate	Saim
Zygophyllum qatarense	Zyga
Tamarix aucheriana	Taau
Haplophyllum tuberculatum	Hatu
Pulicaria undulata	Puun
Haloxylon salicornicum	Hasa
Cistanche tubulosa	Citu
Ifloga spicata	Ifsp
Filago pyramidata	Fipy
Hordeum marinum	Homa
Plantago boissieri	Plbo
Sonchus oleraceus	Sool
Medicago laciniata	Mela
Gypsophila capillaris	Gyca
Emex spinosa	Emsp
Convolvulus cephalopod	Coce
Gynandriris sisyrinchium	Gysi
Trigonella stellata	Trst
Stipagrostis plumose	Stpl
Polycarpaea repens	Pore
Moltkiopsis ciliata	Moci
Pennisetum divisum	Pedi
Lycium shawii	Lysh
Cistanche colocynthis	Cico
Heliotropium bacciferum	Hebe
Fagonia glutinosa	Fagl
Helianthemum lippii	Heli
Astragalus spinosus	Assp
Fagonia bruguieri	Fabr
Convolvulus pilosellifolius	Copi
Diplotaxis harra	Diha
Scrophularia deserti	Scde
Reseda arabica	Rear

1 INTRODUCTION & LITERATURE REVIEW

1.1 Geomorphology and Environmental Background of Study Areas

1.1.1 Kuwait: study area location and the Iraqi invasion in 1990's

The state of Kuwait covers 17,850 km^2 of the north eastern part of the Arabian Peninsula, between latitudes 28˚30′ and 30˚ 05′ N and longitudes 46˚ 33′ and 48˚ 30′ E, sharing terrestrial borders with the Republic of Iraq to the northwest and Kingdom of Saudi Arabia to the south. The highest point of Kuwait's topography is located in the northwest of Kuwait in Jal'Azor area, which reaches 300 m above sea level, whilst the lowest point is at sea level in the shoreline habitats of the Arabian Gulf (Al-Ateeqi 2006). Kuwait is a major oil producer with large oilfields located in both the southern and northern areas of the country (Fig. 1-1).

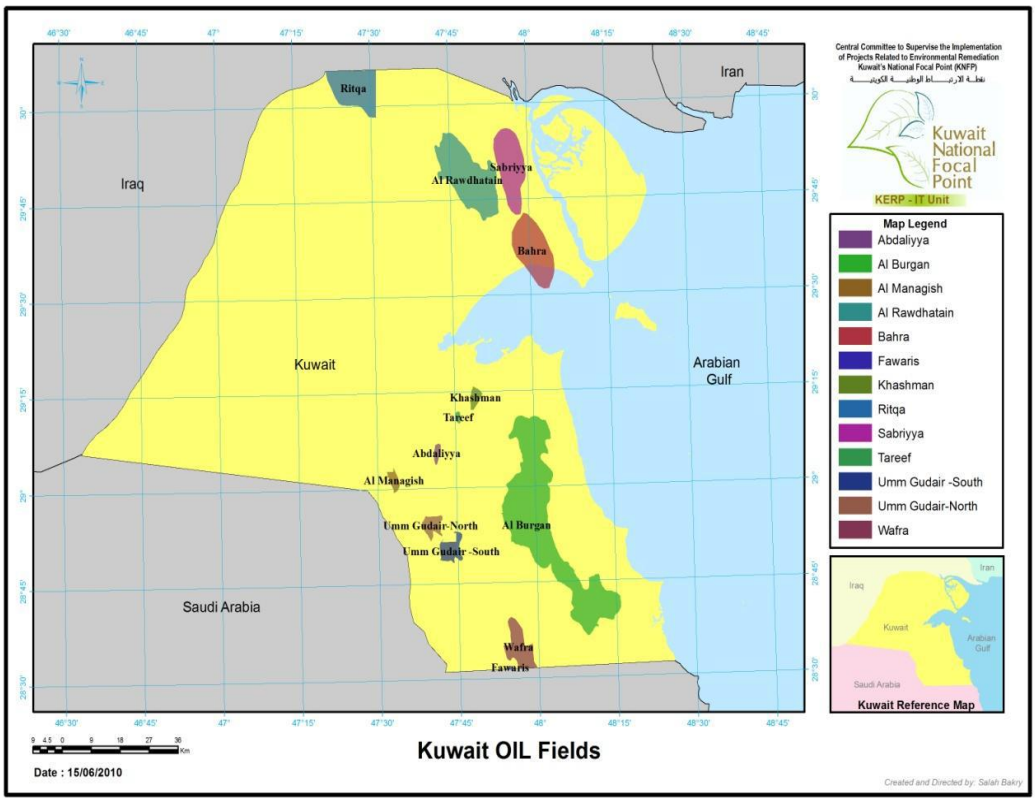


Figure 1-1: Map of Kuwait depicting oil field locations (KNFP, 2010).

Major environmental damage came about as a result of extensive hydrocarbon pollution, caused by damage to oilfield installations during the First Gulf War in 1990-1991:

> "Like in all armed conflicts, the desert and coastal environments of Kuwait were the main victims of this aggression" (Omar 2009).

Desert soils were contaminated as a result of deliberate oil spillage, and gushing from the burning of some 700 oil-wells sabotaged by Iraqi forces. A massive amount of by-products polluted the environment including sulphur oxide, hydrocarbons, and carbon monoxide, together with a lot of unknown particles and metabolites (Dewitt 1991). The United Nations Compensation Commission (UNCC) granted Kuwait and other Claimant Countries environmental remediation awards to undertake environmental cleanup and restoration projects.

Impacts of such contamination on the desert ecosystems of Kuwait continue to this day. The use of suitable phytoremediation management procedures could provide a useful low-cost and ecologically-friendly option to assist with some of the clean-up operation (Ramos 2009), particularly if suitable native plant species could be identified and utilized for this purpose. For example, a study by Sinha (2007) showed that Bermuda grass (*Cynodon dactylon*) and vetiver grass (*Vetiveria zizanioides*) are excellent plant phytoremediators. As for Kuwait, an initial study (Al-Ateeqi 2010) identified potential native species growing in oil-polluted soils: *Haloxylon salicornicum*, in that study then in further studies *Cyperus conglomeratus* and Rhanterium epapposum was identifed, are all widespread in Kuwait, and appear to be able to survive in highly hydrocarbon-polluted soils. The possibility of using such indigenous plant species to help remediate the damage inflicted upon the ecosystem by human actions has great potential, and one which could materially aid the clean-up programme.

1.1.2 Climate:

Kuwait is an arid country (Fig. 1-2) with low humidity and precipitation. The prevailing wind (Fig. 1-4) directions are either from the southeast or the northwest (Khalaf 1989). According to the Meteorological Department (2010) the temperature reaches up to 50˚C in July, (summer) in Kuwait, and could drop to slightly below 0 C˚ in January (Fig. 1-3) during the winter season (2 to 3 months).

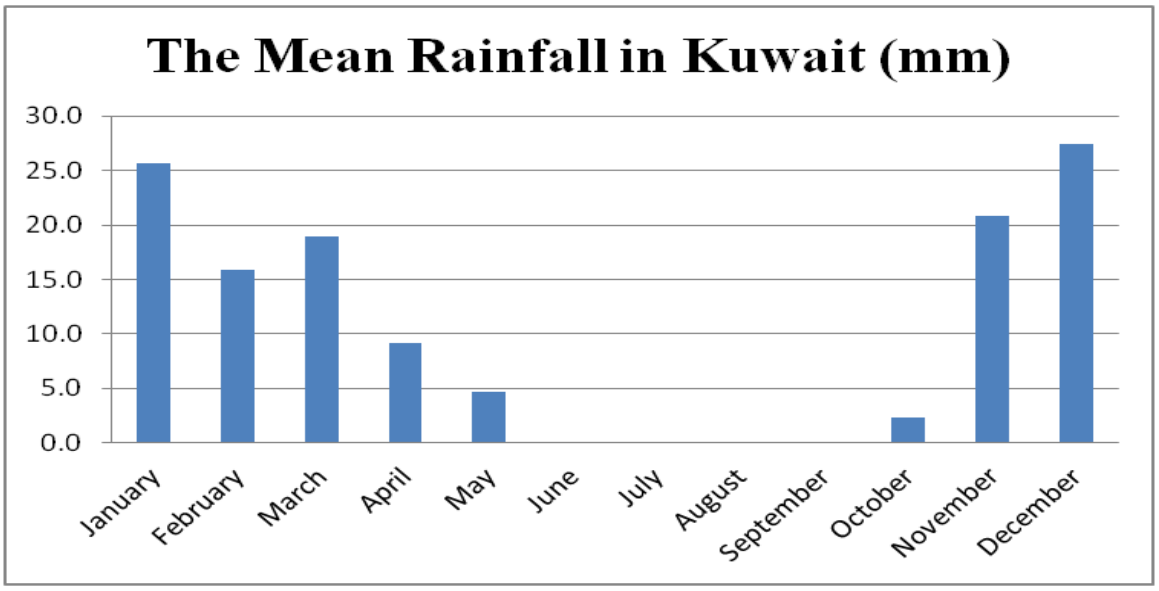


Figure 1-2 Mean rainfall (mm) in Kuwait from 1980-2013 (Meteorological Department 2010).

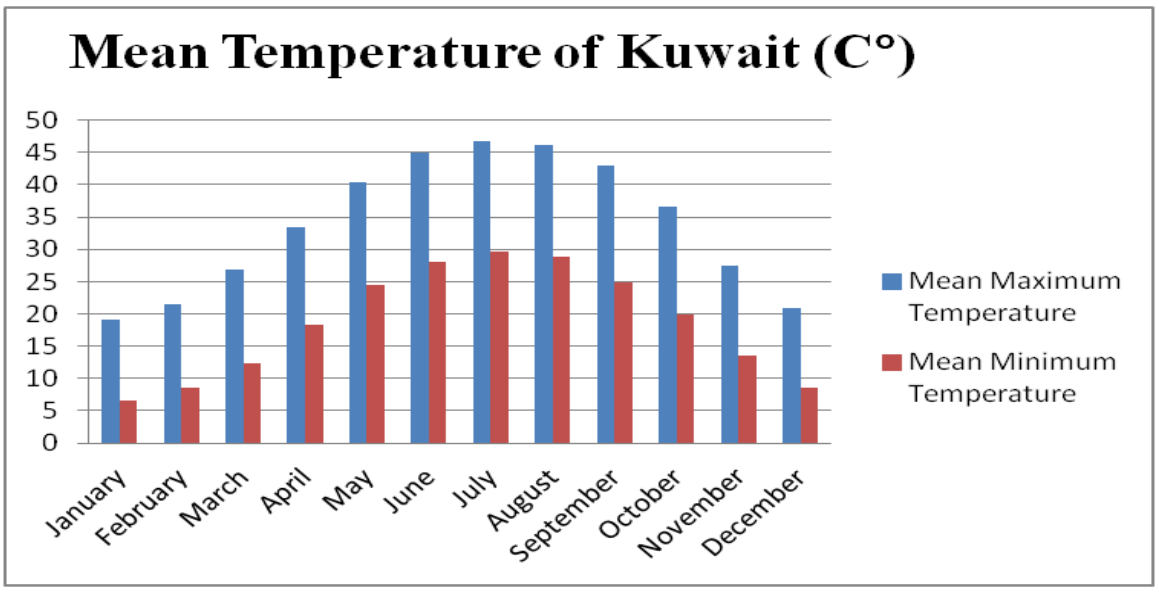


Figure 1-3 Kuwait mean temperature (°C) during the years 1980-2013 (Meteorological Department 2010).

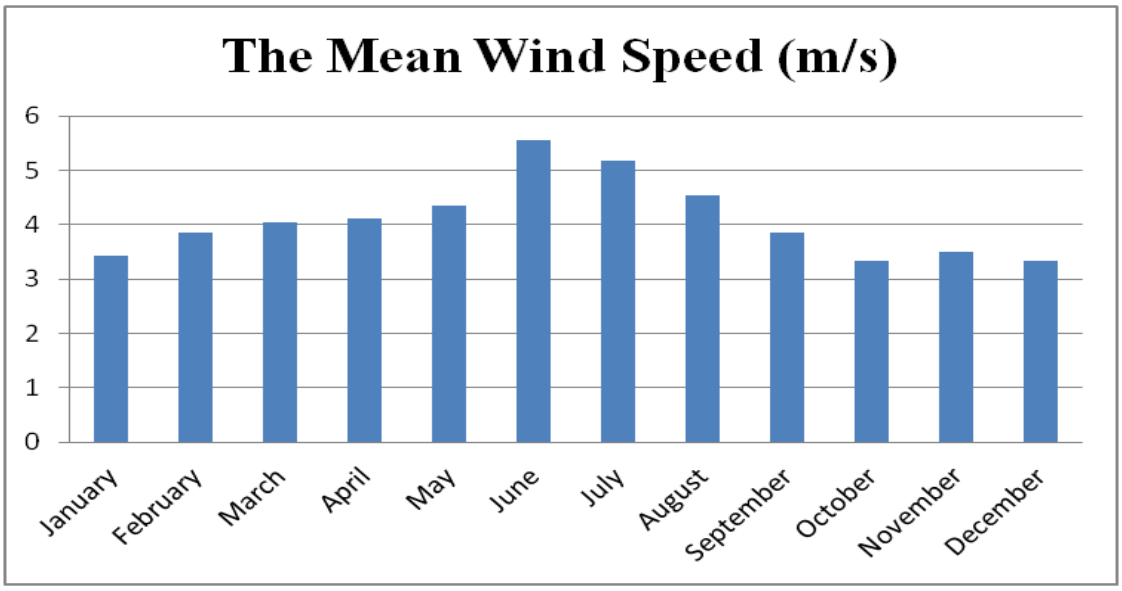


Figure 1-4: Mean wind speed (m/s) in Kuwait from 1980-2013 (Meteorological Department 2010).

1.1.3 Soils

The soil types of the selected study locations have already been characterised. In 1999 a detailed map of soil types in Kuwait was prepared (KISR 1999)see Chapter 2). The survey found that the Sabriya and Alrawdatain area (north of Kuwait) has Typic Haplocalcids that occur in drainage depressions as well as lower flats; Typic Calcigypsids gypsic soils on the crests and slopes, Leptic Haplogypsids and Typic Haplogypsids in drainage depressions (Typic Haplocalcids) that consist of deep sandy calcic soils as a soil continuation of the landscape; a mix of calcic and gypsic soil is usually found further up slope (Typic Calcigypsids) while at the top gypsic (Typic Haplogypsids) can be found, where gypsum frequently occurs close to the surface (Leptic Haplogypsids). Large amounts of gravel (15 to 35%) are associated with gypsum horizons. A hardpan that consists of gypsum or gravels can commonly be found at around 1 m depth, or sometimes at shallower depths (KISR 1999)see chapter 2).

In the south of Kuwait, where the Burqan oil field is located (covering a huge part of southern Kuwait, being the largest oil field in Kuwait), the landscape is flat or gently undulating, and the substrata consists mostly of sandy soils with areas of deeper sand sheets. All of the oil fields in Kuwait are fenced and restricted to the public and protected from grazing (KNFP 2010). Roughly 65% of the area in south of Kuwait and the Burgan oil field is covered with *Cyperus conglomeratus*, *Stipagrostis plumosa*, and *Rhanterium epapposum* which used to dominate the whole landscape (Brown 2003).

1.2 Oil pollution resulting from deliberate burning and spillage of oil from oil wells during the First Gulf War

Kuwait has 15 oil fields (Fig. 1-1), divided into southern and northern sectors (Oil 1991) with 914 operational oil wells prior to the onset of the Iraqi invasion (PAAC 1999). The northern sector includes four fields: Bahra, Sabriya, Alrawdatain, and Alratqa (Oil 1991). The southern sector is divided into 11 fields (encompassing 81.6% of the total Kuwaiti oil wells) south of Alfawars: Alwafra, southern Um-Ghadair, Khashman, Alabdylia, Altarif, Almnakesh, Alahmadi, Almukawa'a, and Burgan, which is one of the biggest oil fields in the world (Oil 1991).

The destruction and the sabotage started when the Iraqi military dumped a huge amount of oil into the Arabian Gulf, much of which reached the shores of Saudi Arabia and other Gulf Corporation Countries (GCC). The dumped oil polluted the seawater in the vicinity of desalination/power plants producing drinking water. Damage to coastal and marine biodiversity was a further impact of such pollution (Dashti 1993), ultimately affecting a major source of food from fisheries.

On land, millions of tons of crude oil were dumped into the desert, even before the UN Desert Storm counter-offensive started, which produced massive oil "lakes". Most of the previously mentioned oil wells were damaged and deliberately set on fire by the retreating Iraqi troops (604 oil wells in total). The Iraqi military used explosives to destroy wells: 149 of them were destroyed completely, and terminally, while 45 of them exploded without catching fire, which meant that oil kept gushing from them for a long time afterwards (PAAC 1999).

Wells set on fire continued burning for up to nine months after the liberation of Kuwait. The fires were very hot as crude oil is highly volatile and extremely flammable and is lighter than water. Fire fighters were put at risk not just from the burning oil itself, but also from airborne oil vapour which formed in huge clouds in the vicinity of damaged wells, posing a constant danger of ignition and explosion (Hirschmann 2005). In addition, un-burnt oil lakes also gave off large amounts of oil vapour (Hirschmann 2005). In order to extinguish the fires a million tons of seawater was used (Oil 1991).

The oil spilt from the damaged wells formed a large number of oil lakes, estimated at 71 oil lakes in the Sabriya Field, 83 in the Alrawdatain Field, 114 in Alratqa Field, and 18 in the Bahra Field, in the northern sector (1991). In the southern sector, Burgan field contains

35 oil lakes, Alahmadi and Almoqawa another 15, Um Ghadair 3 oil lakes, and Alwafra 33 oil lakes: in total 86 oil lakes (Oil 1991). PAAC (1999) estimated the amount of oil spilled from the wells at, on average, two million barrels per day: the total amount spilled was estimated at 1.0 to 1.5 billion barrels. When the fires were at their peak, it was estimated that spillage reached 6 million barrels a day, which was 10-15% of world daily oil production. At the oil price at that time, Kuwait was losing US$1,300 per second (at US$19 per barrel): i.e. US$115 million per day in lost oil revenue (Hirschmann 2005).

According to UNEP (28030 November 1991), the burned oil smoke emissions contain 1% carbon monoxide, 95% carbon dioxide, 4.2% organic non-methane fumigants, 0.35% methane, 0.45% soot, and 0.65% organic particles. The smoke changed the ambient temperature of some parts of the Arabian Gulf as it blocked the sun's rays from reaching the ground, with a temperature drop ranging between 5 and 10˚C (Dashti 1993).

1.3 Initial countermeasures: fire-fighting, saltwater lakes in the desert and oil cleanup operations

With freshwater supplies in Kuwait being extremely limited, the only option for extinguishing the oil well fires was to use seawater. More than 300 large artificial lagoons were constructed in the desert to hold the required amount of seawater, each containing between 500,000 - 1 million gallons of sea water. To bring this amount of water to those areas, 400 km of pipeline was built to suck 25 million gallons per day from the sea (Hirschmann 2005).

To extinguish the fires, the fire fighters used a combination of different techniques. High-powered pumping of seawater (some of these pumps could pour 4,000 gallons of water per minute onto a fire) helped the temperature to drop to less than the ignition point, and pushed fuel away from the seat of the fire. In addition, sometimes chemical suppressants (dry chemical mixtures and liquid nitrogen) were used mixed with the water to extinguish the fire (Hirschmann 2005). Spraying water on the oil well even after extinguishing the fire was usually required to prevent re-ignition. Sometimes explosives were used to push away the flames and the vapours from the wells. Also, the explosion will consume all the oxygen around the well, giving a chance to cap the well and put out the flames. Furthermore, a process called "stinging in" was used: pumping mud into a gushing well thus blocking the oil from gushing/ igniting again (Hirschmann 2005).

Specially equipped vacuum trucks were used to suck up the oil pools and slicks along the sea shoreline, and transfer it to large holding tanks. In total, nearly 1.5 million barrels of pure oil were skimmed from the Arabian Gulf (nearly 640 km was covered with oil slicks on the Kuwaiti shores). On land, vacuuming operations succeeded in removing some 20 million barrels of crude oil from the soil, with much of the remaining liquid oil evaporating by 1993 (Hirschmann 2005). Subsequent refinery operations with this oil succeeded in extracting 18-24% of the oil removed from the shoreline zone in this way. Initial use of chemical dispersants on slicks proved controversial due to damage to marine ecosystems, which can be caused by the dispersants themselves (Hirschmann 2005). Another controversial method which was used to clean up shoreline habitats was blasting hot water onto oil-covered rocks and sediment to loosen the oil, but with a high risk of damage to fragile marine life.

Once the fires were extinguished, the environmental damage to wildlife was revealed in the form of oiled birds and other wildlife, and deaths due to consumption of oil-polluted plants (Hirschmann 2005). Many environmentalists and organizations from all over the world tried to help in dealing with this crisis but only 1,000 birds were saved from an estimated mortality of around 40,000 birds killed by the oil pollution (Hirschmann 2005). In the *Exxon Valdez* oil spill rescue and clean-up of otters, the estimated bill was $80,000 for each animal saved (Hirschmann 2005). In Kuwait, due to lack of available finance in the aftermath of the invasion and war, the initial environmental cleaning process was halted nearly immediately after extinguishing the last oil well fire in November 1991, and the available governmental contribution towards this crisis was only $150 million (Hirschmann 2005).

1.4 Oil pollution of soils

Satellite images taken in early 1992 showed that around 10% of the land area of Kuwait (1,810 km^2) was covered with soot, tar and oil lakes. Three years later the satellite images showed an apparent reduction of this contaminated area, to only about 390 km^2. However, the visual imagery needs further thermal analysis, as blown sand rapidly covered oil-polluted soils and made the pollution invisible to visual channels of remote sensing. In the worst affected areas, it was thought that the pollution will likely persist for decades (Hirschmann 2005).

Researchers found blooming wild flowers in the spring of 1995 growing in soils lying on top of badly oil-contaminated areas (Hirschmann 2005). In addition, the presence of “oil-

eating bacteria" growing associated with the root systems of some of these plants was detected, leading one Kuwaiti scientist (Hirschmann 2005), who marvelled at this fact, to say:

> "We think that God has laid in Nature a huge amount of resilience that you cannot destroy easily" (Hirschmann 2005).

Because the oil was fresh at that time, the government was incentivized to remove it from the environment so it could be refined and used. With the passage of time this is no longer possible, as the remaining unusable oil has weathered out and is partially degraded: hence the economic incentive to remove it has gradually declined over time. Pollution impact studies, and pollution cleanup research and operations were generally deemed too expensive, and relatively little was done on a large scale even though $108 million had been awarded to Kuwait from UNCC for environmental studies extending for up to 5 years (Hirschmann 2005). The compensation funds were regulated with the aim of producing causal evidence of war-related destruction, and did not allow for much needed scientific research. However, some limited international funding was available. One of these internationally funded projects was to showcase bioremediation methods, and was set up in the Al-Ahmadi area. Completed in 2000, this study used bottlebrush trees, bougainvillea, saltbushes, and desert grass planted on top of oil-blackened soil (Hirschmann 2005). Accoding to a personal communication I had with some experts they said it has some success but but not suitable for large-scale implementation to clean up the desert.

1.5 Effects of petroleum hydrocarbons on the environment and applicability of phytoremediation techniques

Given that the amount of environmental destruction in Kuwait was staggering, and there were different types and levels of oil contamination, the idea of using native flora as phytoremediators for the environment was thought promising (see Chapter 3 for more on phytoremediation).

In order to design proper clean-up and restoration, it was important to classify the existing types of oil contamination. "Wet oil lakes" were defined as lakes made up of a mixture of liquid oil contamination and water (over 10% salt): over 6 billion gallons of seawater (4% salt content) were used to extinguish the fires. At the start of this study, the lakes had been weathering for the last 23 years. Most lakes have been removed, however still a few remain inside the oil fields, along natural depressions (Web site #1 2013). Al-Daher (1998) reported that the crude oil was mostly pumped out, but most of the soil down to 30 cm or

more adsorbed the petroleum (Web site #1 2013). The Kuwait Environmental Public Authority (KEPA) reported that oil lakes changed the soil texture, killed the wildlife, and some of it seeped down to the fresh water aquifers. Nowadays, those lakes are merely weathered oil with a semi-solid sludge layer at the top that can handle the weight of a person (Web site #1 2013). A total of 19% petroleum hydrocarbon (TPH) was in this layer while the soil contamination underneath it contains a mean of 3.4% (Web site #1 2013).

Dry oil lakes consist of a semi-solid layer of oil contamination with a dark brown soil underneath it. The tar-like surface contains nearly as much as 7.2% of the oil contamination, whereas the contamination underneath contains around 2.5% TPH (Web site #1 2013).

Oil contaminated piles were created during the Kuwait Oil Company's (KOC) efforts to restore field operations, resulting in pushing the contamination aside in an effort to minimize the size of the contamination. These acts accumulated the wet and the dry petroleum-contaminated soils into mounds, (Web site #1 2013) and those piles, with time, exhibited some re-colonization and growth of native plants on top of them, especially *Cyperus conglomeratu*s.

There are also the areas covered by burnt tar, termed "tarcretes", which consist of a thin hard layer (1-2 cm) of partially burned petroleum with no contamination underneath. The tarecrete is a hard layer which can easily be broken up with hand tools and tilling machines to allow re-colonization by the native vegetation. Such broken-up tarcretes also improve the microclimate and the soil humidity for plant growth (Brown 2001; KNFP 2010).

1.6 Aims of the study

The most important aim was to find potential phytoremediator species. (*Haloxylon salicornicum* had previously been identified as one (Alateeqi 2010)) and in order to do that it was s important to establish the approach and methodology for quadrat-based sampling of vegetation recovery from sudden-impact disturbance events (deliberate oil release in this case). Hence there was a need to collect and analyze data about Kuwait native plant communities growing in oil-polluted soil, and to establish the habitat tolerance of *Haloxylon salicornicum* and other potential phytoremediator species in oil-impacted soil conditions. The results showed that *Haloxylon* had the high potential to survive in oil-polluted areas, together with *Cyperus conglomeratus*, and *Rhanterium epapposum*.

Having confirmed the potential of *Haloxylon* as strong candiadte species for phytoremediation purposes, it was then important to establish experimentally the tolerance of this species for growth in desert soil contaminated with petroleum hydrocarbons. A random block experiment was carried out with different oil contamination levels in each pot (100%, 75%, 50%, 25%, and clean) in a green house, which proved that *Haloxylon* plants can survive under 100% oil contamination. A second experiment was undertaken but in the field, in oil-polluted and nearby clean soils, to assess factors such as ease of handling for operational purposes, and requirements for any environmental amendments(s) to assist plant growth, which showed that it was easy to handle the plants under field conditions, and that transplanted *Haloxylon* plants can easily grow in weathered oil-polluted soils, in two locations, in the north and the south of the country.

And finally it was also important to collect and identify types of bacteria and fungi from the rhizosphere of wild and cultivated *Haloxylon* plants challenged by oil-polluted soil conditions, in Kuwait. And the results showed the presence of a number of bacteria and fungi, isolated from rhizosphere soils, which are known to be associated with oil degradation and oil bioremediation.

2 VEGETATION AND ENVIRONMENTAL CHARACTERISTICS OF OIL-POLLUTED AREAS

2.1 Introduction to vegetation analysis methodology in dry land ecosystems

Quantitative field analysis techniques for the study of vegetation were pioneered by Alexander von Humboldt, in plant geography studies conducted in the early 19th Century (Randall (1978). Since then, the techniques have become standardized, with measures of frequency, density and cover being the most commonly used non-destructive methods of sampling to assess spatial and temporal variation in vegetation (Bainbridge 2007).

Dry land plant communities pose their own problems for sampling purposes. These plants are strongly linked to the variability of seasonal (or year-on-year) rainfall, which can produce huge and very rapid changes in ephemeral vegetation when the soil seedbank is stimulated to germinate after rain events. In addition, and again because of water limitation, desert vegetation tends to be sparse with individual plants widely spaced to minimize competition for available soil water. Bainbridge (2007) is one of many authors (e.g. (Dickinson 1994; Springuel 1997; Springuel 1996) who emphasize that the optimal sampling time for desert plant communities is at the end of the rainy season (if there is one), or alternatively some 3 – 4 weeks after an individual rain event which has deposited 1 – 5 cm of water at the sampling location. This sampling strategy optimizes the chance of finding species which would otherwise only be present in the seedbank. In addition, because they are so strongly adapted for stress-tolerance (*sensu* Grime 1979), desert plant populations have little or no genetic ability to combat intense disturbance, for example due to over-grazing (e.g. (Springuel 1996), so sampling regimes must take account of such pressures, ideally by assessing disturbance intensity, and/or finding undisturbed control areas for comparison (e.g. (Ali 2000).

Given these issues, Bainbridge (2007) suggested that to provide an accurate estimation of desert vegetation abundance, three approaches can be used. These are: pilotless methods, distance (transect) methods, and quadrat methods, which can all be used to provide an appropriate sampling framework for estimation (either randomly or systematically) of frequency, cover and/or density of the plant populations present in a target area. All these approaches yield data which are valid for statistical analysis, so long as appropriate replication of sampling units is utilized.

The use of quadrats as a standard sampling unit to estimate plant cover of individual species (regularly, randomly or subjectively) is a very common procedure in vegetation assessment (Bainbridge 2007), but both density and frequency measurements are also commonly used in dry land ecosystem analyses of vegetation; whilst remote sensing (e.g. aerial photography) can also be useful in such ecosystems, particularly given that plants tend to be widely spaced, with little or no canopy overlap (Bainbridge 2007).

Frequency is a measure of the probability of finding individual species within the sampling unit, and is measured as the:

> "Percentage of quadrats containing at least one rooted individual of a given species" (Bainbridge 2007; Web site #3 2013).

A major issue with the use of frequency assessment is the quadrat size. Obviously with a bigger sampling unit the chance of finding a given species increases. Hence, an optimal quadrat size for the particular vegetation type must first be assessed prior to commencement of sampling, though normally this will already be known from the literature (Bainbridge 2007).

> Quadrat size should be "large enough to include significant number of individuals but small enough so that plants can be separated, counted, and measured without duplication or omission of individuals and at modest cost" (Barbour 1987; Cox 1990).

To determine the size of quadrats needed in a study, the curve of total cumulative species count versus increasing sample unit size is usually plotted, with the optimal quadrat area being the point on the graph where the curve begins to show an asymptote (Bainbridge 2007).

The examination of numerous variables simultaneously is very important in vegetation ecology studies, hence the popularity of multivariate analysis approaches (Gauch 1982). This approach permits examination of plant and environmental variables together in order to detect relationships between them (Krzanowski 1972).

Many authors have emphasized the importance of ordination because it can efficiently represent the true relationships between species and samples, in relation to environmental factors, in a low-dimensional space (Anderson 1971; Everitt 1978; Noy-Meir 1971; Orloci 1974; Orloci 1978). Species and/or samples, which are located close to each other on a

typical ordination plot (in 2 dimensions), are likely to be closely similar also in the multi-dimensional plot which represents their true environmental similarity (Gauch 1982).

According to Gauch (1982), Detrended Correspondence Analysis (DCA) is an ordination method which arranges the species (or samples) in a single sequence across the plot, and is appropriate for use with desert vegetation community analysis where there is likely to be one or more substantial environmental gradients (in my case due to the varying presence of soil pollutants), leading to substantial spatial changes in plant community composition. In a DCA analysis, a full change in plant assemblage membership is indicated by a distance of approximately 2 standard deviations of species turnover along the ordination major axes, and it was considered that DCA would likely be an appropriate method for detecting such variation in Kuwaiti desert plant communities affected by various types of petroleum hydrocarbon pollution. In my study, I also used an allied hierarchical divisive classification procedure (Two-Way Indicator Species Analysis TWINSPAN: (Hill 1979; Hill 1975), which utilizes the same underlying algorithm as DCA, but groups samples (and species) to bring together samples with similar vegetation, and species that tend to occur in the same samples, to produce an ordered samples x species table of the data. The use of classification and ordination approaches together is very common in the plant community ecology literature, across a very broad spectrum of ecosystem types (contrasting examples are Ali (2000) on desert plant communities; (Lang 2012) on upland stream plant communities) and can usually provide useful insight into the structure of the dataset in relation to environmental factors.

2.1.1 Introduction to PAH pollutants

Polycyclic Aromatic Hydrocarbons (PAHs) are a group of organic compounds made up of three or more fused aromatic rings in a range of structural configurations. Generally speaking there is a wealth of PAHs in the environment, usually derived from emissions, tar oil, coal gasification, combustion processes, industrial processes, vehicle emissions, open burning and heating and power-plants. PAHs can be found in the soil or air, sediments, ground water, and road runoff. The amount of PAH in cities is 10 times more than that found in rural areas (Wagrowski 1997), and the expectation is that more will also occur in oil-producing areas such as oil fields.

Because PAH molecules are hydrophobic and have high molecular stability they can persist for a long time in the environment, with a high possibility for biomagnification due

to their lipophilic nature, which increases the chance of transfer and persistence in living organisms through trophic levels (Desai 2006). Juhasz (2000) reported that the amount of PAHs in a petrochemical area in general can reach 821 μg/ kg, and 451 μg/ kg at a gas manufacturing plant area. PAHs released into the environment could be removed through many processes, including volatilization, photo-oxidation, chemical oxidation, bioaccumulation, biodegradation and adsorption (Balachandran 2012).

The PAHs are classified into two groups based on ring structure (Alternate and a Non-alternate): the former includes Chrysene and Phenanthrene which are derived from benzene and they have less than eight benzoid rings (Harvey 1998). The latter, such as Fluoranthene come with six membered rings (Harvey 1998). There is evidence that plants can generally take up members of the former group better than the latter. Based on the toxicology profile from the Agency for Toxic Substances and Disease Registry (ATSDR) (1995) Chrysene (CH) is probably carcinogenic. Chrysene is a chemical that can be released from incomplete combustion, and it is usually associated with the soil and sediment in the environment. It tends to adsorb onto soil particles, and is poorly leached into ground water; nor does it evaporate or hydrolyse readily, though it may be subjected to biodegradation. In general, exposure to sunlight can produce a photolysis effect on it with a half life of 4.4 hr at latitude 40˚N in midsummer's day conditions near the surface of water; while it is been estimated to have 1.25 hr as a half life as a gas phase in the atmosphere, there is little in the literature concerning its rate of degradation in the soil (Web site #2 2013). Organisms lacking microsomal oxidase will suffer from bioconcentration of it in their system (Web site #2 2013).

Phenanthrene is a colourless, crystal-like solid PAH under room conditions, produced (like CH) from incomplete combustion of oil, and also a known carcinogen (Web site #3 2013). It can be found in coal tar pitch, diesel fuel exhaust, or in a natural gas particle emission (Fang 2006; Registry 1995; Rehwagen 2005). It can volatilize (like other PAHs with low molecular weight) from the soil quite readily (Coover 1987; Southworth 1979; Wild 1993).

In their study on the high amount of phenanthrene and pyrene in weathered contaminated soil using bioremediation, Uyttebroek et al. (2007) found that PAH volatilization and degradation was high within the first 30 days then slower afterwards. As a matter of fact, they used coal tar from an old manufactured gas plant for the purposes of their study, which lead them to conclude that in order for high biodegradation to occur, those organisms need to be in an aqueous phase to gain access to PAHs as a substrate. The

limitations of this in desert soil are obvious, as water availability is likely to be a limiting factor for the potential usage of similar microorganisms to biodegrade PAHs in such soils (Bosma 1997; Ogram 1985; Rijnaarts 1990; Volkering 1992).

Fluoranthene can also be released by anthropogenic effect, as a breakdown product of petroleum hydrocarbon. It is very stable in the soil as it is strongly adsorbed by soil, and can persist for many years, especially in the top few centimetres; though it can be biodegraded by microorganisms (Web site #3 2013).

According to Wick (2011) the presence of PAHs in plants is due to accumulation from the atmosphere by above-ground structures of the plants as well as by uptake from the soil via roots. Edward (1986) suggests that plant tissues could contain 50-80 μg/ kg of PAHs, though Salanitro (1997) emphasizes that the amount of PAH in plants depends on species, environmental conditions and the PAH types to which they are exposed. Both Edwards (1986) and Sims (1983) agreed that plants can absorb PAHs through the roots, but perhaps cannot translocate them very well to the upper structures via the xylem, because of the PAHs hydrophobic nature. In this context it should be noted that Yu (2006) found no sign of plant stress impacting on white clover (*Trifolium repens*), corn (*Zea mays*.) or perennial ryegrass (*Lolium perenne*) when they were grown in pots spiked with phenanthrene and pyrene, at rates up to 375 μg/ kg of soil.

2.2 Aims

(I) To establish the approach and methodology for quadrat-based sampling of vegetation recovery from sudden-impact disturbance events: in this case deliberate oil release.

(II) To collect and analyse data about Kuwait native plant communities growing in oil-polluted soil, and to establish the habitat tolerance of *Haloxylon salicornicum* and other potential phytoremediator species in oil-impacted soil conditions.

2.3 Methodology

2.3.1 Selection of Sites

Field survey was undertaken during January – March 2011 to establish the range and abundance of *Haloxylon salicornicum* and alternative potential phytoremediator species in selected areas of Kuwait, across gradients of oil pollution stress, and anthropogenic disturbances. This survey also sought to identify any other plant species/ assemblages

which appear to have the capacity to survive severe hydrocarbon pollution of desert soils, possibly as associates of *Haloxylon salicornicum*, or as separate communities. Plant leaves and soil samples were taken for subsequent laboratory analysis to assess the degree of soil pollution, and take-up of petroleum hydrocarbons by populations of the target plant species under varying conditions.

From my previous study (undertaken in the Sabriya oil-spill area: (Al-Ateeqi 2010) an optimal sampling unit-area (quadrat) size for frequency-sampling in this open herbaceous desert vegetation type was established at 4 m^2 and this was utilized in the fieldwork (the point at which a plot of quadrat area against cumulative number of species recorded begins to asymptote is the optimal quadrat-size for the vegetation: start with an area of 0.125 x 0.125 m^2, doubling to 0.125 x 0.25 m^2; doubling again to 0.25 x 0.25 m^2 .

In that study a stratified quadrate was used from the middle of the weathered dry oil lake to the outside of it to see what type of perennial plants are actually there, and the results came out that the *Haloxylon salicornicum* is the only species who can live at the top of the oil contamination at that place, which made it the strongest potential phytoremediator species that can be found there.

Sampling sites covered a wide range of geographical locations and intensity of soil hydrocarbon pollution throughout Kuwait. In total, 200 quadrat samples were collected from sampling sites of oil field areas such as Burgan, Um Al-Aish, Um-Ghadair, and Sabriya, as well as other less impacted areas with relatively high vegetation cover such as Sabah Alahmad Protected Area, and in clean areas inside the oil field perimeters as control. Seven areas were sampled (Bahra, Sabah Alahmad Protected Area, Burgan oil field, Um Al-Aish oil field, Sabriya oil field, Um-Ghadair, Um Al-Rros Military Base: Figs. 2-1, 2-2, 2-3, 2-4, 2-5, 2-6).

The frequency was measured by Species abundance was assessed using frequency methods at two scales (i) area scale (% frequency measured as abundance of each plant species, present within sample areas (%F=100 x number of quadrats with species/ total number of quadrats sampled per area). (ii) quadrat scale, for every species present (within a 4 m2 quadrat sample unit, subdivided to give 400 sub-units each 10 x 10 cm in area, with % frequency measured as number of hits within 400 sub-units/ 4), for all species present in the sampled quadrat.

Fieldwork had to be undertaken as early as possible due to the seasonal factor: the vegetation dries out by the end of May. Accordingly, the field survey took place from January 2011 to the end of March 2011. In total, 9 field trips were needed to complete the survey phase in 2011. The number of samples varied between each area but was never less than 5 quadrats per area (Bahra had 30 samples, Protected area 45 samples, Burgan 30 samples, Um-Al-Aish 35 samples, Um-Al-Ross 10 samples, Sabriya 30 samples, and Um-Ghadair 20 samples). Contamination conditions in the sample areas varied between dry oil lake, fresh oil lake, tarcretes (burned oil, forming a hard shallow layer just below or at the soil surface), oil pile/pit, and uncontaminated clean soil.

The primary purpose of the initial field sampling work was to attempt to confirm that at least one native plant (the prime candidate being *Haloxylon salicornicum*) can survive on and possible remediate, hydrocarbon-polluted topsoil in Kuwait desert ecosystems. Other candidate species then have been later on identified during the course of the field study, and they were *Cyperus conglomeratus* and *Rhanterium eppaposum* (which rather appropriately is the national flower of Kuwait).

The fieldwork programme was integrated (in selecting sampling locations) with the work carried out to date by Kuwait Institute for Scientific Research (KISR) and other international consultants (CIC and Ecology and Environment Inc.) commissioned by the Public Authority for Assessment of Compensation (PAAC) and Kuwait National Focal Point (KNFP), in assessing the extent of damage caused by oil lakes resulting from oil-well fires in 1991, as well as the possible remediation alternatives. The sampling sites were selected across a combination of natural environmental gradients (e.g. soil type, and/or desert ecosystem type, both of which vary throughout Kuwait (Al-Ateeqi 2006), and gradients of oil-pollution stress plus other human disturbance (namely grazing and off-road driving) in order to cover a sensible range of the likely pressures affecting survival of potential phytoremediator species in Kuwait.

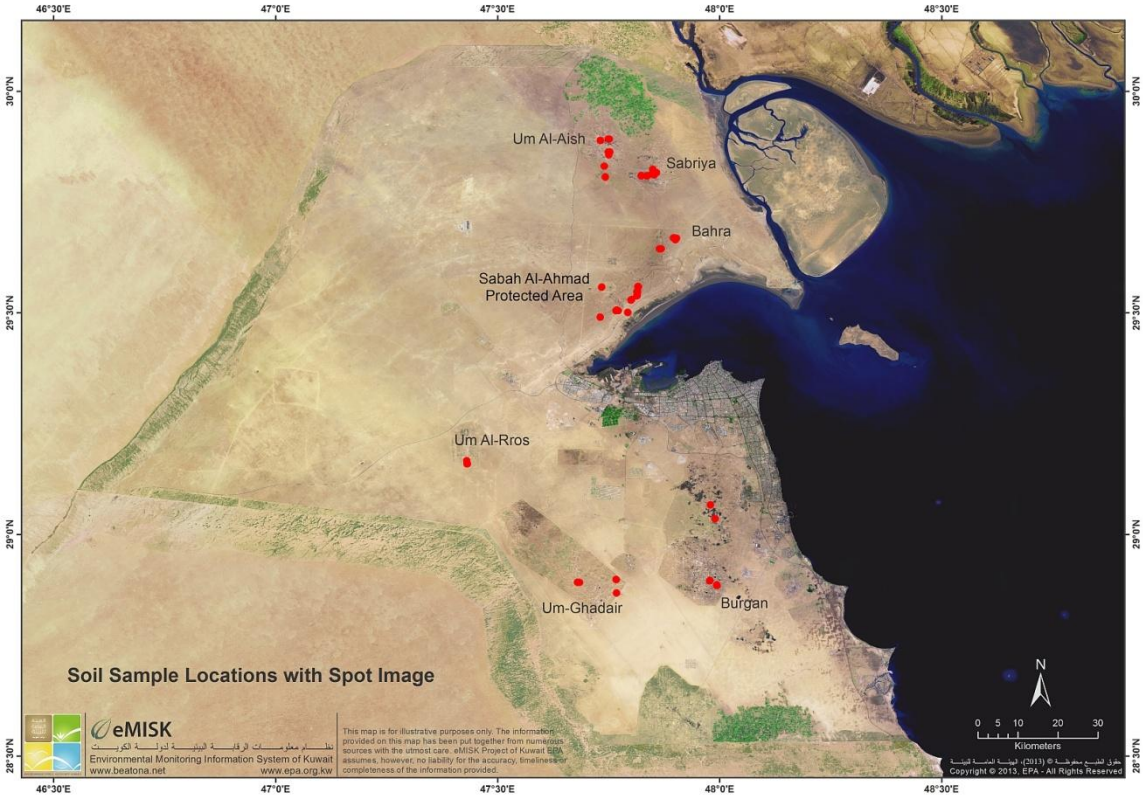


Figure 2-1: Sampling sites in Kuwait, 2011. (Base map: ((EPA1) 2013).

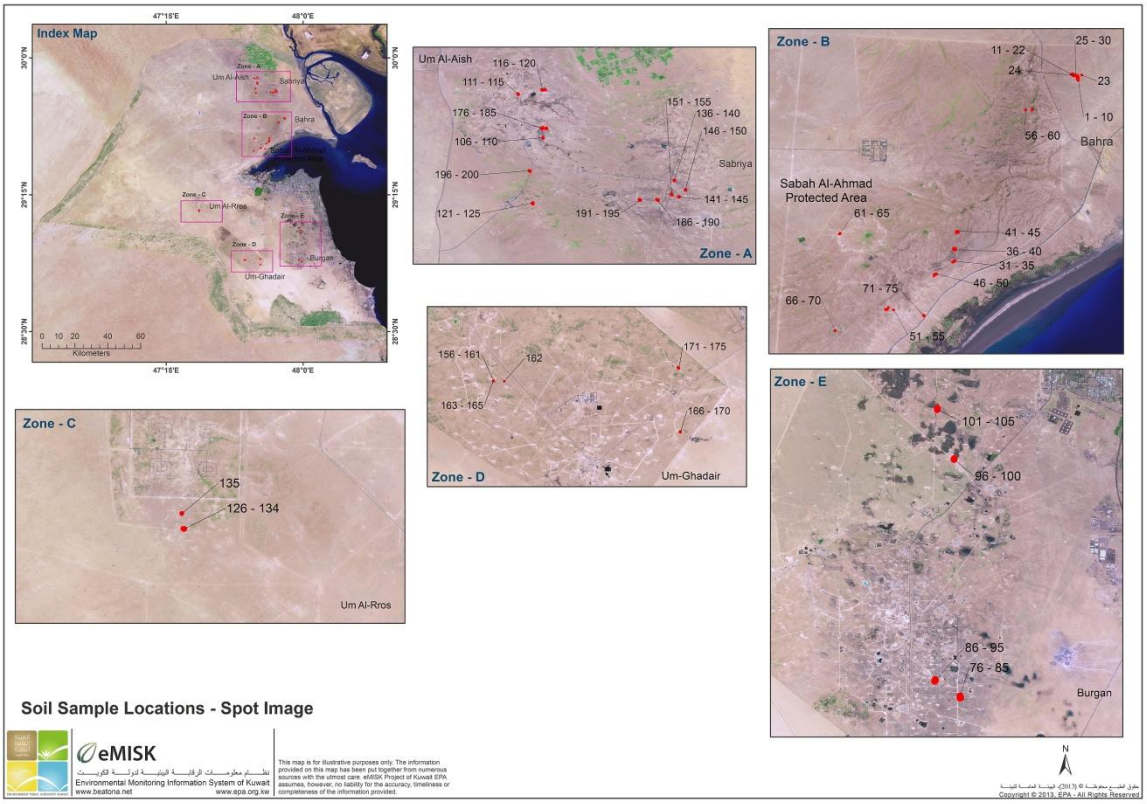


Figure 2-2: Sampling areas with more visual details. ((EPA2) 2013). Zone A is Bahra, Sabriya, and Um Al-Aish oil fields, Zone B is Sabah Alahmad Protected Area, Zone C is Um Al-Rros Military Base, Zone D and E is Burgan and Um-Ghadair oil field.

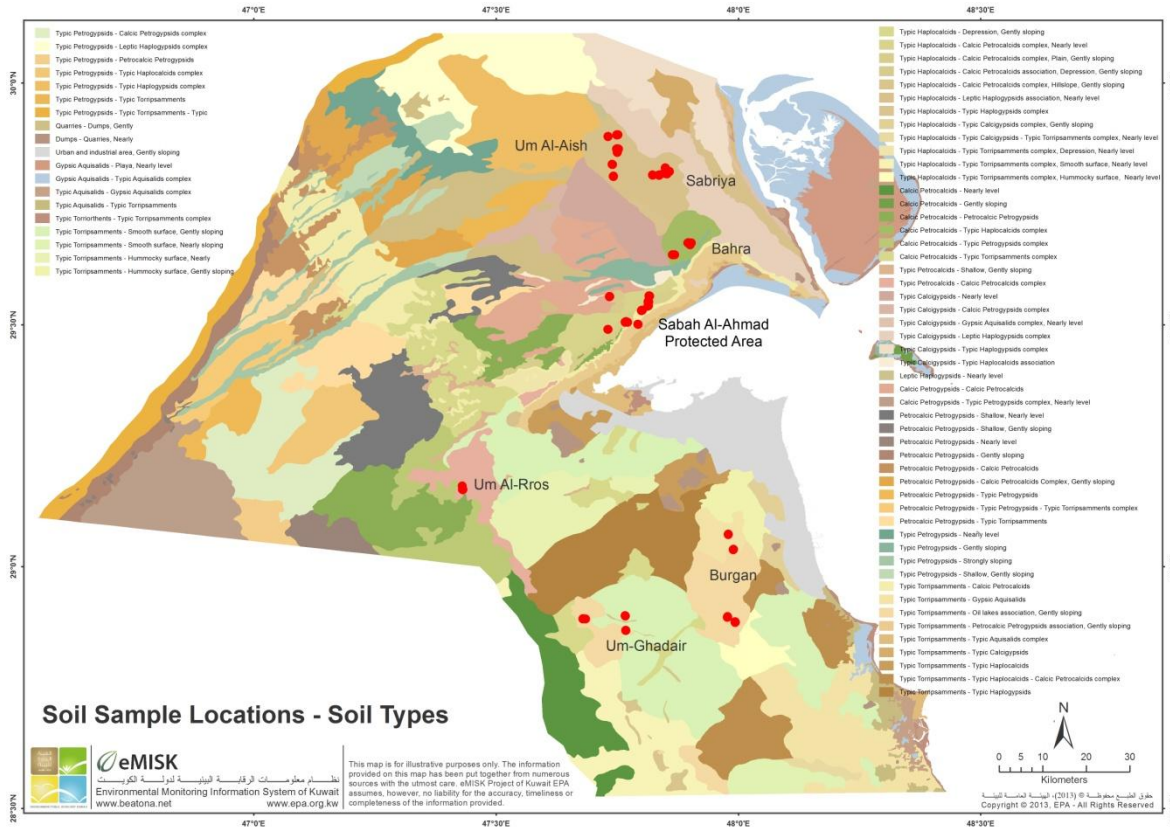


Figure 2-3: Sampling areas including the soil type. Base map: ((EPA3) 2013).

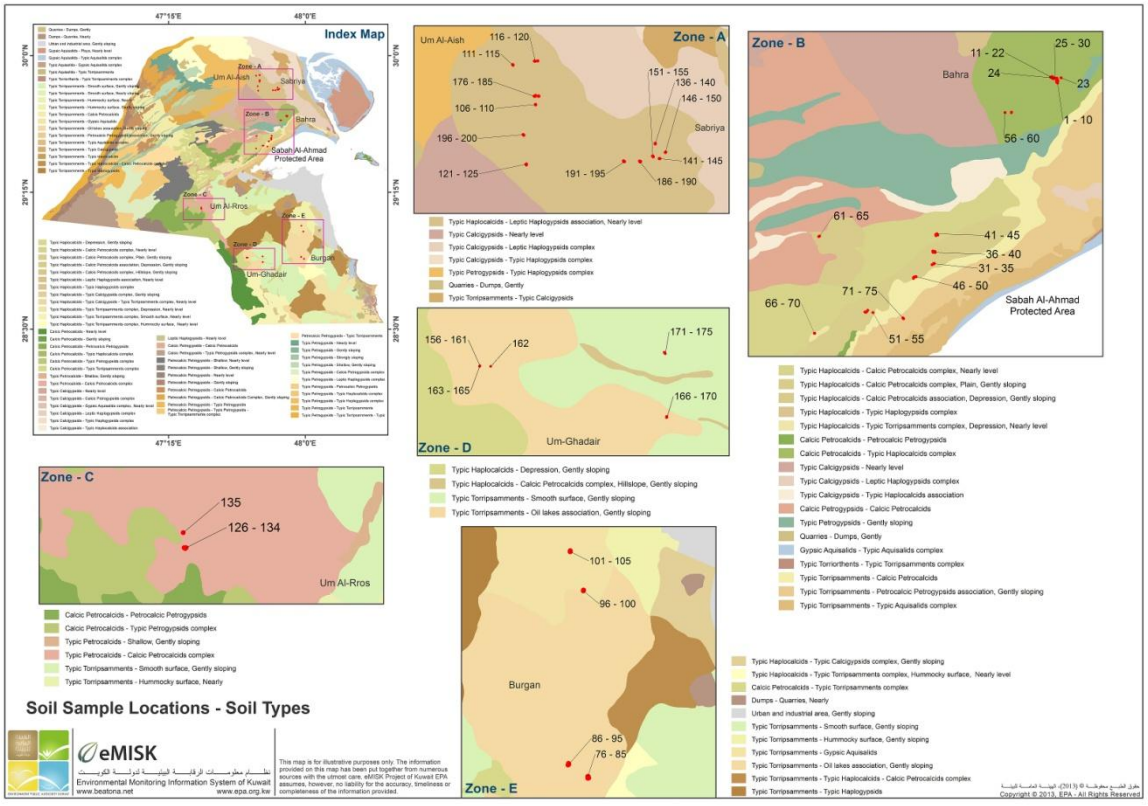


Figure 2-4: Sampling areas for soil with details of the soil type. Base map ((EPA4) 2013). Zone A is Bahra, Sabriya, and Um Al-Aish oil field, Zone B is Sabah Alahmad Protected Area, Zone C is Um Al-Rros military area, Zone D and E is Burgan and Um-Ghadair oil field.

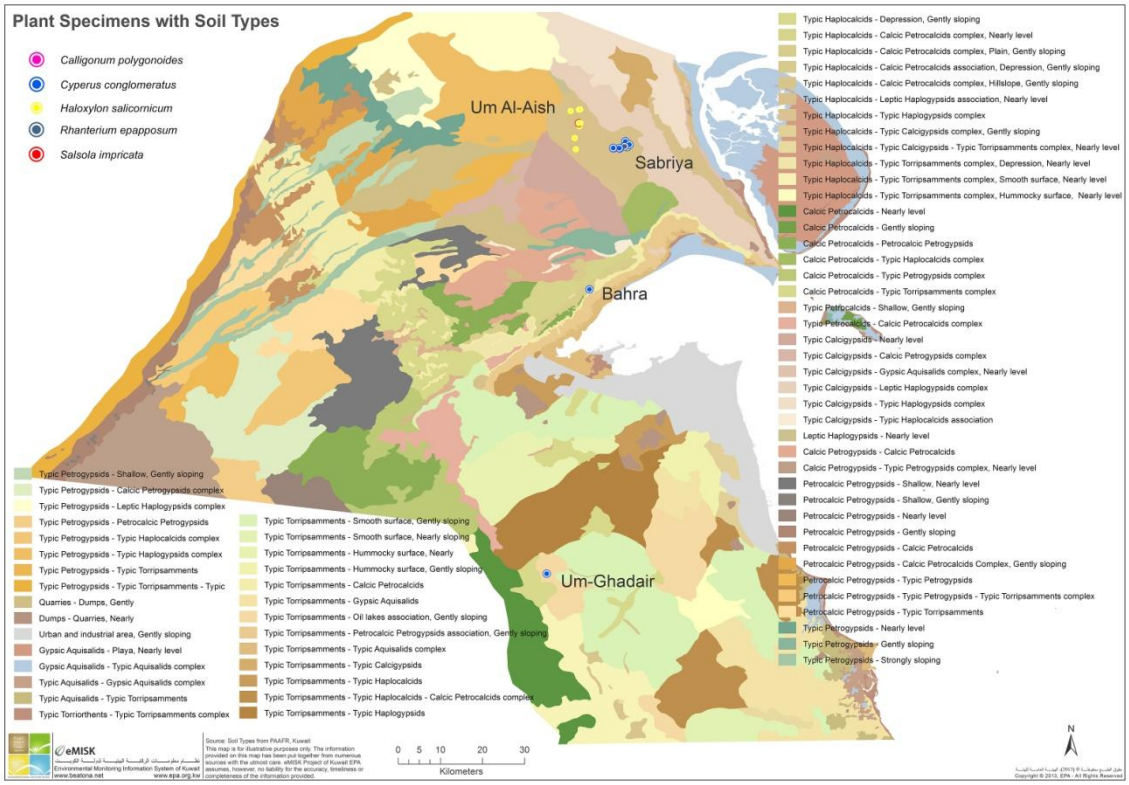


Figure 2-5 :**Sampling areas for the plant specimens. Base map ((EPA5) 2013).**

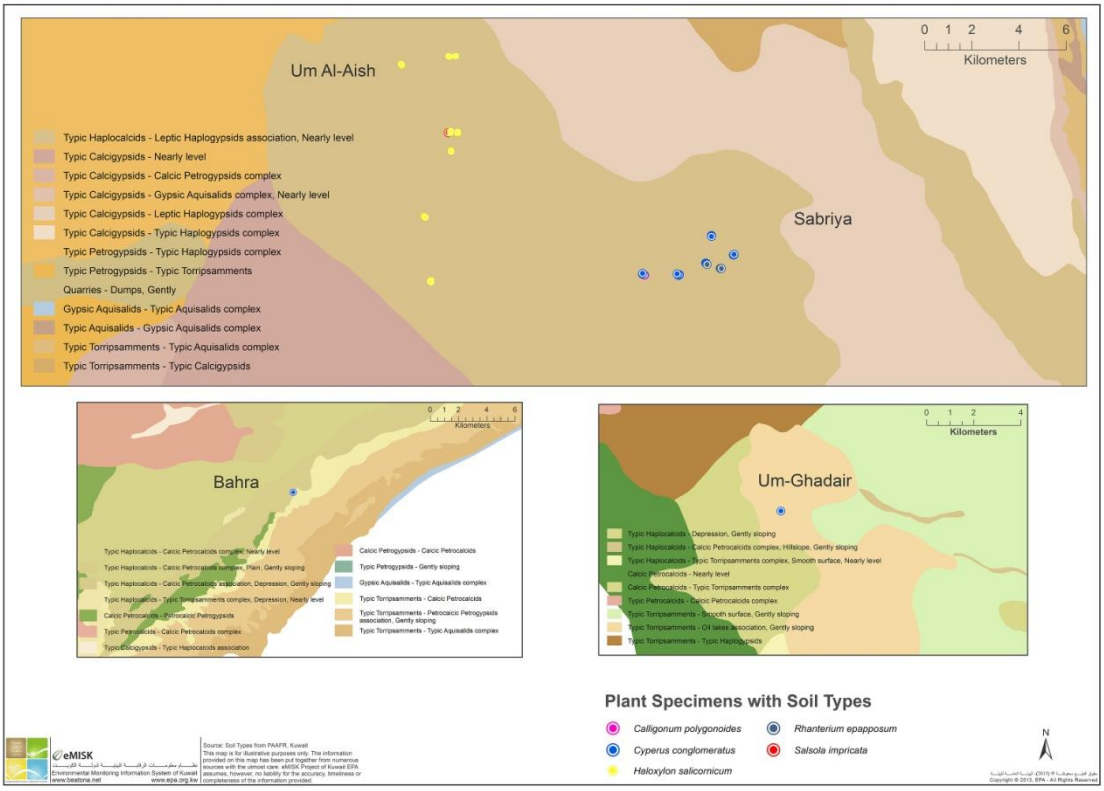


Figure 2-6: **Sampling areas for plant specimens exhibiting details of the soil type. Base map ((EPA6) 2013).**

2.3.2 Sampling Protocol

For each selected site, precise Universal Transverse Mercator (UTM) geo-coordinates (as well as recording the latitude, longitude and altitude), were established using GPS (Garmin Etrex: Basic), and a minimum of 5 randomly-positioned quadrats (Fig. 2-7) were sampled around the established sampling point. For each quadrat species present, frequency and mean vegetation height (cm) were recorded. Soil and plant samples were collected for subsequent lab analysis (Central Environmental Laboratory (CEL), ISO 17025, Faculty of Science, Kuwait University) of a range of relevant chemical parameters. The data collected were:

(i) abundance of each plant species present (F);
(ii) mean vegetation height (cm);
(iii) species diversity (S m^{-2});
(iv) altitude (m above sea level);
(v) latitude/longitude;
(vi) semi-quantitative Oil Damage Score (ODS: on a 1 – 3 scale, with 1 = no visible damage, to 3 = major visible evidence of oil damage to soil); and
(vii) soil and within-plant concentrations (the latter only for the dominant plant species present in the quadrat) of Polycyclic Aromatic Hydrocarbons (PAH), and (viii) soil moisture.

66 frozen plant tissue (leaf) specimens (Fig. 2-5, 2-6) were sent in March 2011 to CEL for laboratory tissue analysis in labelled sealed glass jars. Three specimens out of the 66 were excluded from the interpretation of the data analysis because their rarity made them unsuitable for data interpretation. These included two specimens of *Salsola impricata* and one of *Calligonum polygonoidis*. The remaining specimens consisted of thirty two *Haloxylon salicornicum* (Fig. 2-8), collected from the Um Al-Aish oil field, and eighteen specimens of *Cyperus conglomeratus* (one from Sabah Alahmad Protected Area, one from the Um-Ghadair oil field and sixteen from the Sabriya oil field). The other thirteen specimens were *Rhanterium epapposum* collected from the Sabriya oil field.

For the soil sample analysis, 184 labelled samples were sent to the CEL in March 2011. There were 26 samples from Bahra oil field, 45 samples from Sabah Alahmad Protected Area, 21 samples from Burgan oil field, 35 from Um Al-Aish, 7 from Um Al-Rros, 30 from Sabriya oil field, and finally 20 from Um-Ghadair oil field.

Figure 2-7: Quadrat sampling technique: Um Alaish area. March 2011.

Figure 2-8: *Haloxylon salicornicum* seeds, picture from Sabah Alahmad Protected Area, Feb. 2011

2.3.3 Data Analysis and Interpretation of Results

The survey data were analysed using (Two-Way Indicator Species Analysis (TWINSPAN: (Hill 1979). This method provides a hierarchical divisive classification of the data matrix (Gauch 1982; Hill 1975), and produces a two way ordered table of both species and samples. It also identifies indicator species which characterize sample-groups produced by the classification (Zahran 1996), and separates species into assemblages based on co-occurrence in samples. In addition to classification analysis a DCA ordination (Fig. 2-19, 2-20) of the species data was undertaken. These multivariate procedures permit assessment of the relative influence of environmental factors in predicting plant community composition in relation to environmental factors, providing insight into plant community response to current environmental conditions. The environmental data received from the CEL were complemented by weather data (temperature, rain, humidity, and wind speed data, acquired from the Kuwait Meteorological Department, Directorate General of Civil Aviation, Kuwait).

Datasets for both the plant-related data, and environmental data were tested for normality (Ryan-Joiner test) and log-transformed if necessary prior to the use of one way or two ways ANOVA to examine differences between plant and environmental variables, both between geographical areas and between sets of samples making up TWINSPAN sample-groups. Where data were not normal, and could not be normalized, non-parametric Kruskal-Wallis Test one-way analysis of variance by ranks was used instead of ANOVA. In Table (2-1) for P.T.A (PH), P.T.A. Benzo(a)anthracene (B), P.T.A (FLAN), P.T.A (PY), P.T.A (CH) Data were log-10 transformed prior to analysis (see Appendix 1). The means and standard deviation that were taken from non-logged-10 data are Plant Height, Species Diversity, *Haloxylon salicornicum, Cyperus conglomeratus, Rhanterium epapposum*, (CH), (PH), (PY), (ANTH), (FLAN), P.T.A (NA), P.T.A (ANTH), P.T.A (FL), P.T.A P-moist, P-moist.

In table (2-2) For Species Diversity, P.T.A (PY), P.T.A Benzo(a)anthracene (B) data were log-10 transformed prior to analysis. Mean values sharing a lower case letter in common are not significantly different from each other (significant ANOVA outcomes only: Tukey mean separation test) Kruskal-Wallis tests were used where data remained non-normal even after attempted log-10 transformation. (Appendix 1 shows full outcomes of significance tests). The means and standard deviation taken from non-logged-10 data are Plant Height, *Haloxylon salicornicum, Cyperus conglomeratus, Rhanterium epapposum*, P-

moist, (CH), (FLAN), (PY), (PH), (ANTH), P.T.A (ANTH), P.T.A (FLAN), P.T.A (FL), P.T.A (NA), P.T.A. (CH), P.T.A P-moist.

2.4 Results

2.4.1. Geographical comparison of environmental and plant variables between sampling areas

Results of one way ANOVA comparisons for individual variables measured at sampling sites between areas (Bahra (1), Sabah Alahmad (2), Burgan (3), Um Al-Aish (4), Um Al-Rros (5), Sabriya (6), and Um-Ghadair (7)) and location (north vs. south) groups are shown in Table 2-1 and Figs. 2-3, and 2-4. For the Oil Damage Score (ODS) data analysis was undertaken by using Kruskal-Wallis Test. Full results are given in Appendix 1 (see page 107). The results indicate substantial variation with geographical location, and are outlined for each plant and environmental variable measured.

Table 2-1: Characteristics of sample groups, defined by areas and location (North or South of Kuwait) for vegetation state variables and environmental variables. Values

are given as mean and standard deviation (in parentheses), Two way ANOVA was used to test the difference between areas and different environmental factors.

Groups	Areas							P	Directions		P
	Bahara	Sabah Alahmad	Burqan	Um Alaish	Um Alrros	Alsabriya	Um Ghader		North	South	
Vegetation state variables											
Plant Height (cm)	8.38 (11.01)b	12.64 (15.75)ab	13.51 (10.63)ab	19.45 (13.62)a	1.34 (0.93)b	17.88 (12.16)a	9.80 (4.01)ab	<0.001	13.67 (13.99)	12.03 (8.75)	n.s.
Species Diversity (S)	1.767 (1.104)bc	2.444 (1.374)ab	1.567 (0.817)c	2.971 (1.543)a	1.100 (0.316)c	2.467 (1.137)ab	1.400 (0.598)c	<0.001	2.347 (1.366)a	1.500 (0.735)b	<0.001
***Haloxylon salicornicum* (%F)**	59.57 (61.09)a	13.13 (50.22)b	0	158.23 (114.58)c	0	0	0	<0.001	No Data		
***Cyperus conglomeratus* (%F)**	3.30 (14.49) c	0.53 (3.14)c	30.47 (35.06)b	0	0	50.57 (48.88)ab	74.10 (60.47)a	<0.001	10.93 (30.09)a	47.92 (51.10)b	<0.001
***Rhanterium epapposum* (%F)**	0	6.09 (25.01)a	0	0.11 (0.68)a	0	88.90 (111.87)b	27.30 (50.64)a	<0.001	19.63 (61.93)	10.92 (34.30)	n.s.
Environmental Variables											
Oil Damage Score	1a	1a	3b	3b	1a	3b	2ab	<0.001	1a	3b	≤0.004
Chrysene (CH) (µg/Kg)	5.9 (19.1)b	0	2.3 (10.2)b	5.2 (11.5)b	0	96.1 (220.0)a	0.6 (1.5)b	≤0.002	No Data		
Phenanthrene (PH) (µg/Kg)	17.1 (71.6)ab	0.1 (0.3)b	2.2 (9.2)ab	1.2 (3.0)b	0	82.2 (240.0)a	0.2 (0.4)ab	≤0.012			
Pyrene (PY) (µg/Kg)	4.59 (16.13)b	0	0.68 (3.01)b	0.57 (1.60)b	0	62.01 (134.14)a	0	≤0.002			
Anthracene (ANTH) (µg/Kg)	0.0455 (0.1687)	0	0.0538 (0.2466)	0	0	0.0809 (0.3137)	0	n.s.			
Fluoranthene (FLAN) (µg/Kg)	0.27 (0.59)b	0	0.99 (4.54)ab	0.20 (0.91)b	0	22.59 (61.02)a	0	≤0.016			
P.T.A. Phenanthrene (PH) (µg/Kg)	No Data	No Data	No Data	4.5902 (0.1959)ab	No Data	4.4442 (0.3560)b	5.2095 (*a)	≤0.009			
P.T.A. Benzo(a)anthracene (B) (µg/Kg)	No Data	No Data	No Data	0.135 (0.447)b	No Data	1.344 (1.508)a	0.00 (*ab)	<0.001			
P.T.A. Fluoranthene (FLAN) (µg/Kg)	No Data	No Data	No Data	3.6533 (0.1517) b	No Data	3.8716 (0.2038)a	3.8607 (*ab)	<0.001			
P.T.A. Pyrene (PY) (µg/Kg)	No Data	No Data	No Data	3.0765 (0.1654)b	No Data	3.2474 (0.2262)a	3.1311 (*ab)	≤0.004			
P.T.A. Chrysene (CH) (µg/Kg)	No Data	No Data	No Data	1.6580 (0.8144)ab	No Data	1.9374 (0.5804)a	0.000 (*b)	≤0.018			
P.T.A. Naphthalene (NA) (µg/Kg)	No Data	No Data	No Data	20.591 (2.928)b	No Data	31.250 (10.788)a	21.1 (*ab)	<0.001			
P.T.A Anthracene (ANTH) (µg/Kg)	No Data	No Data	No Data	1.735 (2.229)b	No Data	5.323 (5.812)a	6.590 (*ab)	≤0.004			
P.T.A. Fluorene (FL) (µg/Kg)	No Data	No Data	No Data	10.72 (4.32)a	No Data	70.95 (92.55)b	8.44(*ab)	<0.001			
P.T.A. P-moist (%wt)	No Data	No Data	No Data	52.47 (12.83)a	No Data	69.79 (5.01)b	59.5 (*ab)	<0.001			
P-moist (%wt)	1.925 (1.222)b	3.844 (3.408)b	1.996 (2.657)b	6.315 (4.094)a	3.570 (1.035)ab	3.550 (2.218)b	1.568 (1.453)b	<0.001			

2.4.1.1 Oil Damage Score (ODS)

Samples taken from the Burgan oil field, Um Al-Aish oil field, Sabriya oil field, and Um-Ghadair oil field, unsurprisingly given the massive oil-pollution disturbance in these areas, had the highest ODS values (Fig. 2-9). There were generally lower scores and little difference between ODS values from the other areas.

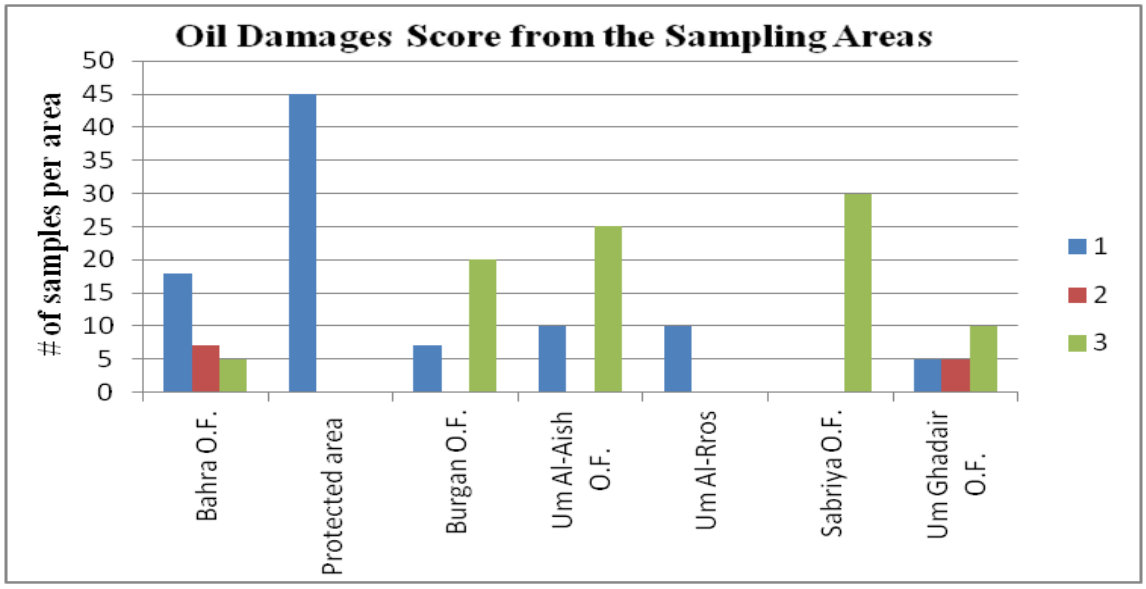


Figure 2-9: Oil Damage Scores for quadrat samples collected in the 7 areas of research regarding the number of Quadrats for each area (O.F. = Oil Field; #1=low or invisible contamination, #2=moderate visible contaminant and #3=high visible contaminant). Vertical axis: number of quadrats with each score category (see Appendix 1 page 107).

2.4.1.2 Species Diversity

Samples from Um Al-Aish and Sabriya oil fields as well as Sabah Alahmad Protected Area were significantly different in terms of species diversity from most of the other areas, especially Bahra oil field, Burgan oil field, Um Al-Rros Military Base and Um-Ghadair oil field, with significantly lower than average diversity, and the Protected Area higher (at 6 species per quadrat) than the average (which is 4.4 species per quadrat)(Fig. 2-10, see Appendix 1 page 107).

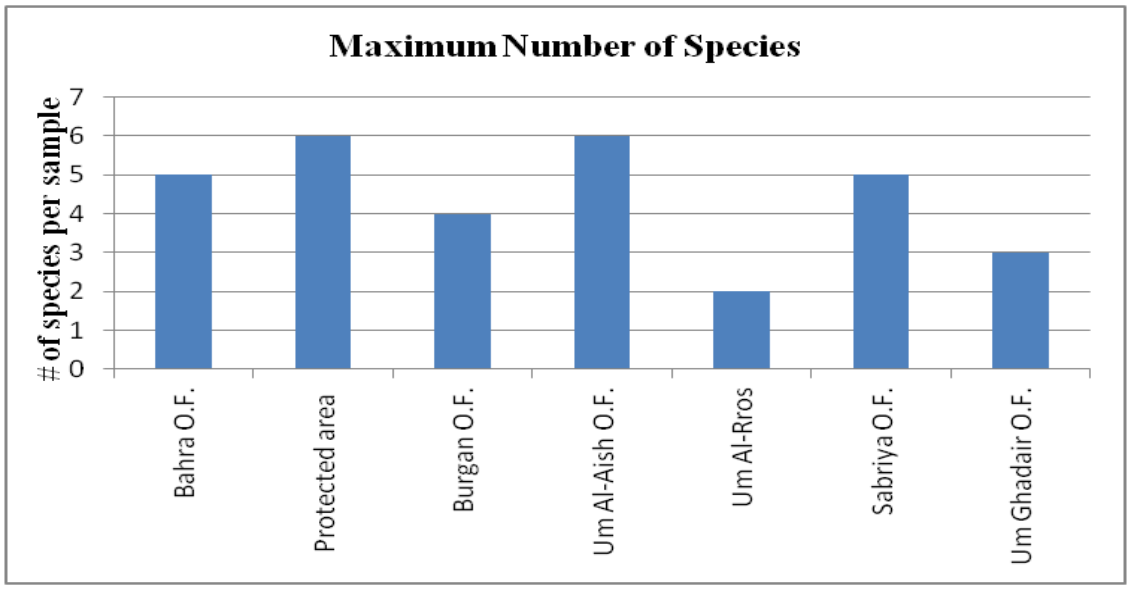


Figure 2-10: Species Diversity where the vertical axis is maximum number of species recorded in the areas of study. (O.F.: Oil Field) (see Appendix 1 page 107)

2.4.1.3 Plant Height

Mean plant height was significantly higher for samples taken from Um Al-Aish and Sabriya oil fields compared with Bahra Oil Field and Um Al-Rros Military Base, although the oil contamination is lower in Bahra Oil Field and um Al-Rros (Fig. 2-11, see Appendix 1 page 107).

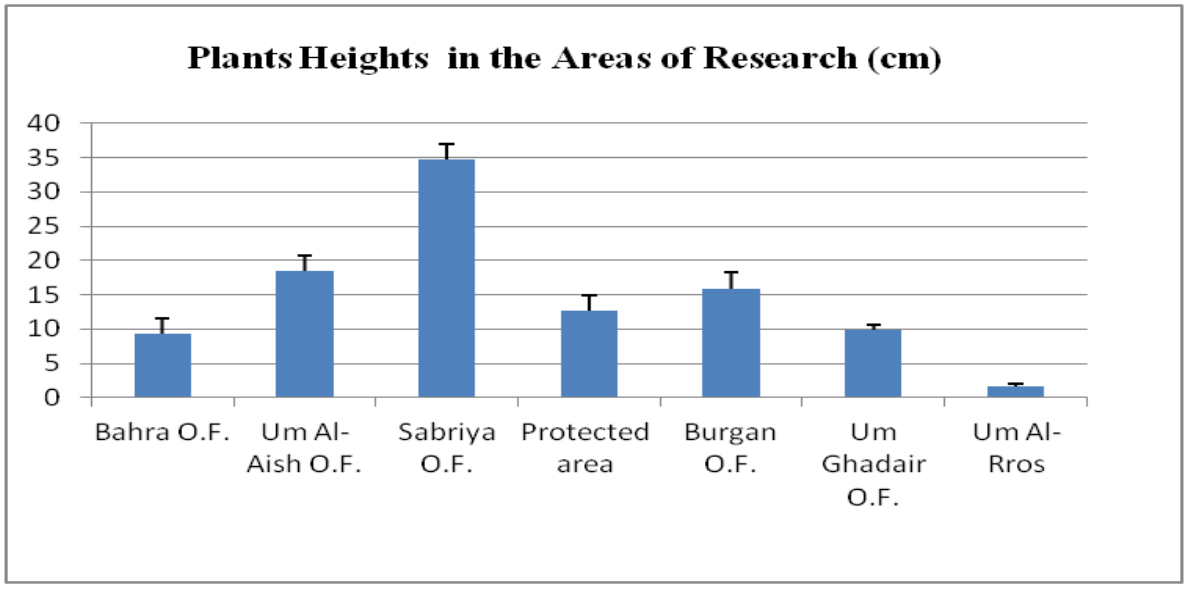


Figure 2-11: Plant Height (cm) for the sample taken in Areas of Research where Sabriya oil field (O.F.) has the higher plant heights per cm (see Appendix 1 page 107).

2.4.1.4 Abundance of *Haloxylon salicornicum*

Although this species generally declines in abundance in the north of Kuwait (due to overgrazing and other anthropogenic effects), the sampled quadrats from north of Kuwait still exhibited the highest abundance of this species (Brown 2003). Ghazanfar (2006) also pointed out that *Rhanterium epapposum*, *Cyperus conglomeratus*, *Convolvulus oxyphyllus*, and *Stipagrostis plumosa* are all non-halophytic communities surrounding the salt marshes in Kuwait, where *Haloxylon salicornicum* is the predominant species. *Haloxylon* proved to be more abundant in Um Al-Aish oil field (north of Kuwait where its community is mostly located), less so in Bahra oil field, and lowest in the other areas— suggesting the potential of this species to survive in oil-polluted soils as well as uncontaminated areas (Fig. 2-12; see also Appendix 1 page 107).

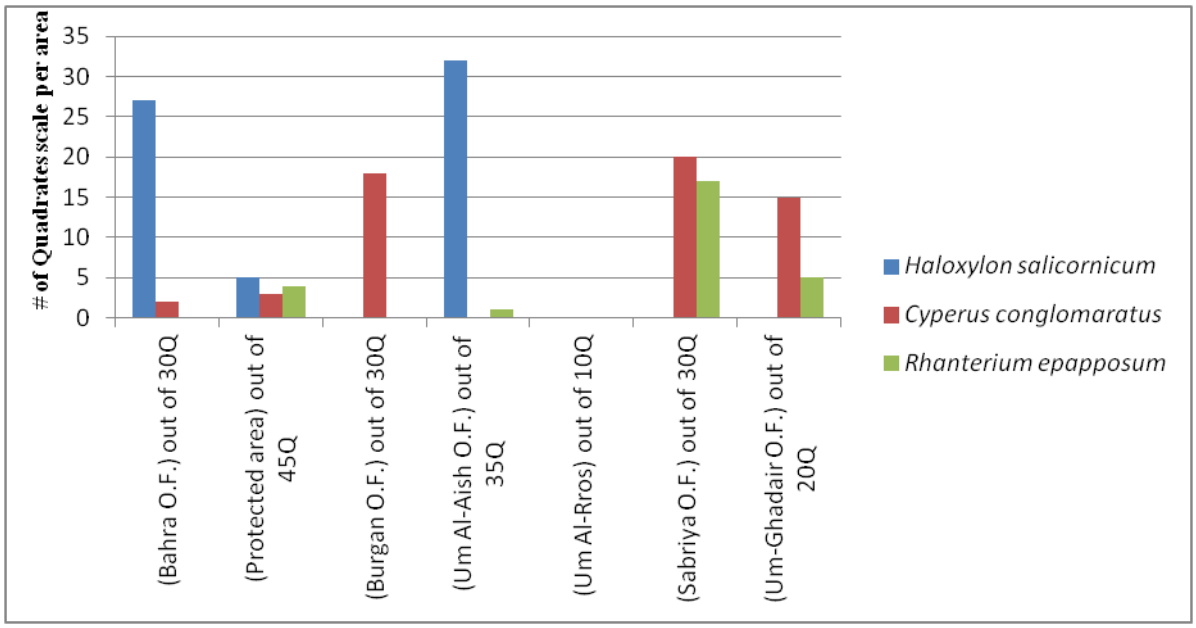


Figure 2-12: *Haloxylon salicornicum. Cyperus conglomeratus,* and *Rhanterium epapposum* quadrate scale, for target species presence in the areas of research, shown as number of quadrat (Q) samples in which it occurred for each area (see Appendix 1 page 107).

2.4.1.5 Abundance of *Cyperus conglomeratus*

Although this species can be found in the north of Kuwait (Sabriya in this case), it is more common in the south, where, according to Brown (2003), it tends to replace normally-occurring species (*Rhanterium epapposum*) of Kuwait in heavily-grazed areas. The species clearly grows well in oil-polluted soils in this part of Kuwait, as shown by its high occurrence in Burgan and Um-Ghadair oil fields respectively (both in the south of Kuwait: Fig. 2-12; see also Appendix 1 page 107).

2.4.1.6 Abundance of *Rhanterium epapposum*

This species is quite widely distributed in Kuwait (Brown 2003), and again there is evidence (Fig. 2-12) that it can survive in oil-contaminated soils. For example, in Sabriya (northern Kuwait) it was found growing in soil lying on the surface of a dry oil lake. It was less common in the Um-Ghadair (south of Kuwait) oil field, and was also found in unpolluted soils in Sabah Alahmad Protected Area (north of Kuwait) (see Appendix 1 page 107).

2.4.1.7 Polycyclic Aromatic Hydrocarbons (PAHs) in soil and plant samples

Results are provided here for the outcome of the sample–analysis by CEL for all 16 of the component PAHs identified by the US Environmental Protection Agency (EPA) as priority pollutants. Of the various PAH component chemicals analysed from soil samples (Fig. 2-13) Chrysene (CH) was usually predominant, especially in Sabriya oil field (present in 30 samples). Next commonest were Phenanthrene (PH) and Pyrene (PY) in soils coming from different conditions (fresh/dry oil lakes, and deposit piles of contaminated soil).

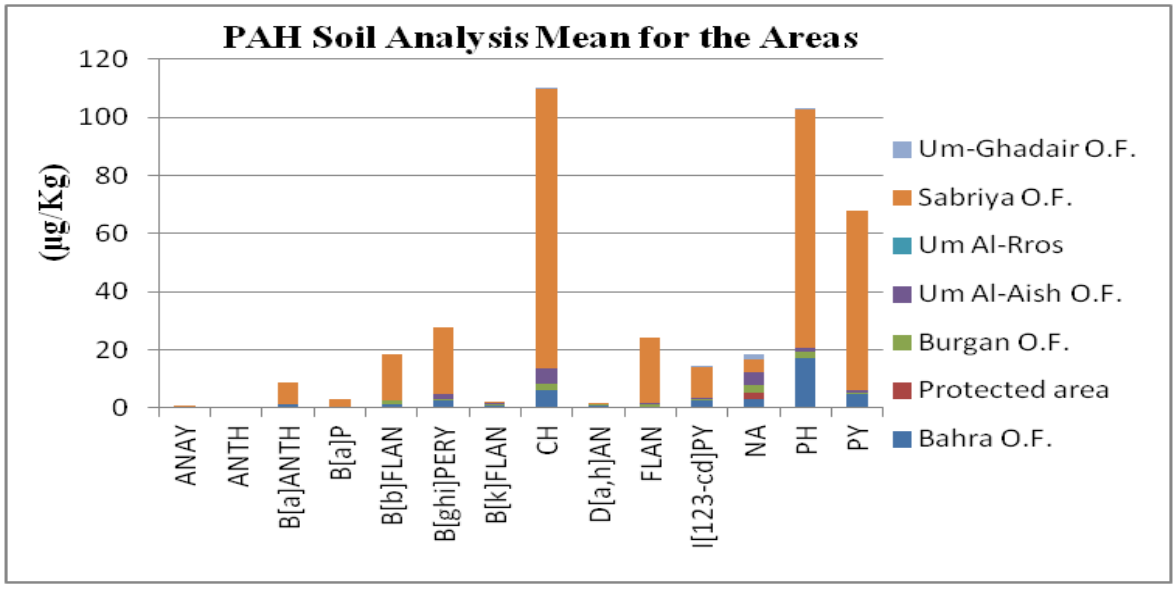


Figure 2-13: PAH soil analysis mean values for the study areas (Note: Acenaphthylene (ANAY); Anthracene (ANTH); Benzo(a)anthracene (B[a]ANTH); Benzo(a)pyrene (B[a]P); Benzo(b)fluoranthene (B[b]FLAN); Benzo(g,h,i)perylene (B[ghi]PERY); Benzo(k)fluoranthene (B[k]FLAN); Chrysene (CH); Dibenz(a,h)anthracene (D[a, h]AN); Fluoranthene (FLAN); Indeno(1,2,3-cd) pyrene (I[123-cd]PY); Naphthalene (NA); Phenanthrene (PH) and Pyrene (PY)). (O.F.: Oil Field) the amount of Chrysene, Phenanthrene, and Pyrene is the highest in Sabriya area. The CH and PY do have high molecular weight, which means they can stay for a long time in the soil (see Appendix 1 page 107).

The amount of Phenanthrene (PH) in plant tissue samples seems to be the highest (Fig. 2-14) in the single plant specimen (*Cyperus conglomeratus* plant) collected from the Um-

Ghadair oil field (in the south of Kuwait), where very high soil PAH occurrence was also recorded. Next highest were the values recorded for 16 specimens of *Rhanterium* from Sabriya, and 13 *Haloxylon* samples from Um Al-Aish. Fluoranthene (FLAN) was the PAH with the second highest content in plant tissues.

Although Chrysene (CH) showed very high occurrence in the soil analysis there is little evidence here that plants were taking it up in anything other than small quantities. On the other hand, Anthracene (ANTH), which had relatively low occurrence in soils, was clearly readily taken up by plants.

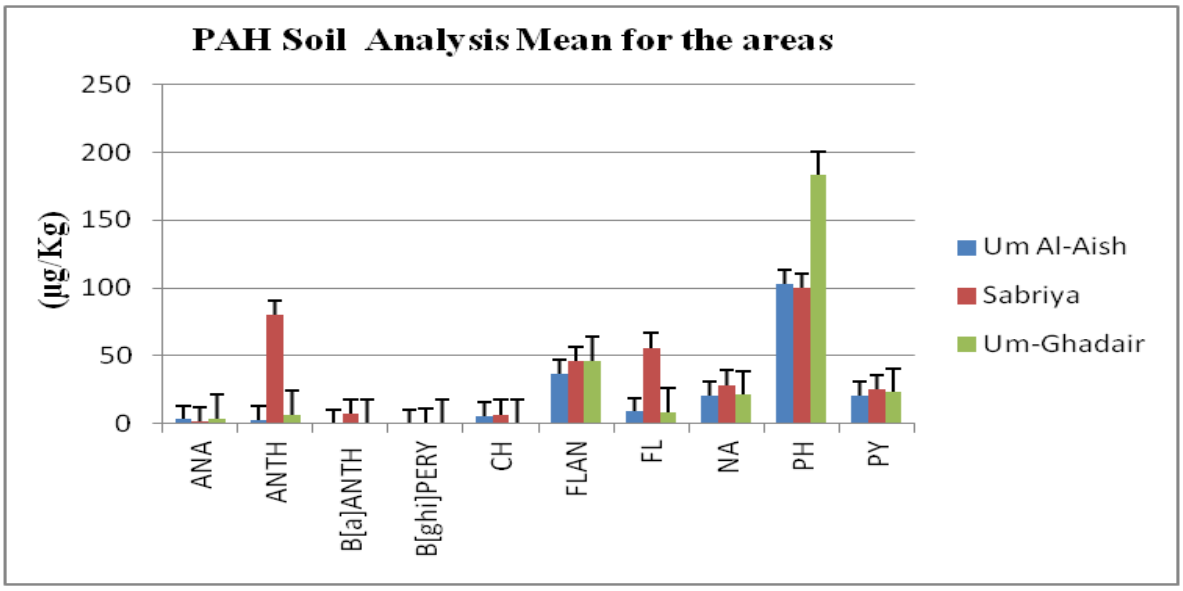


Figure 2-14: Polycyclic Aromatic Hydrocarbons (PAH) found by Plant Tissue Analysis (P.T.A) of plant samples collected from the areas of study. (Note: Acenaphthene (ANA); Anthracene (ANTH); Benzo(a)anthracene (B[a]ANTH); Benzo(a)pyrene (B[ghi]PERY); Chrysene (CH); Fluoranthene (FLAN); Naphthalene (NA); Phenanthrene (PH) and Pyrene (PY) with standard error. The plants do contain amount of PAHs in its tissues, some of them with high molecular weight, and some with low molecular weight which might indicate absorption from the air (see Appendix 1 page 107).

2.4.1.8 Plant moisture content

Plants sampled from Sabriya oil field generally had higher moisture content than the plant populations studied in the other oil fields (Fig. 2-15). This is probably might be due to the morphology of the species present in the three oil fields. *Cyperus conglomeratus* and *Rhanterium eppaposum* (comprising the Sabriya samples) have proportionately more leaf tissue, and are less woody, than the *Haloxylon salicornicum* plants mainly comprising the Um Al-Aish sample (one *Cyperus* plant was also present in the Um Al-Aish samples analysed).

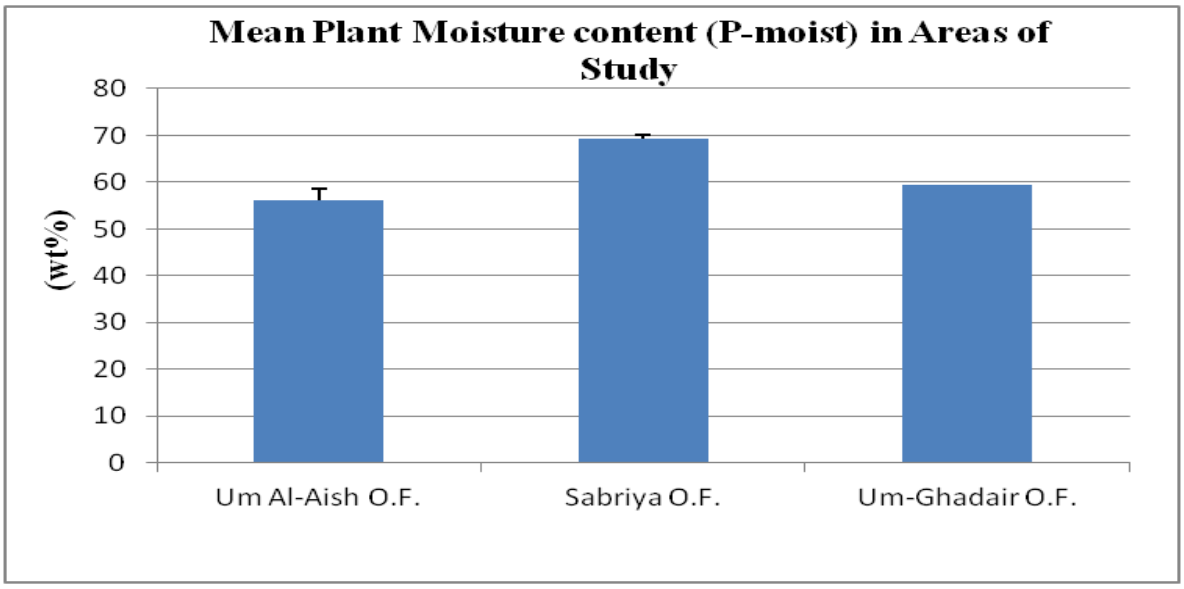


Figure 2-15: Percentage of moisture in the Plant Tissue Analysis (P.T.A), where the moisture content is higher in Sabriya oil field followed by Um-Ghadair oil field then Um Al-Aish, with standard error bar where Um-Ghadair is just one point. (O.F.: Oil Field).

2.4.1.9 Soil moisture content

The Um Al-Aish area, where most of the samples were taken (20-30 cm deep) from a fresh oil lake (mixture of oil and water), had the highest soil moisture content (Fig. 2-16). Other samples collected from this area ranged from tarcretes through to dry oil lakes (contaminated soil which can reach 2 m deep in some places), and oil deposits to clean soil areas.

The soils of the Protected Area also had high moisture content, followed by Um Al-Rros: a military area that is contaminated not by petroleum hydrocarbon but by heavy metals, as it was used to destroy unexploded ordnance. Sabriya oil field soils also had reasonable soil moisture content (3.54 wt.%) compared to the average of both areas equalling 2.88 wt.%. Samples here were mostly taken from dry oil lakes, which tend to retain some moisture underneath the top soil, though samples were also taken from oil deposit piles, as well as a wet oil lake.

The soils of the remaining areas were all very arid. Bahra, Burgan, and Um-Ghadair oil fields are close to each other in terms of moisture content, with samples from Bahra taken from a mixture of oil pile and fresh oil lake. In Burgan oil field the samples were from tarcretes, from an oil lake, and finally from a slightly polluted area. In Um-Ghadair oil field most of the samples were taken from a mixture of tarcretes and dry oil lake areas, with the rest from clean areas.

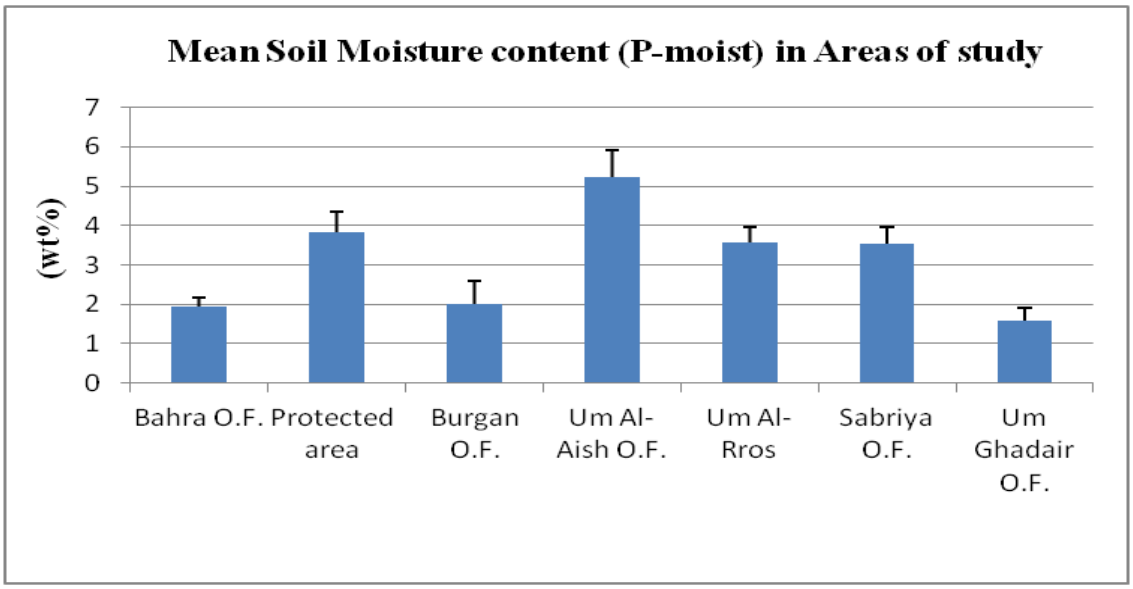


Figure 2-16: Soil moisture content in the areas of study, with an average of 2.88 wt.% for all of them together. Where the higher is in Um Al-Aish oil field area in north of Kuwait. With standard error bars (O.F.: Oil Field).

2.4.2 Comparison of mean values for environmental and plant variables between sample groups defined by multivariate analysis of vegetation data (TWINSPAN/ DCA analyses)

The results of multivariate analyses of the data using ordination and classification (Figs. 2.17 and 2.18) provide strong evidence to suggest that there is non-random variation in the vegetation, not solely associated with geographical location (as was assessed in Section 2.4.1).

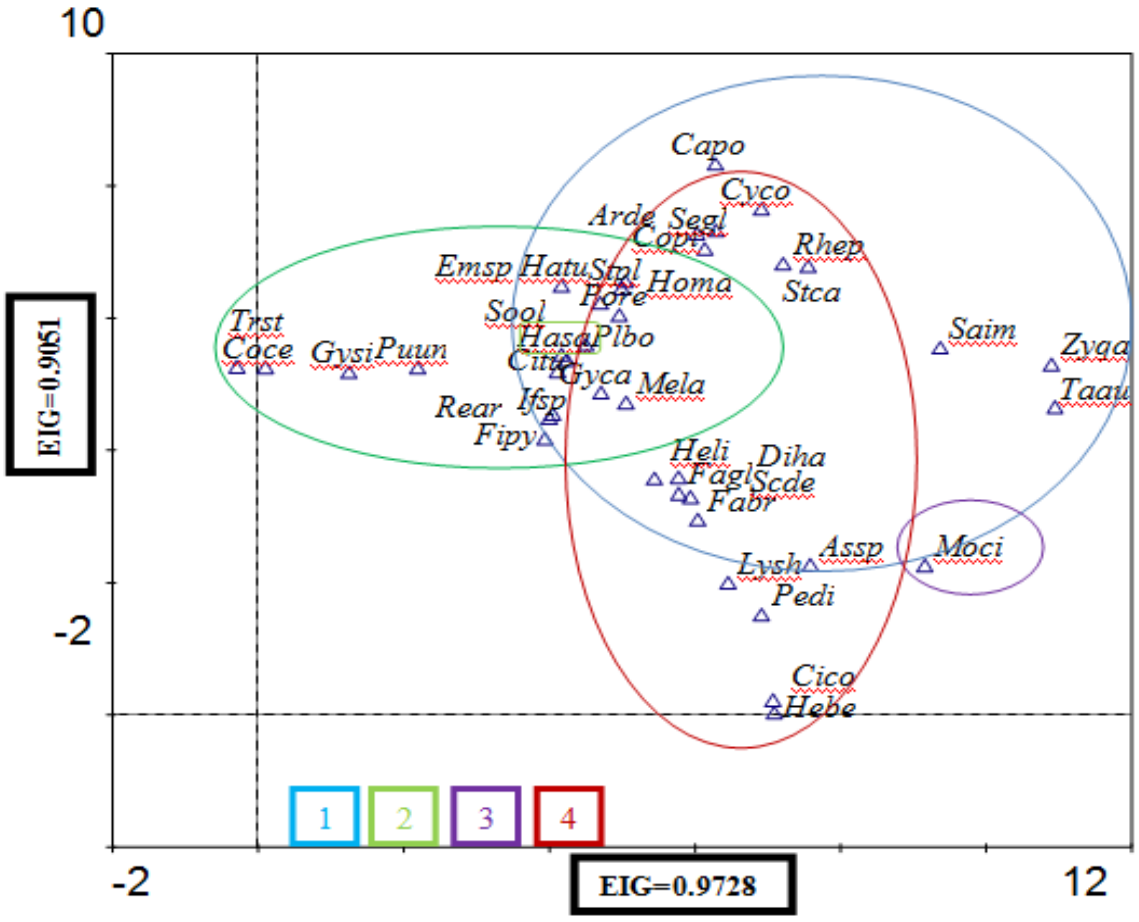


Figure 2-17: DCA species ordination plot with TWINSPAN species assemblages (I, II, III, IV) overlain. Assemblage 1 consists of Cyco: *Cyperus conglomeratus;* Rhep: *Rhanterium epapposum;* Capo: *Calligonum polygonoides*; Arde*: Arnebia decumbens*; Stca: *Stipa capensis*; Segl*: Senecio glaucus ;* Saim: *Salsola imprecate;* Zyga: *Zygophyllum qatarense;* Taau: *Tamarix aucheriana*; Hatu: *Haplophyllum tuberculatum;* Assemblage 2: Puun: *Pulicaria undulata;* Hasa: *Haloxylon salicornicum*; Citu: *Cistanche tubulosa*; Ifsp: *Ifloga spicata*; Fipy:*Filago pyramidata*; Homa: *Hordeum marinum*; Plbo: *Plantago boissieri*; Sool: *Sonchus oleraceus*; Mela: *Medicago laciniata*; Gyca: *Gypsophila capillaris*; Emsp: *Emex spinosa*; Coce: *Convolvulus cephalopod;* Gysi: *Gynandriris sisyrinchium;* Trst: *Trigonella stellata;* Stpl: *Stipagrostis plumose*; Pore: *Polycarpaea repens;* Assemblage 3: Moci: *Moltkiopsis ciliata*;>Assemblage 4: Pedi: *Pennisetum divisum*; Lysh: *Lycium shawii*; Cico: *Cistanche colocynthis;* Hebe: *Heliotropium bacciferum*; Fagl: *Fagonia glutinosa*; Heli: *Helianthemum lippii*; Assp: *Astragalus spinosus;* Fabr: *Fagonia bruguieri*; Copi*: Convolvulus pilosellifolius*; Diha: *Diplotaxis harra*; Scde: *Scrophularia deserti.* Rear: *Reseda arabica* is the species that has been removed by the TWINSPAN for rarity (see also Appendix 1 page 107). Axis units: standard deviations (SD) of species turnover. Where *Cyperus* and *Rhanterium* are associated with sample groups C and D, also *Haloxylon* is related to the sample groups A and B, where all of the areas had high oil contamination

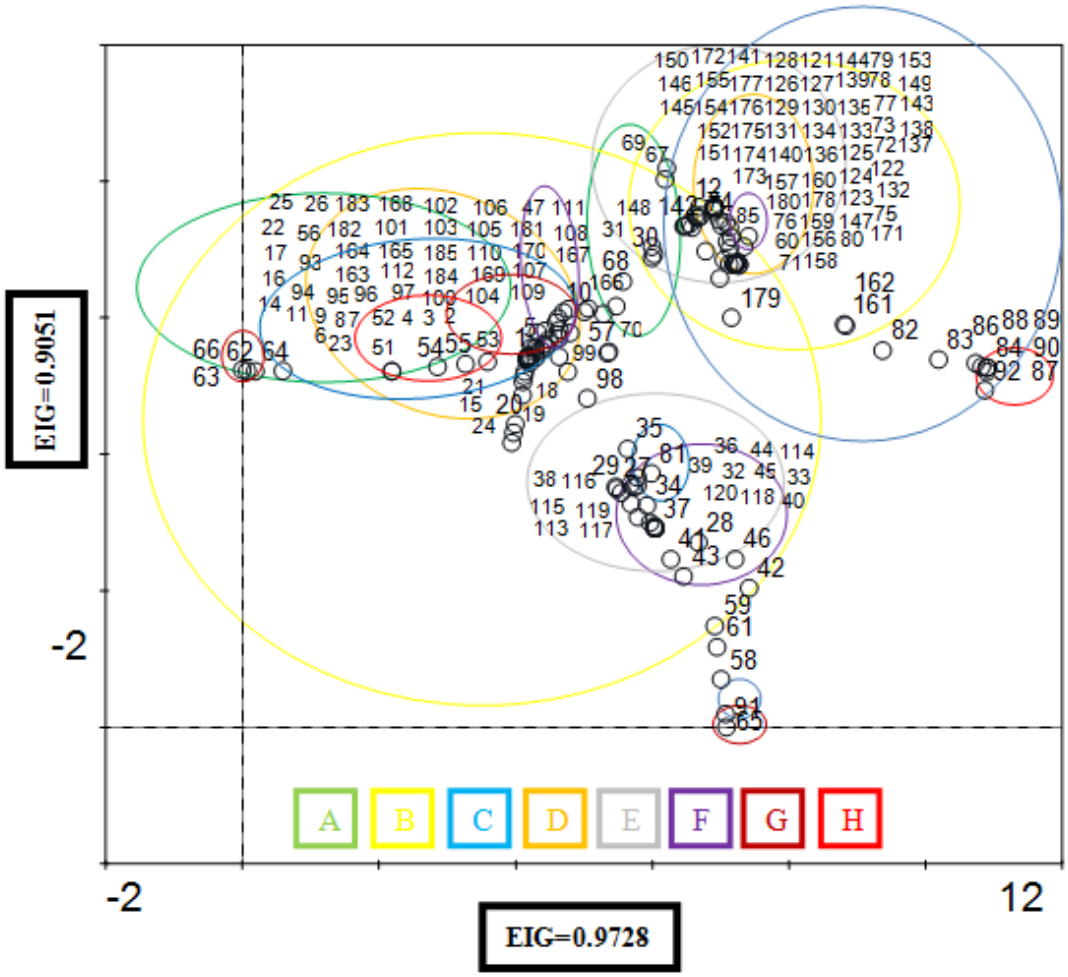


Figure 2-18: Detrended correspondence analysis (DCA) sample ordination for the vegetation analysis, with 7 sample-groups (A – G), containing vegetation, identified by TWINSPAN overlain. Samples in Group H are those which contained no vegetation. Number of samples per group (A=6, B=75, C=66, D=12, E=23, F=9, G=4, and H=5). Axis units: standard deviations (SD) of species turnover. Sample groups A and B are mostly related to the areas in north of Kuwait where there is a high amount of oil contamination, while sample groups C and D related to areas in the north and the south with loweramounts of oil contamination, while the rest are more related to areas with less oil contamination impact (see Appendix page 107).

In total, 8 sample groups (A-H) were identified by the TWINSPAN analysis, which showed reasonable separation in the ordination sample plot (Fig. 2.18). In rough terms a separation of a distance of 2 – 3 SD of species turnover along the DCA ordination axis corresponds to a near-complete turnover of community composition of the sample (i.e. change in species composition in samples). Accordingly, the long ordination axis lengths seen here correspond to major variation in plant community composition. Groups tended to be separated with high to very high eigenvalues in the TWINSPAN analysis, again indicative of substantial differences in plant assemblage between the sample groups (Fig.2-9, 2-10, Appendix 1 page 107). In total, 7 vegetation sample groups were identified by TWINSPAN at level 2 of the classification, and an 8^{th} group contained samples entirely lacking vegetation. Indicator species were identified for each sample group containing plants. Also it is worth mentioning that group E and F have no indicator species but what we would call it preferential species and see if any of those come up as being "next best" characteristic species for the groups, and we found two of them, one for each.

Furthermore in sample group G the group is a small one which does not further subdivide below level 11, so there is no corresponding division-group for this one at any subsequent hierarchical level. All the others are the product of divisions a level lower than this: so A is coded 000, B 001, C 010, D 011, E 100 and F 101, and that analysis also provided evidence for separation of species assemblages supported by the sample groups. Results of one way ANOVA comparisons (or non-parametric Kruskal-Wallis Test, for non-normal variables) between TWINSPAN-defined sample groups for individual environmental and vegetation variables are shown in Table 2-2 and outlined individually below.

Table 2-2: Characteristics of TWINSPAN sample groups, showing sample-group indicator species, vegetation state variables and environmental variables.

Variables	A	B	C	D	E	F	G	H	P
Number of Samples	6	75	66	12	23	9	4	5	
Eigenvalue	0.79	0.79	0.828	0.828	0.583	0.583	0.716	_	
Indicator Species	*Convolvulus cephalopod*	*Haloxylon salicornicum*	*Cyperus conglomeratus*	*Zygophyllum qatarense*	*Fagonia bruguieri*	*Pennisetum divisum*	*Heliotropium bacciferum*	no plants present	
	Trigonella stellata	*Stipagrostis plumosae*	*Rhanterium epapposum*	*Salsola imprecata*			*Cistanche tubulosa*		
	Gynandriris sisyrinchium								
	Pulicaria undulata								
Vegetation state Variables									
Plant Height (cm)	4.80 (2.69)b	14.44 (13.06)ab	14.21 (10.74)ab	23.08 (10.38)a	2.53 (2.45)b	16.78 (24.77)ab	19.40 (23.32)ab	Not Availabel	<0.001
Species Diversity (S)	1.0769 (0.6128)ab	1.1873 (0.3872)a	1.0003 (0.3175)ab	0.9776 (0.2717)b	0.9940 (0.3475)ab	1.2832 (0.4197)ab	0.8959 (0.2341)ab	Not Availabel	?0.017
Haloxylon salicornicum (%F)	0	26.44 (26.28)a	0	0	0	0	0.06 (0.13)b	Not Availabel	?0.050
Cyperus conglomeratus (%F)	0	8.53 (27.34)a	53.08 (49.55)b	0	0	0	0	Not Availabel	<0.001
Rhanterium epapposum (%F)	0	14.20 (36.39)b	52.73 (49.86)a	0.33 (1.15)b	0	0	0	Not Availabel	<0.001
Environmental Variables									
Oil Damage Score	1a	2 ab	3b	1a	1a	1a	1a	1a	<0.001
P-moist Soil (%wt)	4.348 (1.907)ab	3.789 (3.643)b	2.408 (2.145)b	5.041 (3.723)ab	7.438 (3.689)a	2.480 (2.076)b	1.237 (1.985)b	1.665 (0.741)b	<0.001
Chrysene (CH) (µg/Kg)	0	3.3 (8.9)b	50.6 (162.9)ab	5.7 (14.3)a	0	0	0	0	?0.037
Fluoranthene (FLAN) (µg/Kg)	0	0.20 (0.73)a	11.84 (44.61)b	0	0	0	0	0	?0.030
Pyrene (PY) (µg/Kg)	0	0.80 (2.91)b	32.91 (99.98)a	1.44 (4.14)ab	0	0	0	0	?0.017
Phenanthrene (PH) (µg/Kg)	0	1.73 (5.37)	15.05 (70.35)	3.97 (12.66)	0.23 (0.44)	0	0	0.21 (0.43)	n.s.
Anthracene (ANTH) (µg/Kg)	0	0.0167 (0.1032)	0.0412 (0.2255)	0.1027 (0.3407)	0	0	0	0	n.s.
P.T.A Anthracene (ANTH) (µg/Kg)	Not Detected	1.638 (2.261)b	5.364 (5.719)a	3.320 (0.141)ab	Not Detected	Not Detected	Not Detected	Not Detected	?0.004
P.T.A Benzo(a)anthracene (B[a]A) (µg/Kg)	Not Detected	0.096 (0.383)b	1.301 (1.502)a	0.782 (1.106)ab	Not Detected	Not Detected	Not Detected	Not Detected	<0.001
P.T.A Fluoranthene (FLAN) (µg/Kg)	Not Detected	38.164 (6.413)b	47.903 (9.292)a	36.200 (4.950)ab	Not Detected	Not Detected	Not Detected	Not Detected	<0.001
P.T.A Fluorene (FL) (µg/Kg)	Not Detected	11.01 (4.28)b	68.93 (91.69)a	5.94 (0.54)ab	Not Detected	Not Detected	Not Detected	Not Detected	?0.002
P.T.A Naphthalene (NA) (µg/Kg)	Not Detected	20.703 (2.936)b	30.923 (10.762)a	18.750 (2.899)ab	Not Detected	Not Detected	Not Detected	Not Detected	<0.001
P.T.A Pyrene (PY) (µg/Kg)	Not Detected	3.1292 (0.1588)b	3.2828 (0.2145)a	3.0057 (0.1398)ab	Not Detected	Not Detected	Not Detected	Not Detected	?0.003
P.T.A. Chrysene (CH) (µg/Kg)	Not Detected	5.983 (3.726)a	6.524 (2.962)a	0 b	Not Detected	Not Detected	Not Detected	Not Detected	?0.034
P.T.A P-moist (%wt)	Not Detected	51.039 (11.750)b	69.461 (5.266)a	76.000 (2.121)ab	Not Detected	Not Detected	Not Detected	Not Detected	<0.001

Values are given as mean and standard deviation (in parentheses). In Groups E and F there are no indicator species those are preferential species "next best" characteristic species for the groups (see Appendix 1 page 107).

2.4.2.1 Comparison of species assemblages occurring in TWINSPAN-defined sample-groups

The species classification produced by TWINSPAN (and shown overlain on the species ordination plot in Fig. 2.19) suggested evidence for four main plant assemblages existing in samples collected from within the study areas.

Assemblage I was found in samples from both the north and south of Kuwait (Burgan, Um Al-Aish, Um-Ghadair oil field and Sabriya oil field). It was characterized by both *Cyperus conglomeratus* and *Rhanterium epapposum*, often growing in very close proximity to severe oil contamination (scoring 2 and 3 in the visual parameter of the ODS, and associated also with the presence of the other halophytes like *Salsola impricata* and *Zygophyllum qatarense*, as well as *Tamarix aucheriana*. This assemblage was characteristic of TWINSPAN sample group C (*Cyperus conglomeratus* and *Rhanterium*

epapposum) and D (for which *Zygophyllum qatarense* and *Salsola imprecata* was an indicator species).

Assemblage II (occurring mainly in areas in north of Kuwait like Bahra, Sabah Alahmad Protected Area, Um-Ghadair oil field and Um Al-Aish) tended to be in oil damaged sites and contaminated soils with high levels (reaching 140 µg/kg for some of the PAHs like chrysene) of polycyclic aromatic hydrocarbon (PAH). It was characterized by *Haloxylon salicornicum*, together with *Trigonella stellata, Convolvulus cephalopoda, Gynandris sisyrinchium, Pulicaria undulata,* and *Stipa capensis.* This assemblage was characteristic of TWINSPAN sample-groups A and B, where A has *Convolvulus cephalopoda, Gynandris sisyrinchium, Trigonella stellata* and *Pulicaria undulata* for indicator species and B has *Haloxylon salicornicum, Stipagrostis plumosa*.

Both assemblages III and IV (perhaps better considered as variants of each other, and mainly found in Sabah Alahmad Protected Area and the Burgan area where there is a less impacted samples) supported the species *Pennisitum divisum* and *Fagonia bruguieri*, together with associates such as *Heliotropium bacciferum* and *Citrullus colocynthis* (Fig. 2-9, 2-10). *Fagonia bruguieri* was a preferential species for sample group E, and *Pennisitum divisum* was a preferential species for TWINSPAN sample group F, while *Heliotropium bacciferum* and *Cistanchus tubulosa* for group G. The occurrence of the main assemblages was less clearly separated for sample –groups A and E.

2.4.2.1 Oil Damage Score compared between TWINSPAN sample-groups

When applying Kruskal-Wallis Test, the results show that mean ODS score for two groups (B, C) were significantly different from the other 6 sample groups. Most of the samples making up B and C are from impacted areas with oil pollution (Bahra oil lake spill (also known as Sabriya Oil Lake), Um Al-Aish oil field, Sabriya oil field, Um-Ghadair oil field, Burgan oil field), while the other sample groups came from less impacted areas like Sabah Alahmad Protected Area (Fig. 2-19).

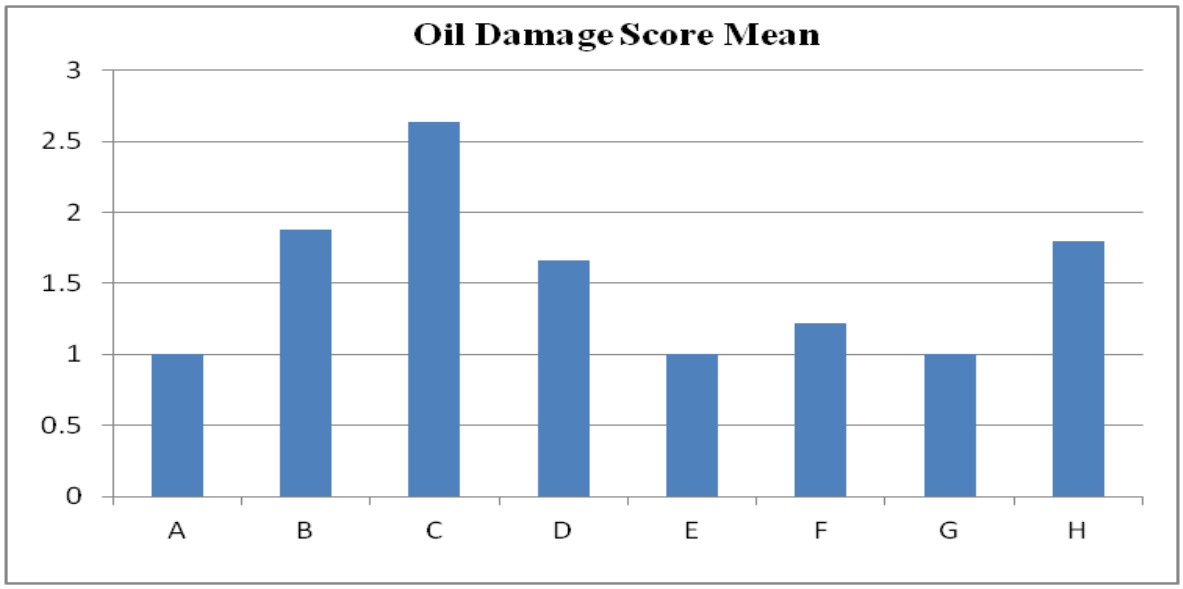


Figure 2-19: Oil Damage Score compared between TWINSPAN Groups. Number of samples per group (A=6, B=75, C=66, D=12, E=23, F=9, G=4, and H=5), where (#1=low or invisible contamination, #2=moderate visible contaminant and #3=high visible contaminant).

2.4.2.2 Species Diversity compared between TWINSPAN sample-groups

The mean plant species diversity per quadrat did not differ greatly between the sample-groups (Fig. 2-20). The low values (nearly three species for the best group) are typical of desert plant communities. The results suggest that oil-tolerant species (provisionally considered to be plants like *Haloxylon* and *Cyperus*) tend to replace pollution-intolerant species in damaged areas, hence maintaining the average species diversity, some 23 years after the pollution events occurred (Fig. 2-21).

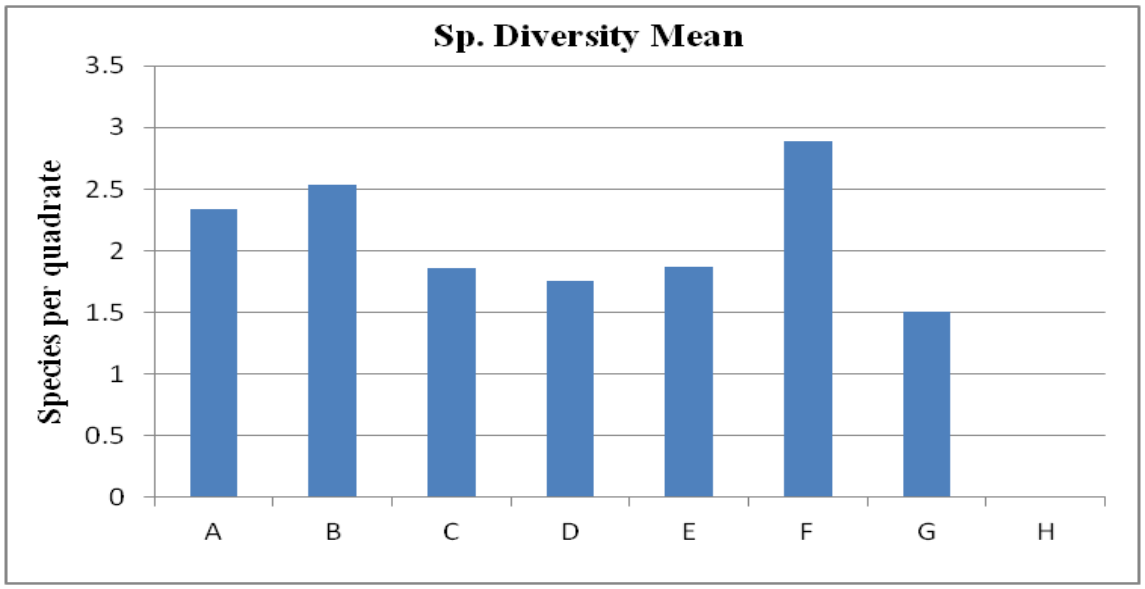


Figure 2-20: Species diversity per quadrat compared between TWINSPAN groups. Number of samples per group (A=6, B=75, C=66, D=12, E=23, F=9, G=4, and H=5).

Figure 2-21: Dr. Kevin Murphy and Sarah Alateeqi working in the Sabah Alahmad Protected Area and identifying species, Feb. 2011(Al-Musawi 2011)

2.4.2.3 Plant height compared between TWINSPAN sample-groups

Surprisingly, the mean plant height of Group D (23.8 cm), samples, mainly drawn from Burgan oil field, is significantly greater than in other sample-groups. Groups A (mean height 4.8 cm) and E (mean height 2.5 cm) of the sample-groups had the shortest–growing vegetation: mainly representing Sabah Alahmad Protected Area and Um Al-Rros Military Base (Fig. 2-22).

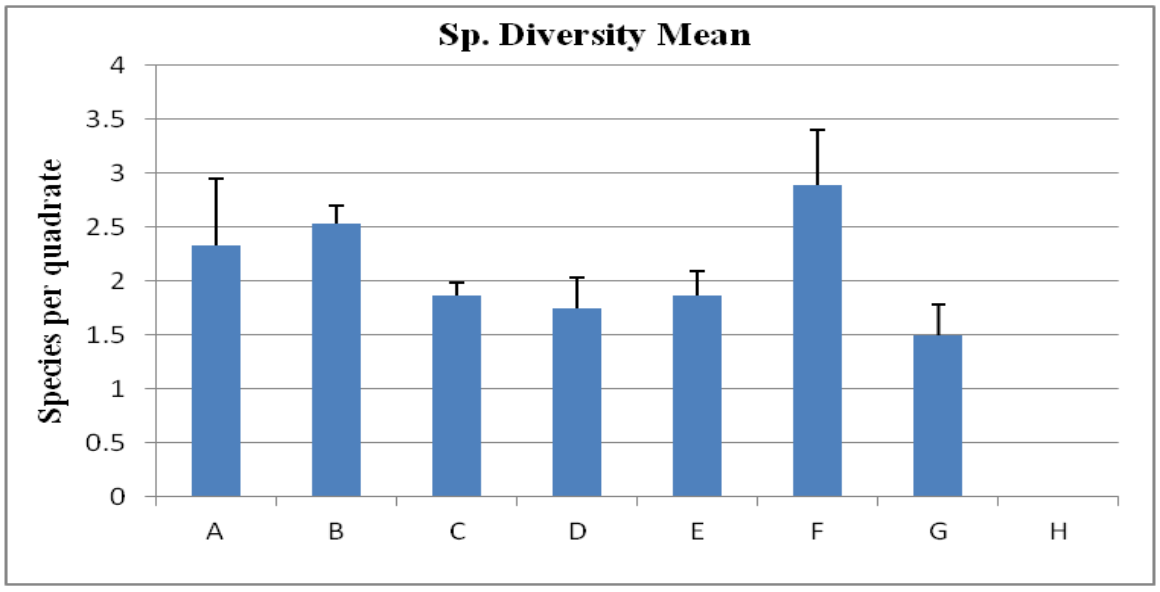


Figure 2-22: Mean plant height (cm) compared between TWINSPAN groups, number of samples per group (A=6, B=75, C=66, D=12, E=23, F=9, G=4, and H=5).

2.4.2.4 Abundance of *Haloxylon salicornicum* compared between TWINSPAN sample-groups

Sample-group B, mainly drawn from Bahra oil lake spill, Um Al-Aish oil field, Sabriya oil field, and Um-Ghadair oil field, contained all the samples that have *Haloxylon* populations in them (Fig. 2-23).

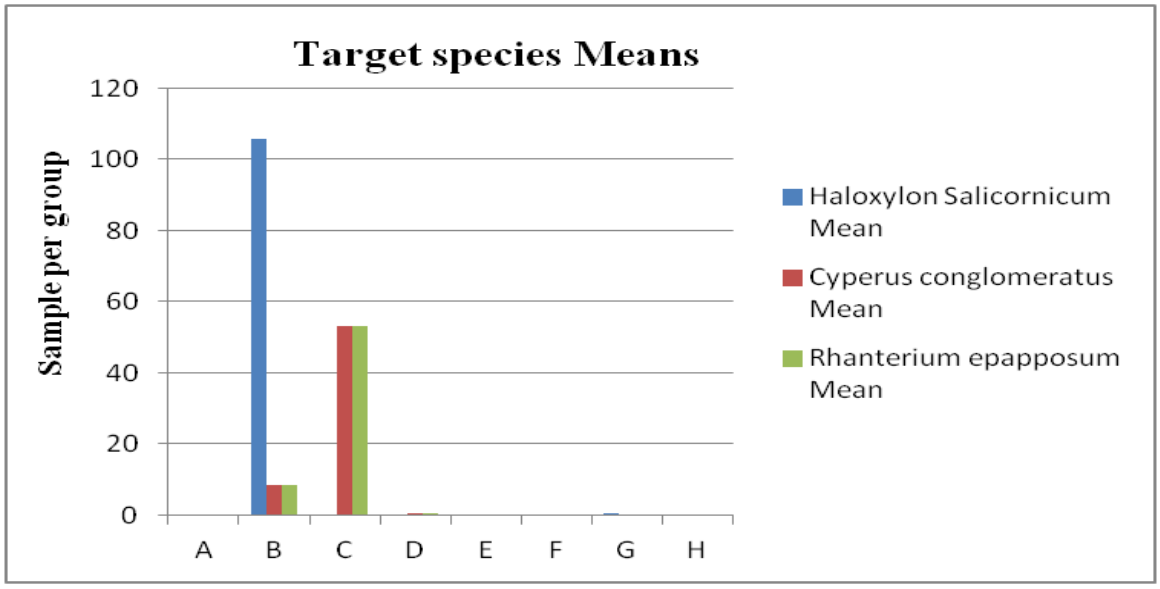


Figure 2-23: *Haloxylon salicornicum* occurrence compared between TWINSPAN groups, vertical axis is number of samples per group.

2.4.2.5 Abundance of *Cyperus conglomeratus* compared between TWINSPAN sample-groups.

Group C (samples from Bahra, Sabah Alahmad Protected Area, Burgan, Sabriya and Um-Ghadair oil fields) is where *Cyperus conglomeratus* was mainly found. However, the plant was also present in some Group B samples (from Bahra, Sabriya, Um-Ghadair and Um Al-Aish). Although this species can be found throughout Kuwait, it preferentially occurs in the south (Brown 2003) (Fig. 2-23).

2.4.2.6 *Rhanterium epapposum* occurrence compared between TWINSPAN sample-groups

Group C (with samples from Bahra, Sabah Alahmad Protected Area, Burgan, Sabriya and Um-Ghadair oil fields) contained all the *Rhanterium epapposum* populations sampled (Fig. 2-23).

2.4.2.7 Soil PAH content compared between TWINSPAN groups

Chrysene (CH), Pyrene (PY), Fluoranthene (FLAN) and Benzo (g, h, i) perylene (B[ghi]PERY) were the predominant PAH compounds found in the soil sample analyses carried out in this study, with sample groups C (Bahra, Sabah Alahmad Protected Area, Burgan, Sabriya and Um-Ghadair oil fields) and Group B (Bahra, Sabriya, Um-Ghadair and Um Al-Aish) being the most heavily polluted (Fig. 2-24).

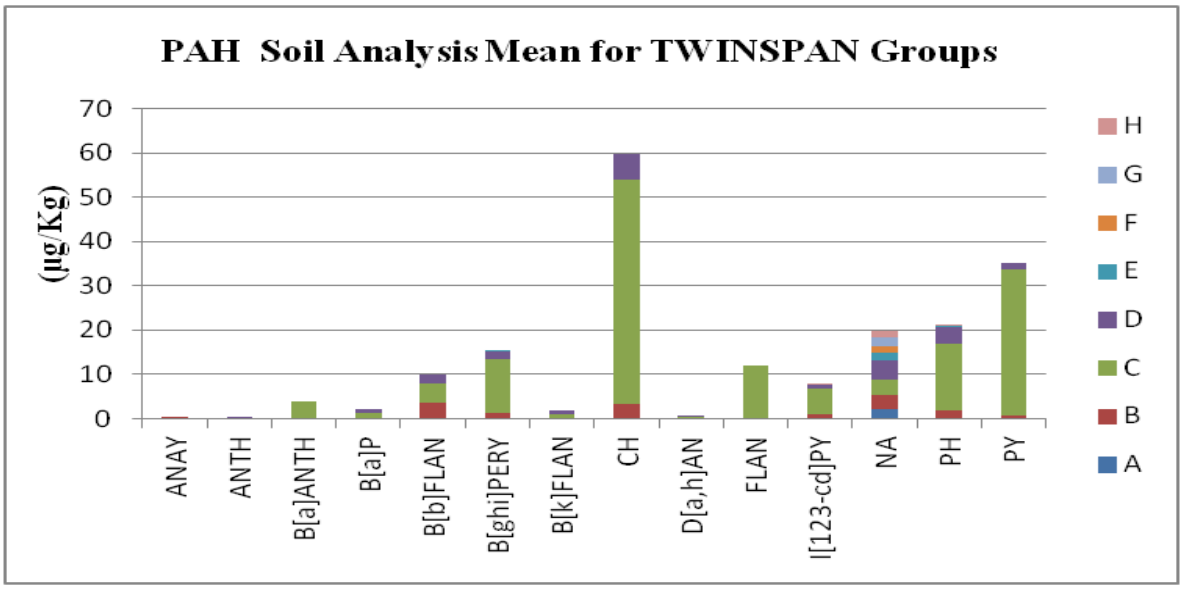


Figure 2-24: TWINSPAN sample group content of soil PAHs (Note: Acenaphthylene (ANAY); Anthracene (ANTH); Benzo(a)anthracene (B[a]ANTH); Benzo(a)pyrene (B[a]P); Benzo(b)fluoranthene (B[b]FLAN); Benzo(g,h,i)perylene (B[ghi]PERY); Benzo(k)fluoranthene (B[k]FLAN); Chrysene (CH); Dibenz(a,h)anthracene (D[a,h]AN); Fluoranthene (FLAN); Indeno(1,2,3-cd)pyrene (I[123-cd]PY); Naphthalene (NA); Phenanthrene (PH) and Pyrene (PY)) (see Appendix 1 page 107).

2.4.2.8 Plant Tissue Analysis (P.T.A) of PAHs compared between TWINSPAN groups B and C

Phenanthrene (PH) was the most abundant PAH chemical with 96.94 µg/kg mean in plant tissues analysed from plant populations occurring in both groups B and C, the sample groups in which suggested possible candidate phytoremediator species occurred. Next was Fluorine (FL) with a 68.92 µg/kg mean in Group C (samples from Bahra, Sabah Alahmad Protected Area, Burgan, Sabriya and Um-Ghadair oil field, consisting of *Cyperus conglomeratus* (18 specimens) and 13 of *Rhanterium eppaposum*, followed by Fluoranthene (FLAN). Fluoranthene (FLAN) is high too for Group B with 38.16 µg/kg (Bahra, Sabriya, Um-Ghadair and Um Al-Aish), with 32 for both *Haloxylon Rhanterium* plants analysed here.

Although Chrysene (CH) is very high in the soil with a mean of 4.16 µg/kg (see Section 2.5.2.7), apparently the plants do not take it up, and there is also little evidence for uptake of Benzo (b) fluoranthene B (b) Flan, or Pyrene (PY). The plants do take up Phenanthrene (PH) especially in plants forming sample groups Group C and D with a mean of 99.07 µg/kg. Fluoranthene (FLAN) shows up especially in Group C. Although Naphthalene (NA), Benzo (a) anthracene (B[a]ANTH) and Anthracene (ANTH) were not detected as abundant in the soil analysis, the plants seem to take up these compounds quite readily (Fig. 2-25).

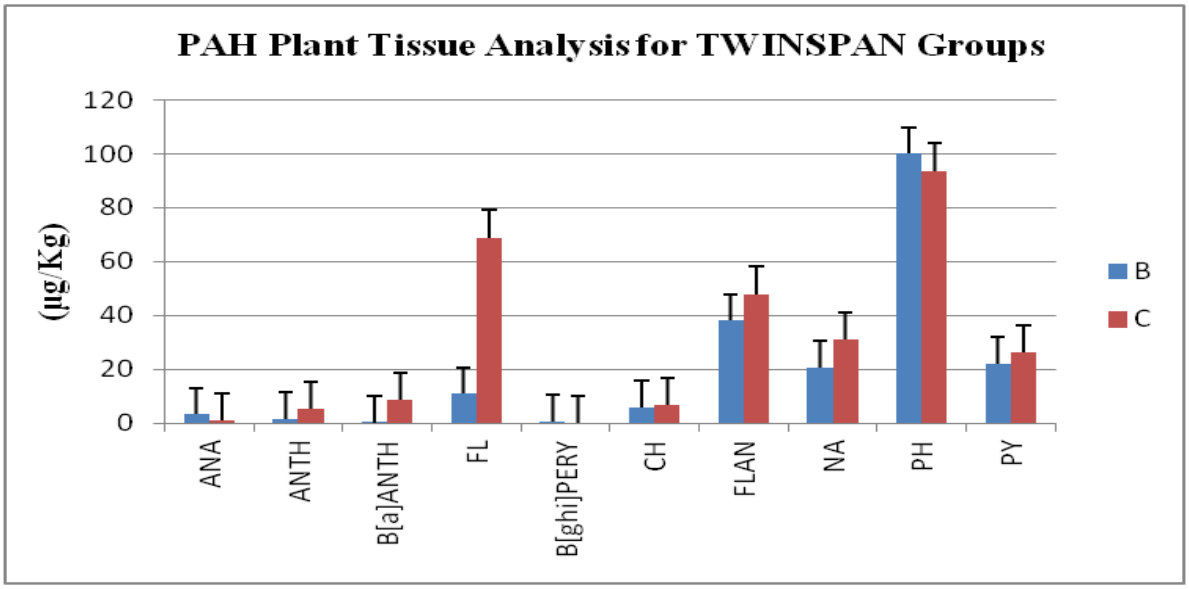


Figure 2-25: **Polycyclic Aromatic Hydrocarbon (PAH) content in plants found from Plant Tissue Analysis (P.T.A) in TWINSPAN sample-groups B and C (Acenaphthene (ANA); Anthracene (ANTH); Benzo(a)anthracene (B[a]ANTH); Benzo(g,h,i)perylene (B[ghi]PERY); Chrysene (CH); Fluoranthene (FLAN); Fluorine (FL); Naphthalene (NA); Phenanthrene (PH) and Pyrene (PY)) (see Appendix 1 page 107).**

2.4.2.9 Moisture content of plant tissues compared between TWINSPAN sample-groups

Plants occurring in sample-group D (two *Cyperus conglomeratus* specimens taken from Burgan oil field, and 20 *C. conglomeratus* from Um Al-Aish) had the highest tissue moisture content. This was followed by group C (Burgan oil field and Sabah Alahmad Protected Area, both with 3 specimens of *Cyperus conglomeratus;* while Sabriya oil field provided another 13 of the same species). Finally comes group B, with samples from Bahra oil field (five *Haloxylon* specimens, plus another 32 from Um Al-Aish; and one *Cyperus* plant from Sabah Alahmad Protected Area (Fig. 2-26).

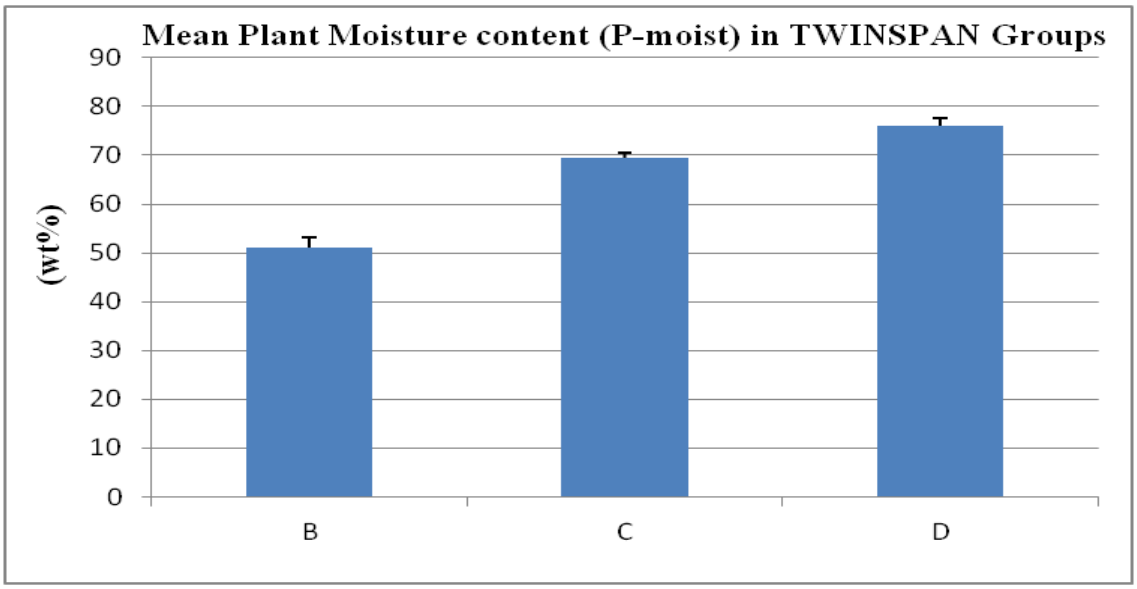


Figure 2-26: **Plant tissue percentage moisture content compared between three TWINSPAN groups. The mean P-Moist in soil for the groups above is (B=3.78 wt%, C=2.40 wt%, and D=5.04 wt%). Number of samples per group (B=75, C=66, and D=12) (see Appendix 1 page 107).**

2.4.2.10 Percentage of moisture in oil contaminated soil compared between TWINSPAN sample-groups

The moisture content in the soil of samples forming Group E is higher than any of the other groups. Group E comprised samples from Sabah Alahmad Protected Area and Um Al-Rros Military Base. Second is Group D, with samples from Burgan oil field (clean area plus an area near a fresh oil lake), also Um Al-Aish samples from near a fresh oil lake. The few Sabah Alahmad Protected Area samples were in Group A. Although the samples were from the same mentioned areas (Sabah Alahmad and Burgan) in Groups G and H, they have the least water content of them all (Fig. 2-27).

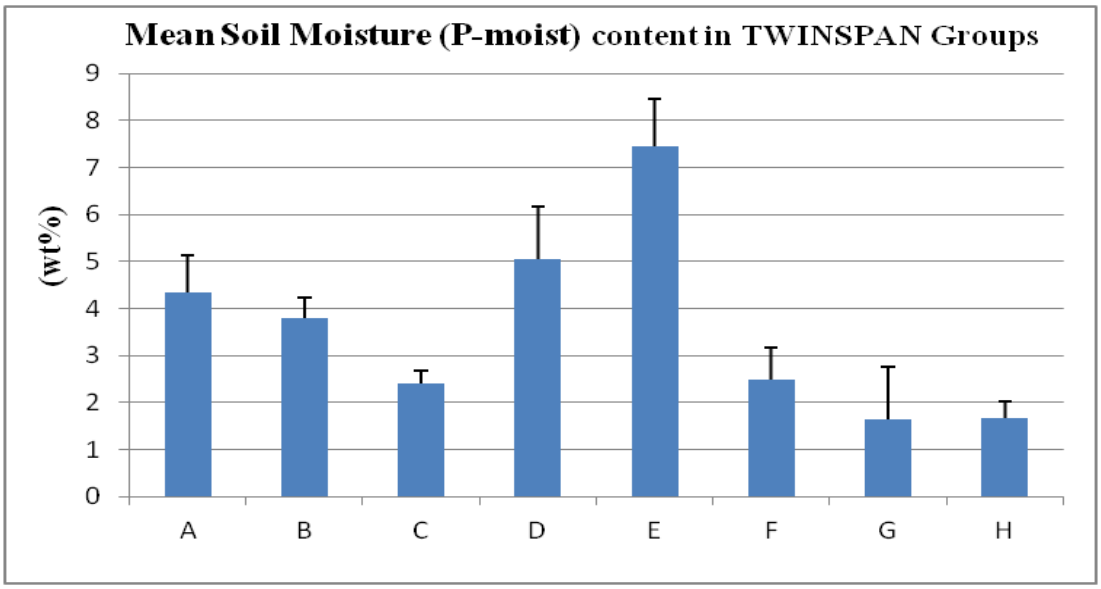


Figure 2-27: **Percentage soil moisture values compared between TWINSPAN groups. Number of samples per group (A=6, B=75, C=66, D=12, E=23, F=9, G=4, and H=5) (see Appendix 1 page 107).**

2.5 Discussion and Conclusion

In my M. Res. thesis (Al-Ateeqi 2010) I pointed out the need to assess the possibility of finding additional native flora that might be potential phytoremediator species, other than *Haloxylon salicornicum*, which appear able to survive in oil-polluted soils in Kuwait, to be used in oil pollution clean-up operations. In this chapter, I have presented evidence from field surveys of plant communities and environmental conditions to suggest that *Haloxylon salicornicum* does indeed appear to be the prime candidate for this role, but additional candidates include *Cyperus conglomeratus* and *Rhanterium epapposum*. All three, though especially *Haloxylon*, were present in different levels of heavy oil contamination including wet/dry oil lakes, where the first two assemblages from the TWINSPAN showed the proximity of those species to the weathered petroleum hydrocarbon, which speaks volumes about how strong those species are.

According to Meng (2011) there is still little known about the phytoremediation process of removing the PAHs and how the cropping patterns and the plant species affect that process. So the questions here are: are those plants blocking out the toxicity in those contaminants? Do they break them down? Do they break them down and block them out of their system? Can the plants absorb them as they are without breakdown? If so, what is the mechanism? And if it didn't how does it survive PAH toxicity? Are there specific mycorrhiza that are working with the plant's root system to help survival? If there is what are they? Are there stress-tolerant genes in those plants? These and more questions need to be answered, and we get close to answering some of them; but still there is a need to do

more and search for more precise answers since these types of studies are still at the beginning.

In order to know some of the answers to the questions above it is important to understand that the plant species may differ in their abilities to metabolize and uptake the PAHs through the root system, which will be either by transpiration, enzymatic pathways, or adsorption (Harvey 2002). Therefore, it is important to select the plant that we are going to use for the phytoremediation because it is an

> "important parameter to optimize for the phytoremediation of PAH-contaminated soils"(Meng 2011)

The idea of using a suitable phytoremediator is not just discussed and researched on terrestrial land, but also in the marine sediment after using e.g. eelgrass (*Zostera marina*) for 60 weeks extensively improved the removal of the PAHs from the sediment (Huesemann 2009).

Bient (2000) suggests that the dissipation of the contamination would increase if the plant growth can be established and maintained in those areas, so the idea here is to do the same thing in our polluted sites since we have the seeds and the means to grow all three species. Also, Joner (2001) says that polluted areas mostly either have very few plants or none at all; we have seen both conditions in our case in this study, where the pollution damaged or left few of the native plants alive. Therefore, Joner (2001) recommends re-planting the damaged areas again in order to reduce the wind erosion and/or to enhance the degradation. Harris (1996) concurs: even if the success of this method would be limited or none at all, it will still protect the soil from surface water run-off, increase water infiltration and reinforce the soil by the root system, and enhance the area's aesthetics, where the physical process is frequently depicted as phytostabilisation (Smith 2006). Likewise, Smith (2006) also believes that the best way to undertake phytoremediation is by using native plants tolerant of the soil conditions in the damaged area. In accordance with all the above experts' opinions, my study proposes the utilization of native species to remediate damaged desert ecosystems in Kuwait, and further investigate this approach experimentally.

In heavy metal pollution studies, it has been well documented (Belimov 2001; Burd 2000; Sheng 2006; Wu 2006) that using a multispecies mixture can improve the removal of heavy metal contaminant from the soil compared to using a monoculture, as multispecies

mixture will provide better rhizosphere conditions that will enhance the degradation and the bioavailability of the contamination (Wenzel 2009). Since we have 3 different species from 3 different families that can be considered potential phytoremediators, this approach seems promising. According to Meng (2011), there are very few studies that actually discuss the influence on PAHs removal using the multicropping approaches. Furthermore, those few studies produced conflicting results, and hence the results cannot be generalized. Smith (2006) reasons that such conflicting results are due to the ways each study was conducted; in the future it will be useful to utilize multispecies mixture of all three species, namely *Haloxylon salicornicum, Cyperus conglomeratus* and *Rhanterium epapposum*, to assess their efficiency in petroleum hydrocarbon removal. Given that every situation has its own variables that would increase or decrease the efficiency of any technique in that matter, the multispecies mixture approach would provide a good tool to simulate the natural recovery already seen in the field.

Because there is an array of factors that will need to be assessed and analysed, we will start by focusing on one species at a time, therefore the focus of this study will be on *Haloxylon*, as it is the strongest and the most abundant species in this survey we have undertaken so far, which also the most important finding up till now. In addition, it was obvious from the plant tissue analysis that *Haloxylon* plants can actually contain some of the Polycyclic Aromatic Hydrocarbons (PAH). This result further supports the basic idea of this project. The PAH contamination could be absorbed through the surface of plant leaves (Slaski 2000), or stem surface or the root system. In this study, the *Haloxylon* might actually be absorbing most of the PAH contaminants from the stem, as the amount of chrysene (CH) for example is higher in the plant than it is present in the soil, which means that the plants might absorb or accumulate this type of PAH on its surface more than uptaking it through the root system. Further studies are needed to determine the uptake mechanism of PAH contamination in these desert species.

3 EXPERIMENTAL STUDIES: *H. SALICORNIUM* OIL CONTAMINATION TOLERANCE

3.1 Background on phytoremediation

Phytoremediation (Greek: *phyton* = plant; Latin: *remediare* = remedy) is emerging as a green bioengineering technology that uses plants to remediate environmental problems. Ramos (2009) states that phytoremediation is a very successful way to remove contaminants from the soil directly or indirectly, as it can be applicable to different types of pollutants. Others, like Harley (1990) and Ekundayo (2000) have investigated the ability of plants like *Echinochloa polystachya* to absorb many pollutants. It is very important to find plant species that can act as phytoremediators for differing forms of soil and water pollution (Merkl 2004).

Both aquatic and terrestrial plant species suitable for environmental restoration and phytoremediation purposes tend to have certain features in common. Notably, they are stress tolerators, which tend to

> "Thrive in very harsh environmental conditions of soil and water; absorb, tolerate, transfer, assimilate, degrade and stabilise highly toxic materials (heavy metals and organics such as solvents, crude oil, pesticides, explosives and polyaromatic hydrocarbons." (Sinha 2007).

Such plants can survive harsh conditions by using one of two strategies: by being excluders or accumulators. Excluders basically take up the contaminant(s) within their biomass, and tolerate its effects on their physiology. Accumulators also take up the contaminant(s), on temporary bases, accumulate it in the tissues, and then transform, or biodegrade, or store the contaminant(s) in the non-active tissue of the plants (Sinha 2007). Relatively few plants can tolerate this kind of soil contamination, as was shown for example by Taser (2002), who observed a huge reduction in ryegrass biomass after nearly a 30 period day growing in oil-contaminated soil.

Phytoremediation techniques have produced somewhat contradictory results. Reports regarding their efficiency in solving soil pollution suggest that the key to resolving such issues is to use a native plant that can tolerate high oil contamination in the soil e.g. Joner (2004) and Zand (2010). In this study, such a plant is *Haloxylon salicornicum*.

3.1.1 Effects of petroleum hydrocarbons on plants and finding native plant phytoremediator species in Kuwait

After the First Gulf War (1991), it became crucial to find appropriate procedures to clean up total petroleum hydrocarbon (TPH) contamination of desert soils of Kuwait, in an environmentally friendly way.

> In a study by Balba (2003) the phytoremediation process for Kuwaiti soil was described as a "...polishing method to further reduce the residual level of TPH in the treated soil and to assess phytotoxicity of residual TPH on the growth and performance of a wide range of domestic and ornamental plant species."

Balba (2003) investigated the impact of cultivation using phytoremediator species on pollutant content of Kuwaiti soil. In Kuwait, three types of treatment were primarily being used to treat TPH-contaminated soil: composting piles, landfarming, and bioventing of soil piles (with irrigation and bioventing). The study used several domestic and ornamental plants to enhance the removal of the PAHs (Balba 2003). Only more recently has phytoremediation using native Kuwaiti plants come into the picture. Since there was no studies focusing solely on native plants and how strong they can be to tolerate weathered oil contamination, it is time to focus on one and start to study it thoroughly, or as best we can at this point.

3.2 Using target phytoremediator species under controlled and field experimental conditions, to assess plant growth in weathered crude oil contaminated soil and clean soil.

3.2.1 Introduction: halophytes and *Haloxylon salicornicum*

> Halophytes are defined as "plants which are able to live under elevated salinities in their growth media**"** (Web site #12 2012).

The halophytes are plants which have evolved from terrestrial ancestors (living in non-marine conditions) to tolerate high sodium chloride concentrations in the soil and water.

Several different strategies have evolved to allow plants to tolerate those harsh conditions. For example, when the plants exist on foreshore habitats (e.g. forward dune and cliffs) they utilize the processes of secretion and exclusion of salts as their main survival strategy. However, when plants are rooted in salty water (for example in a salt marsh), such

mechanisms are less successful and the plant exists by maintaining its internal osmotic conditions in equilibrium with the salt concentration in the soil water (Crawford 1989). These two main approaches to living in salty conditions are classified physiologically into two groups: osmoregulators and osmoconformers, where the halophytes are the former (Wyn Jones 1983). There are two types of halophytes (hydro-halophytes and xero-halophytes): the former is typified by the mangroves, which can live in the sea, while the latter can grow in salty soils where water is very scarce like sabkha in desert areas. *Haloxylon salicornicum* is an example of the latter type.

Haloxylon salicornicum (synonym: *Hammada salicornica;* Rimth in Arabic) is a member of the Amaranthaceae family. It is a succulent undershrub (which acts as an efficient sand-binder). It lives in a range of different types of desert habitats, including sandy plains, wadi terraces, and gravel desert (Al-Qurainy 2007).

According to Al-Qurainy (2007) this species is the most common plant in the sandy desert of Kuwait, with a good ability to resist overgrazing. Ghazanfar (2010) also pointed out that it dominates where the sand is abundant. It has been considered most promising for rehabilitation and dune fixing purposes, as well as a good food source for range animals (Brown 2003). It's been estimated by Aldawsari (2014) that it can hold up to 4.9 m^3 of sand underneath it, making it an excellent plant to keep sand from drifting, which can damage/cover the roads. The need to remove drifting sand in Kuwait costs huge amounts of money, which can reach several hundred thousand Kuwaiti dinars in one year (Al-Dawsari 2014). Furthermore, Halwagy (1982) mentions that the soil humidity underneath *Haloxylon* is higher than that underneath *Rhanterium epapposum*, while the latter has higher soil humidity than *Cyperus conglomeratus.*

As part of the present study a small scale phytoremediation experimental program was undertaken in Kuwait comprising first a controlled-conditions block experiment in a greenhouse (60 specimens of one year old *Haloxylon*), and second field experiments in the following year, conducted in two areas, one in the north of Kuwait (Bahra) and the other in the south (Burgan area), utilizing two-year old *Haloxylon* plants (60 specimens for each site). In each location, 30 specimens were planted at ten points along a row, with 3 replicates planted into weathered contaminated soil of an old dry oil lake, and with a second set of specimens similarly planted into nearby clean soil as a control.

3.2.2 Aims of experiments

(I) To establish experimentally the tolerance of *Haloxylon salicornicum* for growth in desert soil contaminated with petroleum hydrocarbons.

(II) To assess factors such as ease of handling for operational purposes, and requirements for any environmental amendments(s) to assist plant growth.

(III) To assess the effectiveness of the plants in assisting clean-up operations.

3.2.3 Methodology

3.2.3.1 Growth experiment in controlled greenhouse conditions

Weathered crude oil-contaminated soil was collected from Bahra oil field (latitude 29˚ 37.013' to 29˚ 37.084' N; longitude E 047˚ 54.585' to E 047˚ 54.703'). To prepare for the experiment a cultivated plants consisted of one year old specimens, grown prior to the experiment in clean soil were used. Then the contaminated soil was weighed and mixed with an appropriate amount of clean soil (with Perlite for aeration), for each treatment, before placing in the pots. Three *Haloxylon* plants were planted in each 30 cm pot, with 5 treatments consisting of 100% weathered crude oil-contaminated soil; 25% clean soil plus 75% contaminated soil; 50% contaminated and 50% clean soil; 25% contaminated soil and 75% clean soil; and 100% clean soil. There were four blocks, each with those 5 treatments.

A block experiment was conducted in Alardyia Greenhouse, Public Authority for Agriculture and Fisheries Affairs (PAAF). The experiment began on 25/01/2012, with a watering regime that consists of 200 ml water, provided for all of them before the beginning of the experiment to make sure that the planted soil was saturated with water as much as possible. Then the plants received 150 ml of water for the first two weeks, then during the experiment the water irrigation was changed to 300 ml on the third week, through to the final week to make sure that the water is sufficient to reach the root system then they received 200 ml water before an hour of the experiment termination (Figs. 3-1, 3-2, 3-3 and 3-4).

Figure 3-1: Block I with different concentrations of contaminated soil pots containing 100%, 75%, 50%, and 25% polluted soil, mixed with clean soil, and clean soil only as a control. Alardyia Greenhouse, Public Authority for Agriculture and Fisheries Affairs (PAAF).

Figure 3-2: Block II with different concentrations of contaminated soil pots containing 100%, 75%, 50%, and 25% polluted soil, mixed with clean soil, and clean soil only as a control. Alardyia Greenhouse, Public Authority for Agriculture and Fisheries Affairs (PAAF).

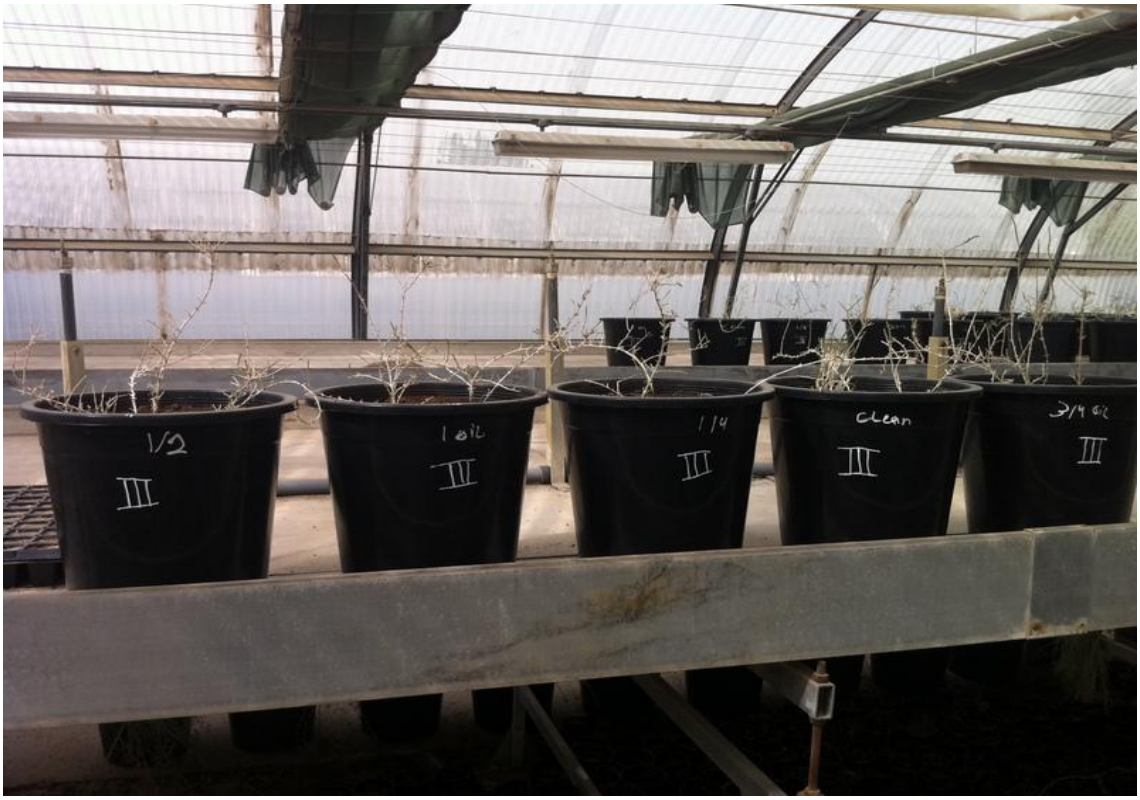


Figure 3-3:Block III with different concentrations of contaminated soil pots containing 100%, 75%, 50%, and 25% polluted soil, mixed with clean soil, and clean soil only as a control. Alardyia Greenhouse, Public Authority for Agriculture and Fisheries Affairs (PAAF).

Figure 3-4: Block IV with different concentrations of contaminated soil pots containing 100%, 75%, 50%, and 25% polluted soil, mixed with clean soil, and clean soil only as a control. Alardyia Greenhouse, Public Authority for Agriculture and Fisheries Affairs (PAAF).

The temperature were measured using a thermometer, along with the humidity by using a hygrometer, and plant height also using a ruler for measurement, and pictures were taken for each block to monitor the plants growth using iPhone 4, Apple (assembled in China), weekly throughout the experiment.

At the end of the experiment on 22/02/2012, then fresh and dry weight was measured (average of the 3 replicas was taken from each pot) using Precision scale Model KERN 572 for all the plants to measure the dry weight and that were made by using a laboratory oven .7 cu .fl, 20L capacity and drying for 12 days at 30^{o}C, and a soil analysis were made for the TPH content in the pots. Furthermore, the soil samples were analysed for TPH (Total Petroleum Hydrocarbon) (Figs. 3-5, 3-6 and 3-7) content at the Kuwait University Lab for Bioremediation Research using the following method:

- 5g of soil was extracted thrice with 10ml aliquots of pentane.
- The 3 extracts were combined and the total volume completed to 20ml with pentane.
- 2µl was injected in a GC (Varian, USA) with:
    - Injector temp. = 270 ^{o}C
    - Detector (FID) temp. = 300 ^{o}C
    - Oven temp. = 45 – 310 ^{o}C (raising 10 ^{o}C/min)
    - Carrier gas = N_2
    - WCOT – fused silica CP-SIL – 5CB capillary column (Varian, USA)

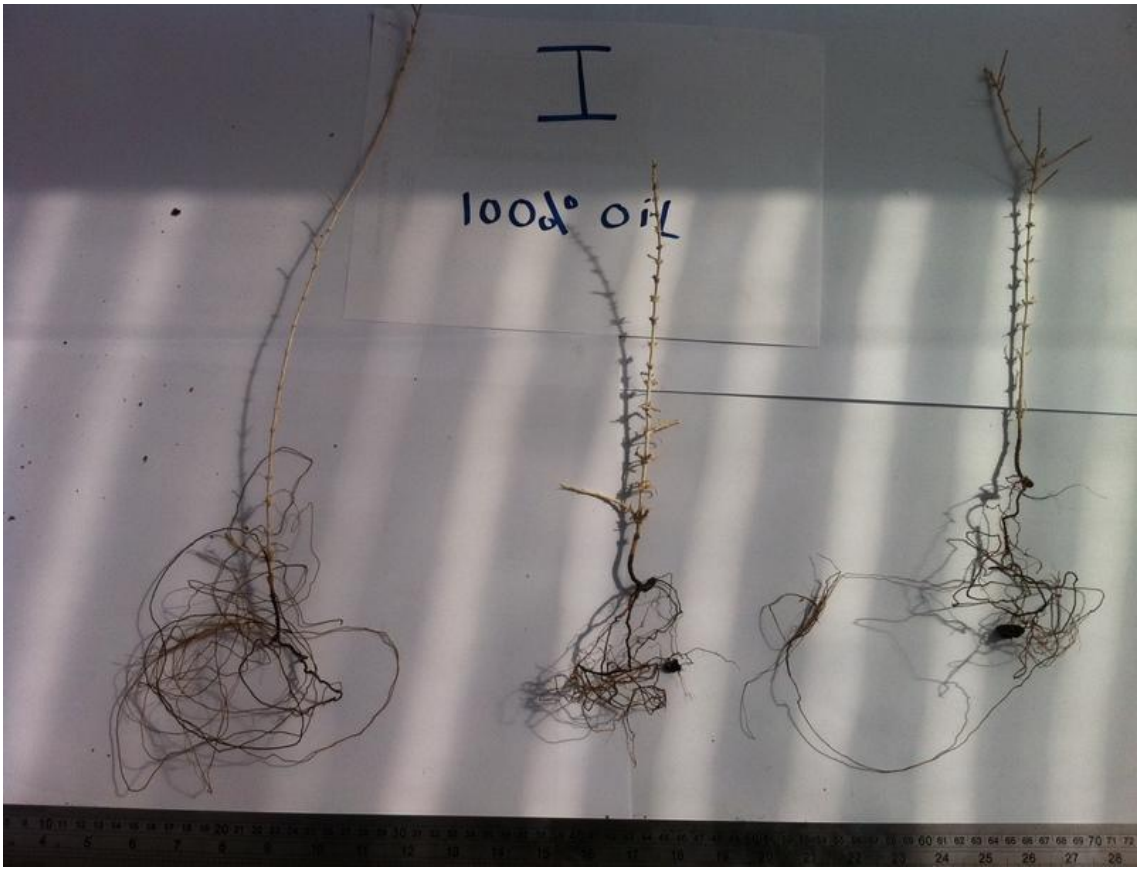


Figure 3-5: Examples of *Haloxylon* removed from contaminated soil at the end of the experiment in 100% oil contamination.

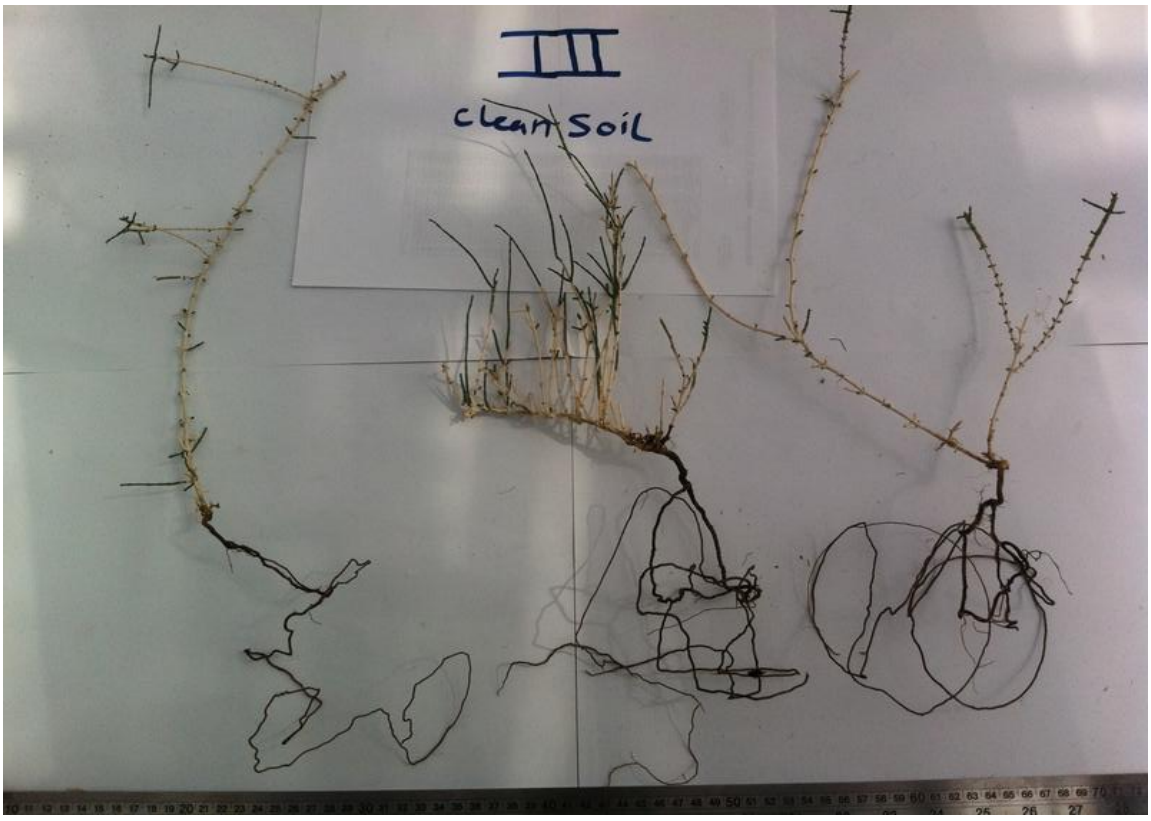


Figure 3-6: Examples of specimens from clean soil at the end of the experiment taken from Block III.

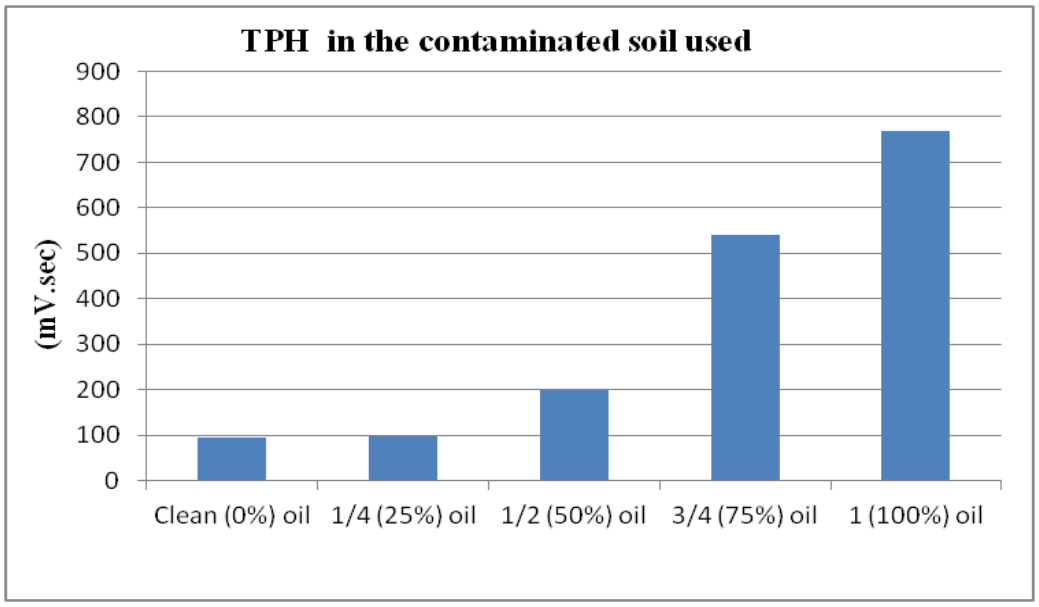


Figure 3-7: The amount of TPH (mV .sec) in the soil samples from the pots at the end of the experiment. No standard error for it is a one sample tested for each treatment.

A long, hollow tube was used to extract the soil cores; in order to measure the water content, and again for all the treatments, for 3 out of 4 Blocks, and pictures were taken for the whole plants after the experiment termination as evidence of there growth throughout the experiment (Fig. 3-8). The analysis or the moisture content was made in PAAF soil and water department. A 0.1 g tin and lid were weighed (W1) then a 300 g soil sample weighed out and placed on the lid (W2). Samples were then transferred to the oven to dry between 105 °C and 110 °C, prior to reweighing (W3) (Web site #10 2013). The soil analysis for the TPH was made in Kuwait University lab for bioremediation research the same way as mentioned before. For the statistical analysis, a General Liner Model (GLM) was used and the name of the program is JMP version 10 (SAS Institute Inc.), and the initial and the final heights were used as an explanatory variable while the Biomass (fresh and dry weight) is a response variable. The block were accounted for but was not used for the model. Regression analysis, considering oil dosage as a continuous trait, while oil content is discrete trait.

Figure 3-8: Soil sample cores for soil moisture content measurements.

3.2.3.2 Growth experiment in the field

For both experimental areas (Bahra and Burgan) were they both have a dry weathered oil lakes from the time of the gulf war that can be used for this experiment, which means that the same techniques were used as the experiment before where: 3 replicate of a 2 years old *Haloxylon* specimens were planted in each of the 10 spots/plots in each one of those dry oil lakes (Fig. 3-9) in order to avoid concerns of pseudoreplication (Hurlbert 1984) those plants were taken from Alardiya green houses where they've never been impacted by drought, heat or contamination. Most of the specimens at the time of planting were woody in some parts and green in other parts: the plants were taken from a greenhouse in PAAF, where they were growing in 30 cm pots the previous year.

The same amount of plants were planted in the control areas, a few meters away from the oil contaminations, with a 1 litre supply of "Driwater" in each plot (Driwater company, USA) (Web site #11 2013) that can reach the roots (fresh water was added to the planting spot before the addition of the Driwater and the specimen). "Driwater" is a non polymer, solid source of water that dissolves at a constant rate for an extended amount of time. It is made of cellulose gum and water and when put into the soil microbial breakdown of the cellulose allows the release of the water to the root system (Web site #11 2013). According

to the manufacturer (Web site #11 2013), it is a standard technique to provide water for up to 70 days without further irrigation.

Figure 3-9: The oil contaminated area where 30 specimens of *Haloxylon* were planted in Burgan Area south of Kuwait.

The study began on 31/01/2013 in Bahra area and ended 28/03/2013 with 9 field trips, while starting on 05/02/2013 in Burgan area and ending 03/04/2013 after 10 trips in the south, though the 10^{th} week wasn't used for the statistical analysis to test the heights (Fig. 3-10, 3-11). In both cases trips were made at 7-9 day intervals, plant heights were measured and notes of their progress were made. But for the statistical analysis only the initial and the final heights was used for statistics.

All the plants were planted in a 20-30 cm deep hole in the ground, and a single soil sample was taken from under each contaminated spots, using the standard procedures to collect the dry oil/soil sample (See Chapter 1).

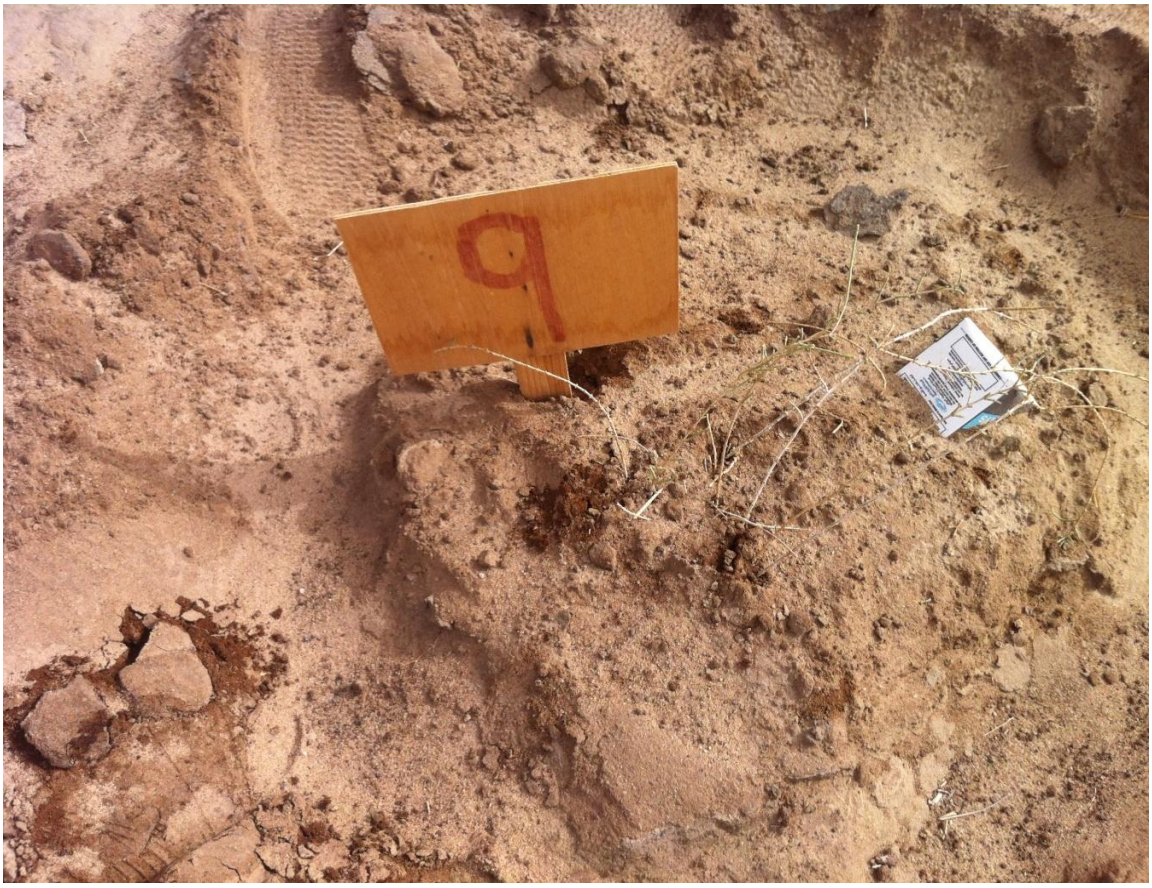


Figure 3-10: *Haloxylon* planted in oil contaminated soil in the southern Burgan area, where 30 plants were used (3 replicates in each plot) and the same number for clean soil as control.

Figure 3-11: *Haloxylon* planted in the clean area in Burgan Area, where 30 plants were used (3 replicates in each plot) and the same number for oil contaminated soil.

In the past both of the areas were heavily affected by overgrazing (by camels and sheep) and other anthropogenic impacts. A fence was erected to protect the experimental areas. At the end of the experiment the plants were removed from the soil and photographed including their root system for record (Figs. 3-12, 3-13). The plants were then been taken from the soil and packed in plastic pages to avoid losing the moisture content from the plants for subsequent biomass measurements (fresh and dry weights for the shoot and the roots and the total of both was taken where the 3 replicas from each spot was measured) in the lab. For the soil moisture content and the fresh and dry weight, the same procedures from the green house experiment were made for the field samples. For the statistical analysis, a General Liner Model (GLM) was used and the program name is JMP version 10 (SAS Institute Inc.), and the initial and the final heights were used as explanatory variable along with the Biomass (fresh and dry weight) and the initial and the final heights were used as an explanatory variable while the Biomass (fresh and dry weight) is response variable.

Figure 3-12: *Haloxylon* specimens from the clean area in Burgan south of Kuwait at the end of the experiment.

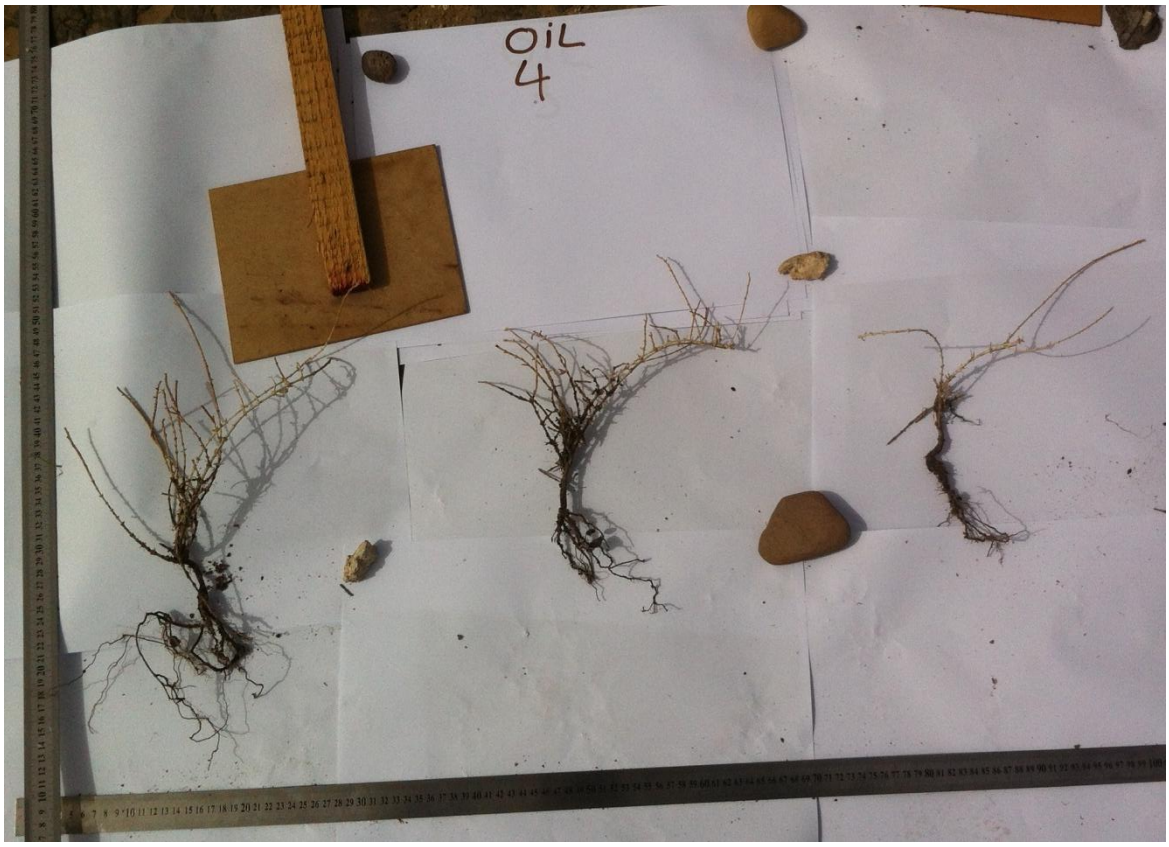


Figure 3-13: *Haloxylon* specimens from the oil-polluted area of Bahra in northern Kuwait at the end of the experiment.

3.2.4 Results

3.2.4.1 Greenhouse growth experiment

The analysis of variance from GLM for experimental data produced the following results (Table 3-1 to 3-4). The interaction was non significant for the oil levels and the blocks, while the oil levels had a significant effect for the fresh weight which means that amount of oil levels actually affected the amount of the biomass. The same thing happened to the dry weight where the oil level affected the dry weight so there will be an increase in biomass much more than the fresh, and the regression is higher, which means that the oil levels is significant and has a stronger effect on dry weight (Fig. 3-14).

For plant height (Fig. 3-15), the initial height was not significant which means that the there was no bias when the model was made, also the final height was not significant which might indicate the it is might not be a very good measure for this type of tests for there is a significant difference in the biomass. While there is no significant interaction the soil layers oddly enough shows significance where the upper and the lower layers are

similar in the amount of water they have, although the amount of water reaching the root system is so low compared to the upper.

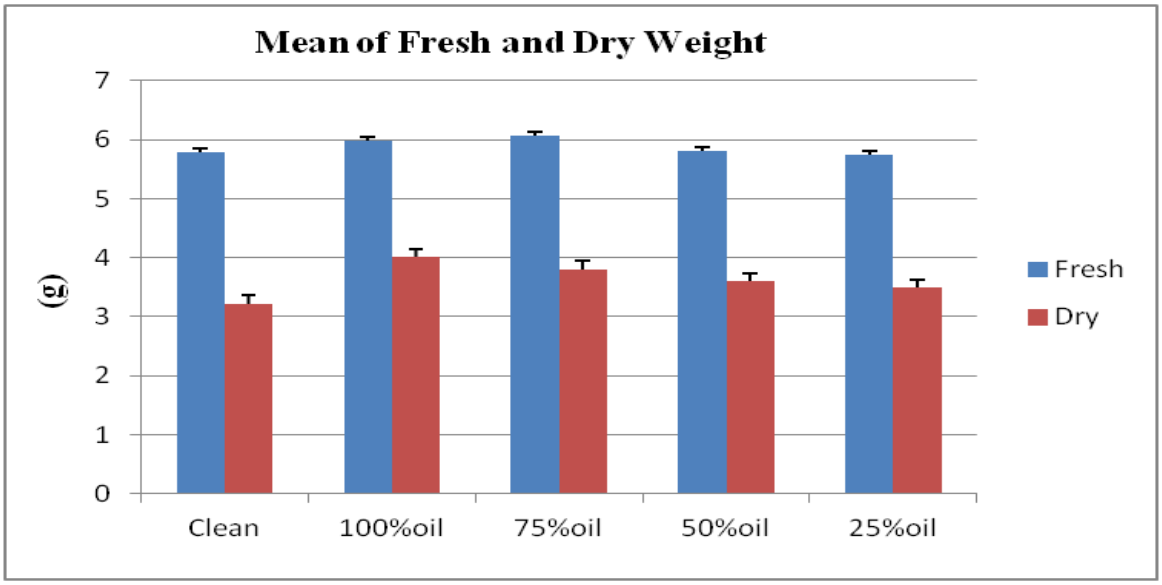


Figure 3-14 Means of the Biomass (each pot with 3 replicas) for fresh and dry weight of *Haloxylon salicornicum* ranging between (see Table 3-1 to 3-2)

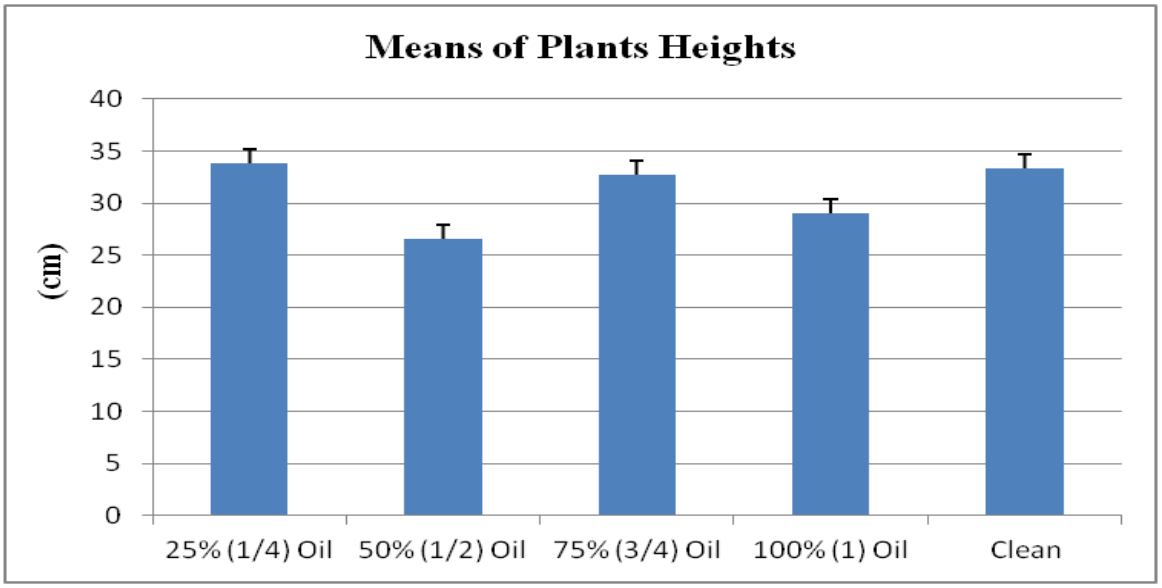


Figure 3-15: Means of Heights of the plants during the time of the experiment for all the Blocks in each treatment. The average of each treatment through the whole time span of the experiment. It is significant (P=<0.01) (Table 3-1 to 3-2).

Table 3-1: Results of GLM comparisons for greenhouse treatments including analysis of the Blocks are shown here:

Regression analysis, considering oil levels as a continuous trait					
			P values		
Response Variable	r2	slope	Oil Level	Block	Oil*Block
Fresh Weight	0.27	0.003	0.0038	0.13	0.24
Dry Weight	0.45	0.007	<0.0001	0.17	0.1
Initial Height	0.2	-0.03	0.2	0.09	0.21
Final Height	0.18	-0.048	0.08	0.16	0.38

Table 3-2: Tukey's Tests for the Treatments

Tukey's Tests				
100%	75%	50%	25%	0%
AB	A	AB	B	B
A	AB	BC	C	C
Not valid because no Significant difference				
A	A	A	A	A

Table 3-3: Moisture content for the soil layers

Source	Nparm	DF	Sum of Squares	F Ratio	Prob > F
Treatment	4	4	7.057036	0.5715	0.6853
Layer	2	2	52.975	8.5802	0.0011
Treatment*Layer	8	8	21.425711	0.8676	0.5539

Table 3-4: Tukey's Tests for the layers

LSMeans Differences Tukey HSD			
Level			Least Sq Mean
Middle	A		4.536
Upper	A	B	3.386
Lower		B	1.886

Characteristics of Fresh and Dry weight, Height and moisture content in the soil by treatments and Blocks for vegetation state variables and environmental variables. L**evels not connected by same letter are significantly different.**

Soil water moisture content is shown in Fig. 3-16 and 3-17 for all treatments (for three out of the four blocks). There was significant between treatments detected by Tukey's test. Upper, middle and lower soil layers in the pots showed no significant difference in water content between different treatments. As expected the water content in the lower layer, where the roots are, is at its lowest, but still exhibited a significant difference between most of the treatments (ranging between 0.64 wt% in the 100% oil contamination to 3.02 wt% in the 75% oil contamination, while it is higher in the upper layer (ranging between 1.2 wt% in 100% oil contamination to 7.81 wt% in the 75% oil contamination). On the other hand, the middle layer shows a variation of water content between low to high (ranging between 1.82 wt% in 100% oil contamination to 7.22 wt% in 75% oil contamination).

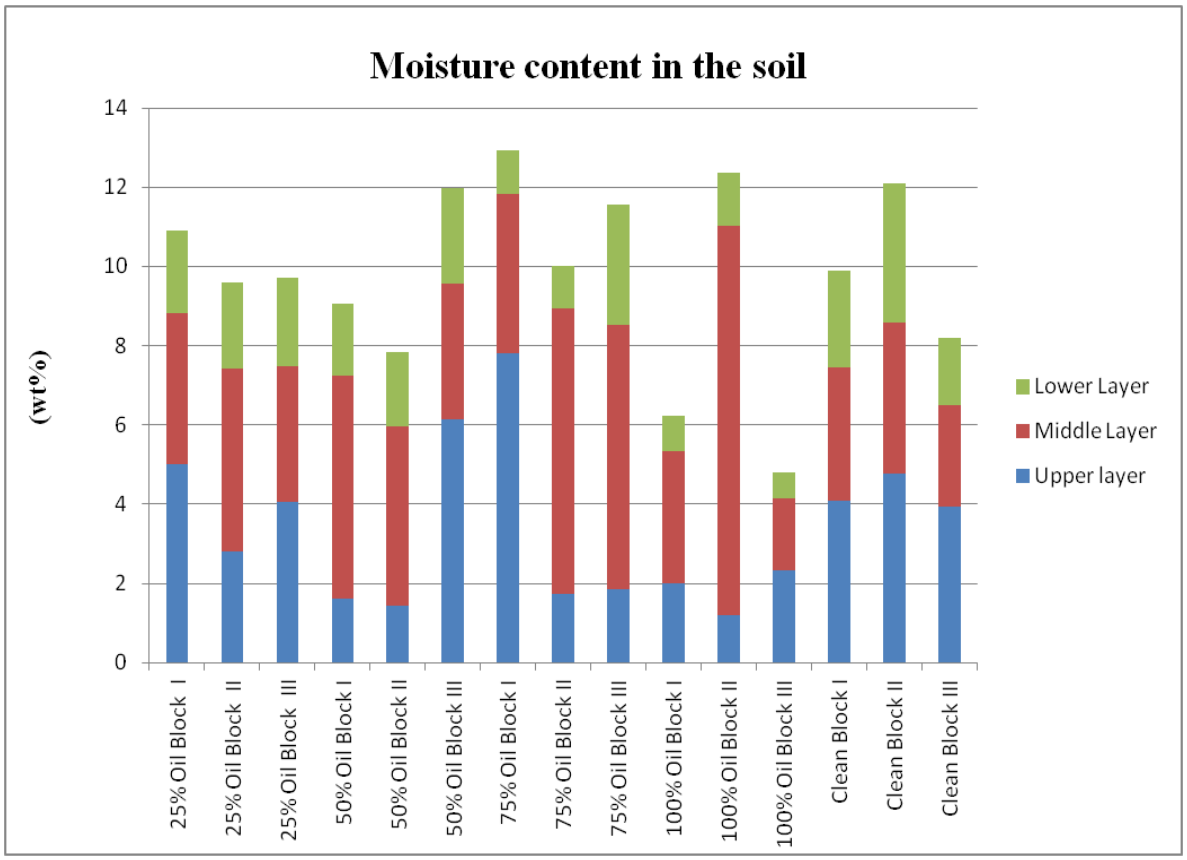


Figure 3-16: Moisture content (wt%) in soil of the treatments for 3 blocks. It is significant variation between blocks (see Table 3-3 to 3-4).

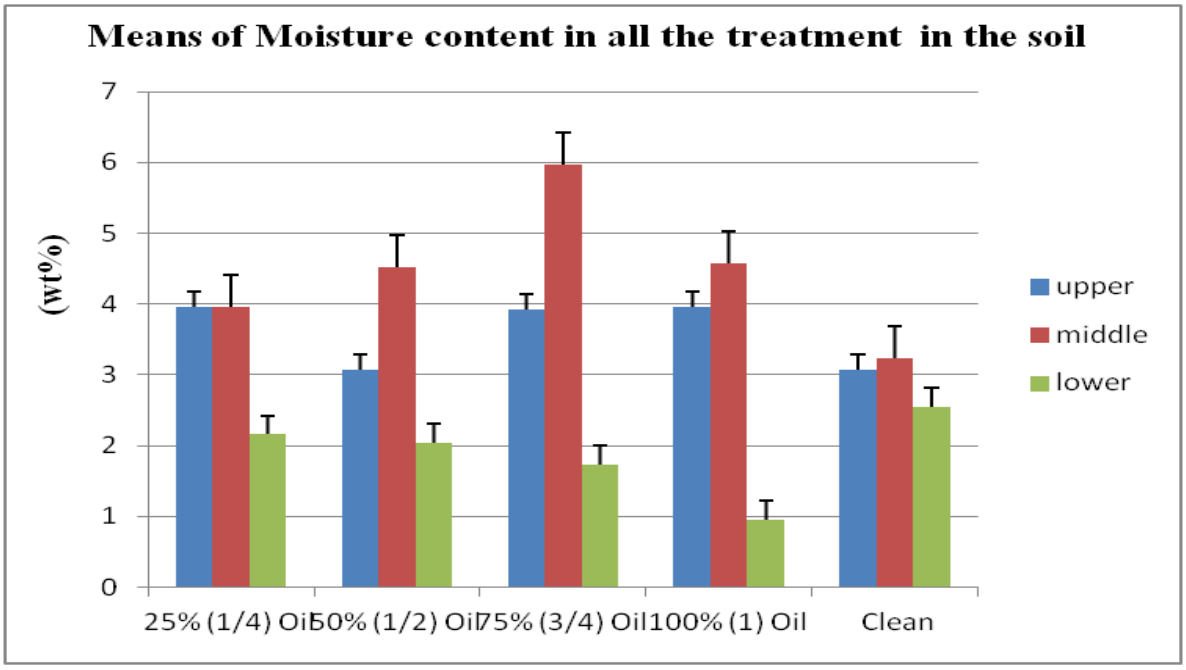


Figure 3-17: Means of Moisture content in the soil (wt%) where the average for the upper layer of 25% oil contamination for all the blocks tested is 3.95 wt%, 3.06 wt% for 50% oil contamination, 3.79 wt% for 75% oil contamination, 1.87 wt% for 100% oil contamination, and 4.27 wt% for the clean soil. While for the middle layer the averages are 3.95 wt%, 4.51 wt%, 5.97 wt%, 4.58 wt% and 3.23 wt% respectively, and finally the averages for the lower layer are 2.15 wt%, 2.04 wt%, 1.72 wt%, 0.95 wt% and 2.54 wt%. It is significant (P=<0.01) for the layers but not for the Blocks (for more details see Table 3-3 and 3-4).

3.2.4.2 Results for the field experiment

3.2.4.2.1 GLM for the data collected for the field experiment:

GLM was used to analyse plant height data but there was a problem with the experiment modelling: the potential bias in plant distribution, owing to differences in starting plant height between the experimental areas (e.g. Bahra clean soil plants were taller than plants in Bahra oil-polluted soil) (Figs. 3-18 to 3-21).

Table 3-5: Results of GLM comparisons for variables measured at sampling sites between locations (Bahra and Burgan) and response variables are shown in here:

	Site (Burgan as reference)				Treatment (Oil as reference)		
Response Variable	r2	coefficient	t-ratio	P value	coefficient	t-value	P value
Fresh Shoot	0.36	0.51	2.47	**0.018**	0.78	3.76	**0.0006**
Fresh Root	0.56	0.13	1.57	0.12	0.52	6.53	**<0.0001**
FreshTotal	0.5	0.64	2.61	**0.013**	1.3	5.33	**<0.0001**
Dry Shoot	0.45	0.17	1.96	0.058	0.43	4.87	**<0.0001**
Dry Root	0.3	0.04	0.31	0.76	0.45	3.87	**0.0004**
Dry Total	0.53	0.21	1.41	0.17	0.88	5.97	**<0.0001**

Biomass	Bahra		Burgan	
	Clean	Oil	Clean	Oil
Fresh Shoot	14.91(0.470)	13.20(0.389)	13.74(0.397)	12.34(0.389)
Fresh Root	3.00(0.159)	1.98(0.194)	2.77(0.113)	1.70(0.163)
Fresh Total	17.92(0.476)	15.19(0.566)	16.51(0.166)	14.4(0.526)
Dry Shoot	6.70(0.161)	6.08(0.154)	6.59(0.226)	5.50(0.147)
Dry Root	2.11(0.109)	1.38(0.092)	2.21(0.147)	1.13(0.054)
Dry Total	8.82(0.201)	7.46(0.228)	8.81(0.061)	6.64(0.194)

Data analysis for the treatments and the location. Values are given as mean and standard deviation (in parentheses). Reference is the level of the factor that is used as the comparison point

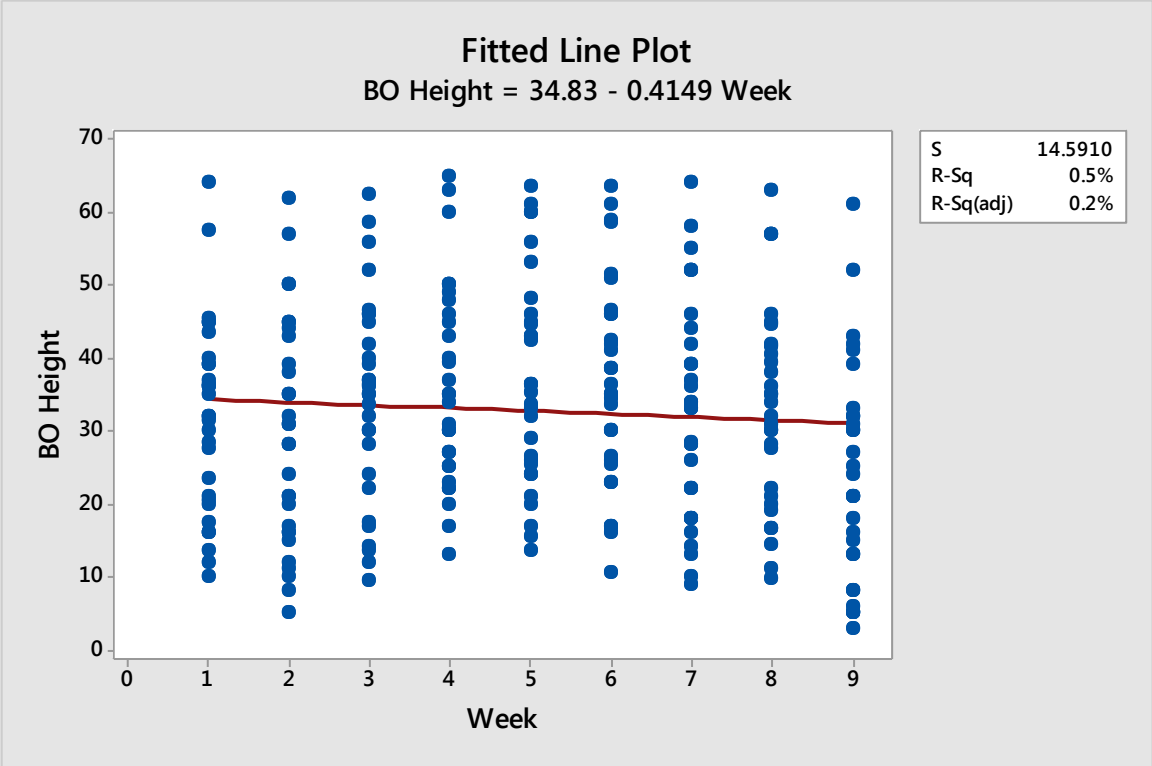


Figure 3-18: Scatter plots with the best-fitting regression of plant height by week indicated for Bahra oil samples with regression means equals to 309.922 with p values 0.229

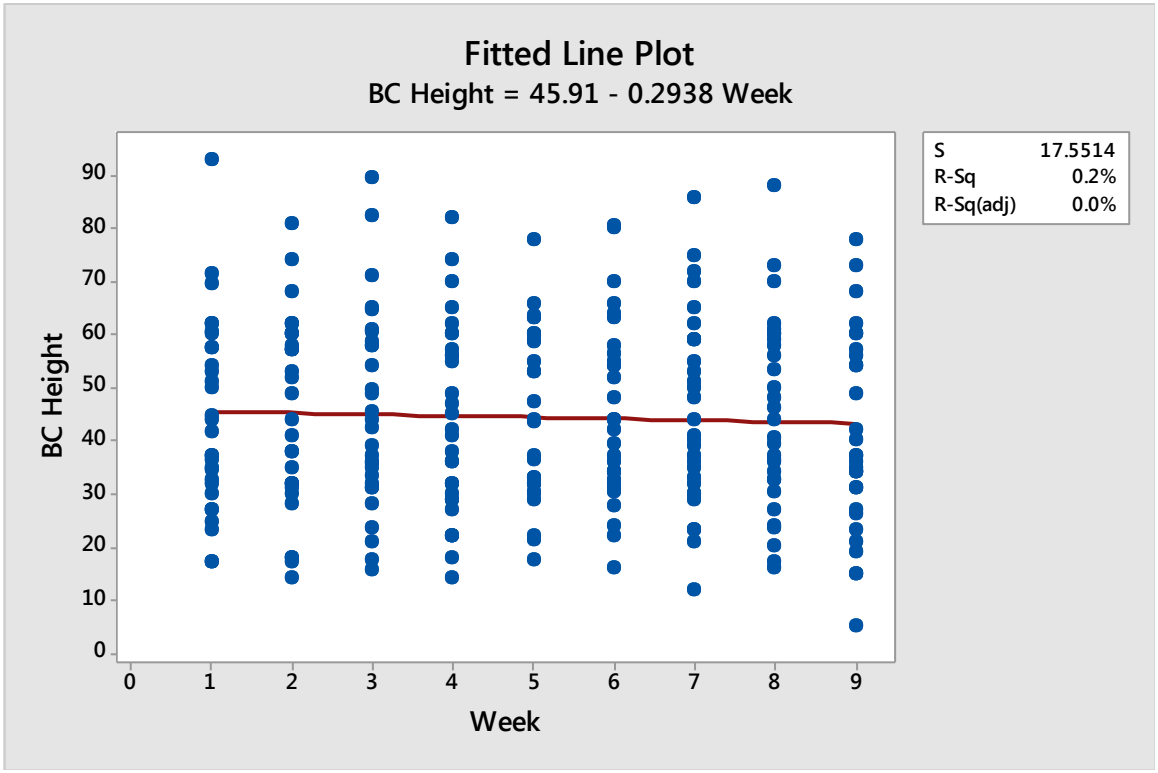


Figure 3-19: Scatter plots with the best-fitting regression of plant height by week indicated for Bahra clean samples, with regression means equals to 155.35 with p value 0.478

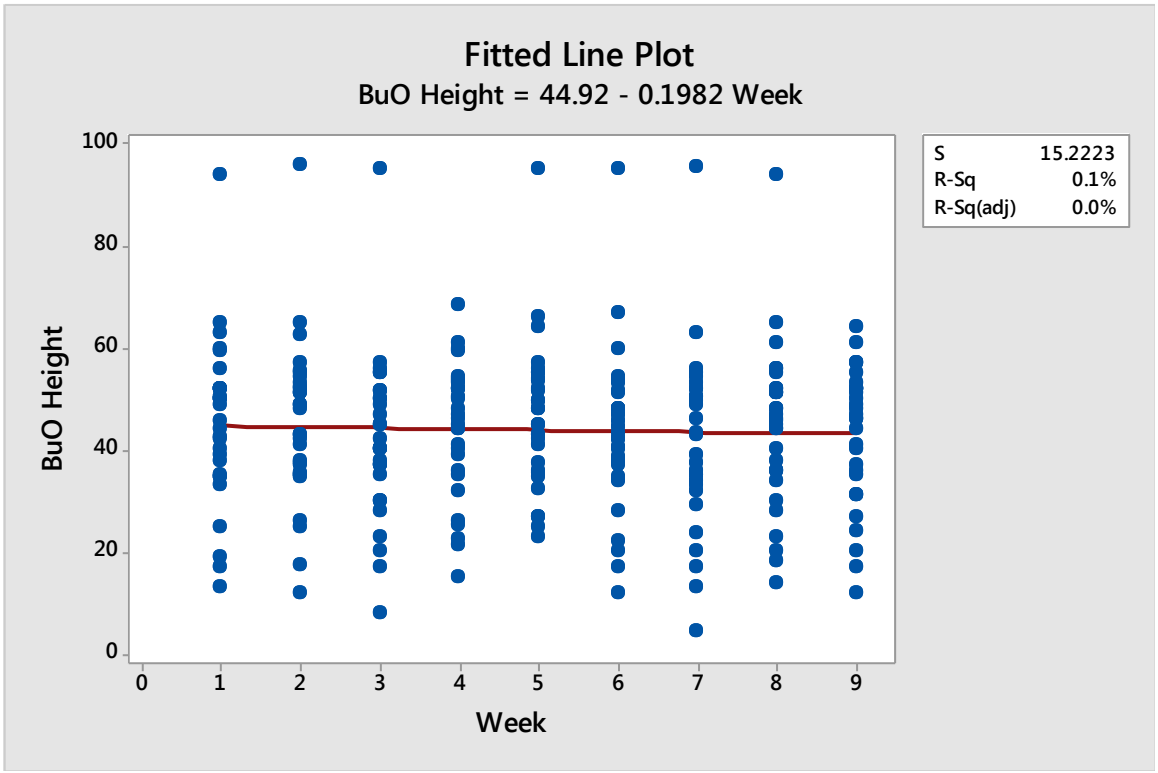


Figure 3-20: Scatter plots with the best-fitting regression of plant height by week indicated for Burgan oil samples, with regression means equals to 20.587 and p value 0.581

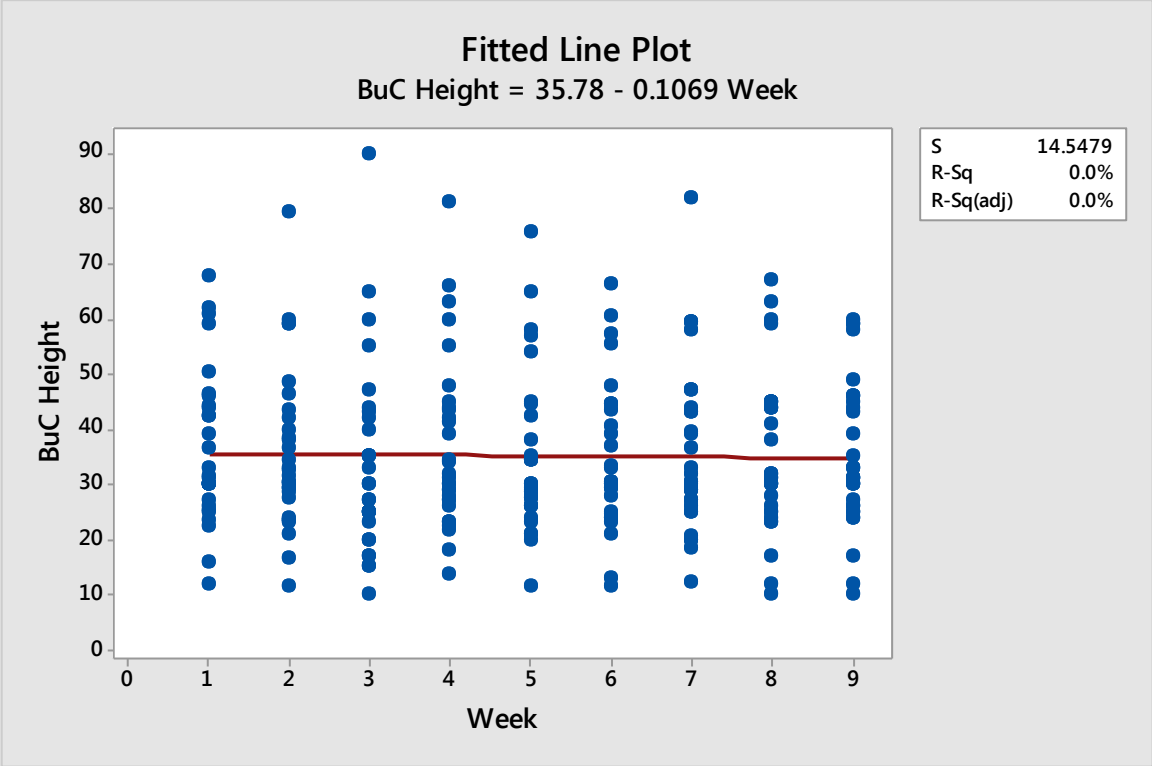


Figure 3-21: The regression scattered plot for the plant heights during the time of the experiment for Burgan clean samples, with regression means equals to 70.726 and p value 0.755

The GLM of the fresh and dry weight data (Table 3-5), showed that there is no significant interaction between sites and treatments. Also the shoots/roots and whole plant (the shoot+ the root together) biomass datasets showed a significant treatment effect in every case. However, location was only significant in two cases: fresh weight shoot (where it ranges in Bahra oil contaminated plots between 11.223g-14.963g, and for the clean plots between 12.253g-16.484g, while for Burgan in the oil contaminated plots between 10.278g-13.785g, and for the clean plots 11.578g-15.245g) and fresh weight whole plant datasets (where it ranges in Bahra oil contaminated plots between 12.279g-17.528g, and for the clean plots between 15.374g-19.736g, while for Burgan in the oil contaminated plots between 11.296g- 15.967g, and for the clean plots 14.702g-17.282g). The results, hence, suggested significance in final biomass due to geographical location of the experimental plots, where the plants in Bahra would do better than the one in Burgan (the coefficient is positive), where the clean plots doing a better in both sites. Also, at the end of the experimental period, the plants produced smaller but significantly lower biomass in oil-polluted settings when compared with plants in clean soils. The final biomass of the plants was impacted by breakage and loss of substantial parts due to strong winds wind damage was noted. Wind damage was observed in both areas and was responsible for breaking off some plant parts, which can be considered as a nondemonic intrusion:

> As defined by Hurlbert (1984): the impingement of chance events on an experiment in progress.

Accordingly, the biomass does not correlate with the documented higher growth rates and vertical elongation (Figs. 3-22).

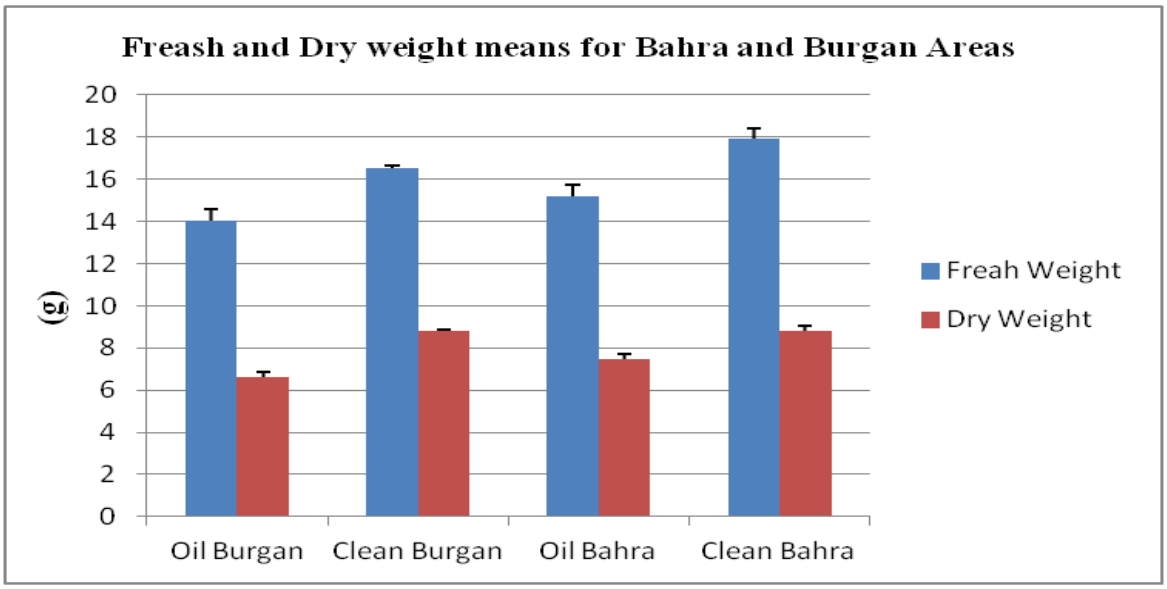


Figure 3-22: Fresh and Dry weight for the total plant in Bahra and Burgan area specimens. Shoot ($P<0.018$) and the total fresh weight ($P<0.013$) is significant between areas, and the others are not which means that the fresh weight in both areas are doing much better than the dry, also the treatment came as significant which means that the oil affected the size of the plants to be smaller than the one in the clean plots (for more details see Table 3-5).

Soil analysis for the amount of TPH (Total Petroleum Hydrocarbon) in polluted soils in the experimental areas showed no significant differences between the soils in which experimental plots were located. Although there was a quite substantial intra-site variation, which means that the amount of TPH contamination in the soil inside each plot varies (for example in Bahra it ranges between 40.163 mV.sec and 358.872 mV.sec, while in Burgan it ranges between 165.536 mV.sec and 257.631 mV.sec (Fig. 3-23), the average for Bahra was 200.35 mV.sec, and for Burgan 210.27 mV.sec, both indicative of moderately high TPH pollution.

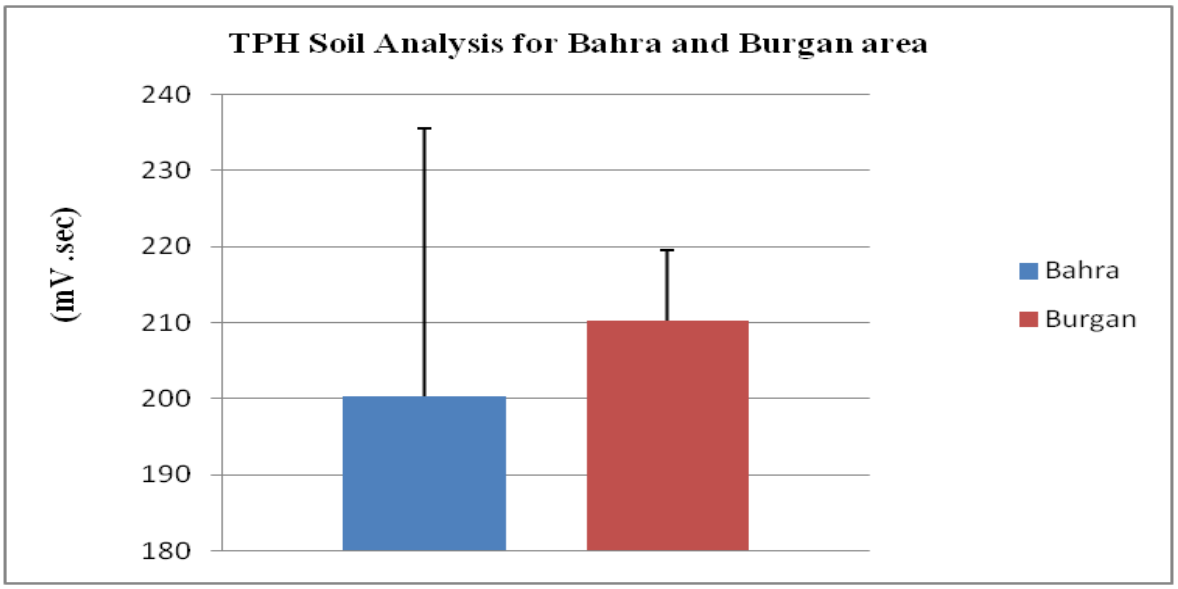


Figure 3-23: Soil TPH (mV.sec) of the oil contamination plots located in Bahra (with average of 200.34 mV.sec) and Burgan (with average of 210.26 mV.sec) areas (20 plots in total), each plot was planted with 3 replicas of *Haloxylon* in both clean and oil areas. It is not significant (for more details see Table 3-2).

3.2.5 Discussion and Conclusions

To conclude this part of the study, the *Haloxylon salicornicum* populations tested under both greenhouse and field conditions showed a very high tolerance of TPH contaminated soils, in terms of biomass response the amount of dry weight from the high amount of oil contamination have increased especially the 100% soil contamination treatment. Plants may in part be responding to the pollution stress by elongating the shoot; with the longest of the shoots being in the 25% (1/4) oil treatment. Banks (2003) emphasized that the plant height and size (bigger shoot biomass) might indicate that it is healthier; yet the health of the plant does not signify the efficiency of remediation. Also the amount of TPH has decreased in the 25% (1/4) and 50% (1/2) pots, which might indicate that the plants might actually phytoremediate the soil (though a several replicas for that test might have gave accurate results, or confirmed this one).

Although natural *Haloxylon* communities are mostly located in the north of Kuwait where Bahra is (which also was doing better than Burgan), the results indicates that this plant can live in any place in Kuwait comfortably. This could also mean that *Haloxylon* could be the species that takes over the south the same as in the north when the *Rhanterium epapposum* stopped being the dominant species there (Brown 2003).

The most important study prior to this was the work undertaken by the Public Authority for the Assessment (PAAC) in collaboration with Kuwait Institute for Scientific Research

(KISR) (KNFP 2010) to restore and accelerate the rehabilitation and the recovery of the environment damaged by tarcrete. Several techniques (furrowing, pitting or hand removing to a depth of 1 - 2 cm) were applied to fragment the tarcrete. Some plots were seeding species like *Rhanterium epapposum* and *Cyperus conglomeratus* (see Chapter 2), while other plots were not. In the follow up study for that same experiment done by Kuwait National Focal Point, of these plots *Rhanterium epapposum* was found to be the dominant species growing in this area, in conjunction with some annual species like *Cakile arabica*. The furrows in particular had the best results in both density and number of species, as they collected and stored seeds and organic matter, provided a sheltered place, and acted as a small scale water sink with the tarcrete acting as an insulator preventing excessive evaporation (KNFP 2010). The seeding seemed to work very well, though with limited success for *Rhanterium epapposum*, with delay to this species' germination at the time of report writing (KNFP 2010). That study is related to my study in the since of targeting the tarcrete and its ability to be rehabilitated, and the results came as positive for this type of oil contamination, while the experiments in this chapter is more focusing on the dry oil lakes as a source of contamination, and it is a positive thing to know that the targeted species were doing very good to survive and live in different types of oil lakes

The most important point was that the plants (plus their associated rhizoflora) could either be used as a primary source of cleaning (though it might take longer to reach an acceptable stage of recovery, in this case) or as a secondary source after probably applying a mechanical cleanup process, which would speed up recovery.

Right from the start of my work with *Haloxylon salicornicum* (Al-Ateeqi 2010) it appeared to be a strong candidate for use as a phytoremediator of oil-polluted soils since it was observed to be successfully growing on top of the oil contamination in Bahra dry oil lake. All the evidence subsequently gathered in my studies has confirmed that the species is highly tolerant of the stress conditions associated with such polluted habitats.

- A major advantage of using *Haloxylon*, (which also applies to native *Cyperus* and *Rhanterium* populations occurring in oil-polluted habitats in Kuwait) is the ease of obtaining seed and growing the plants (as appropriate) prior to transplanting into desired clean-up locations. The Public Authority of Agriculture and Fisheries Resources (PAAF) has a specific department to deal with native plants in Kuwait, and they already have the facilities, seeds, manpower, know-how and other resources required to plant those target species: indeed this is already a priority for this department and for the rehabilitation program. The UN is working with other governmental agencies to achieve the maximum of what those plants can do, and

they are using the protected areas and protected area (e.g. Sabah Alahmad) to collect the seed and store them appropriately, according to the international standard for storing seeds, in a seed bank.

- My study has provided further evidence that *Haloxylon salicornicum* should be added to the set (which also includes *Cyperus* and *Rhanterium*) of potential phytoremediator species for use in oil-polluted areas in Kuwait. Further work is now needed to examine the physiology of these species in more detail, in order to ascertain pathways and fates of petroleum hydrocarbons and breakdown molecules in these plants. In addition, it would also seem essential that future work should address the question of how well these plants are equipped with anatomical and physiological mechanisms to deal with toxicity, and what type(s) of toxin they can defend themselves against. Are they absorbing and metabolizing toxins, or do they possess defense mechanisms which prevent or minimize toxin entry into the plant? A new technology called two-photon excitation microscopy coupled with autofluorescence (TPEM-AF) has been developed that seems relevant. This allows researchers to observe in real time PAHs travelling through plant tissues, and the interactions inside tissues as well as bacterial interactions with the contaminants. The technique does not alter the way the target chemicals behave (as earlier procedures tended to) because the fluorescence utilized is the natural autofluorescence of the living matrix (Wild 2007). Techniques such as this, and other scientific advances may well improve our ability to find, utilize and understand better the ecology of phytoremediator species aimed at assisting clean-up of oil polluted soils in Kuwait, and other areas affected by petroleum hydrocarbon pollution.

4 USING BACTERIA AND FUNGI FOR OIL-POLLUTED SOIL BIOREMEDIATION

4.1 Introduction

Alongside the use of plants as phytoremediators, a second theme in tackling oil pollution cleanup by biological means is the application of microbe-based procedures. These can supplement or enhance phytoremediation (for example, the presence of appropriate rhizosphere microfloras in association with plant roots). Microorganisms are well known for their ability to degrade and utilize the components of petroleum hydrocarbon pollutants Singh (2009). Such pollution incidents are increasingly becoming common environmental problems in different parts of the world, whether caused by accident and mishandling, or by intended sabotage and destruction (Radwan 2000; Steliga 2012). Therefore, it is a necessity to establish environmentally-friendly ways to deal with the problem. One of the most important ways will be using microbes as bioremediators of oil pollution, for both terrestrial and aquatic ecosystems. Singh (2009) notes that there are also now considerable research interests, within the oil industry around the world, in using microbes, not just for bioremediation, but also to improve oil recovery and enhance the quality of products extracted from oil.

In Balba's (2003) study, TPH was reduced drastically using landfarming treatment. In lightly TPH-contaminated soil, TPH was reduced by up to 80% within 6 months; and in heavily contaminated soil it was reduced to 80% within a 12 month span. Balba (2003) concluded that the rate and percentage suggests that the reduction of the TPH was not due to volatilization but was caused by microbial biodegradation. But the bioremediation is still not the whole answer for the remediation process because it is known that the microbial communities are less active on their own in unplanted soil than when within the power of the plant root systems (Anderson 1993; Jhonson 2005; Joner 2006; Mueller 2006). According to Escalante-Espenosa (2005), the presence of microbial communities can help the plants in solubilization and recycling of the mineral nutrients along with providing gibberellins to stimulate the growth of the plants and supply auxins and cytokinins. That is why it is important to focus also on knowing what types of microorganisms are living on top of the target species in this study which is *Haloxylon salicornicum*.

4.2 Aim

(I) To collect and identify types of bacteria and fungi from the rhizosphere of wild and cultivated *Haloxylon salicornicum* challenged by oil-polluted soil conditions, in Kuwait, and knowing their rules in bioremediation/phytoremediation

4.3 Methodology

4.3.1 Bacterial and fungal extraction from the rhizosphere of wild and cultivated *Haloxylon salicornicum*, and the block experiment.

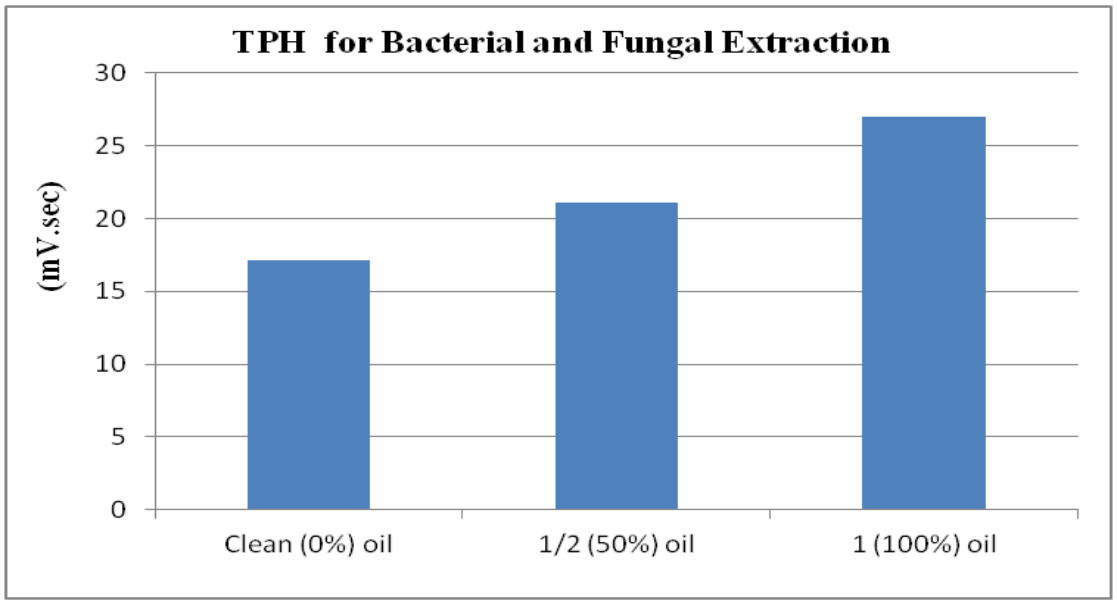
Wild *Haloxylon* plants with intact root systems were dug up from Bahra area were the root system was cut and used. I took the plant samples to Kuwait University lab, for microbial/fungal extraction. No further treatment was applied to wild plants before extraction of microbial/fungal populations immediately (same day) on returning to the lab.

Furthermore, 3 *Haloxylon* replicas in each treatment were grown for 20 days in a greenhouse, at a temperature range between 15-25 °C and 55% humidity. Watering (using tap water) regime started with 200 ml per pot for two days, then 150 ml every week to mimic the large block experiment (Fig. 4-1). The experiment commenced on 03/01/2012 and ended on 22/01/2012.

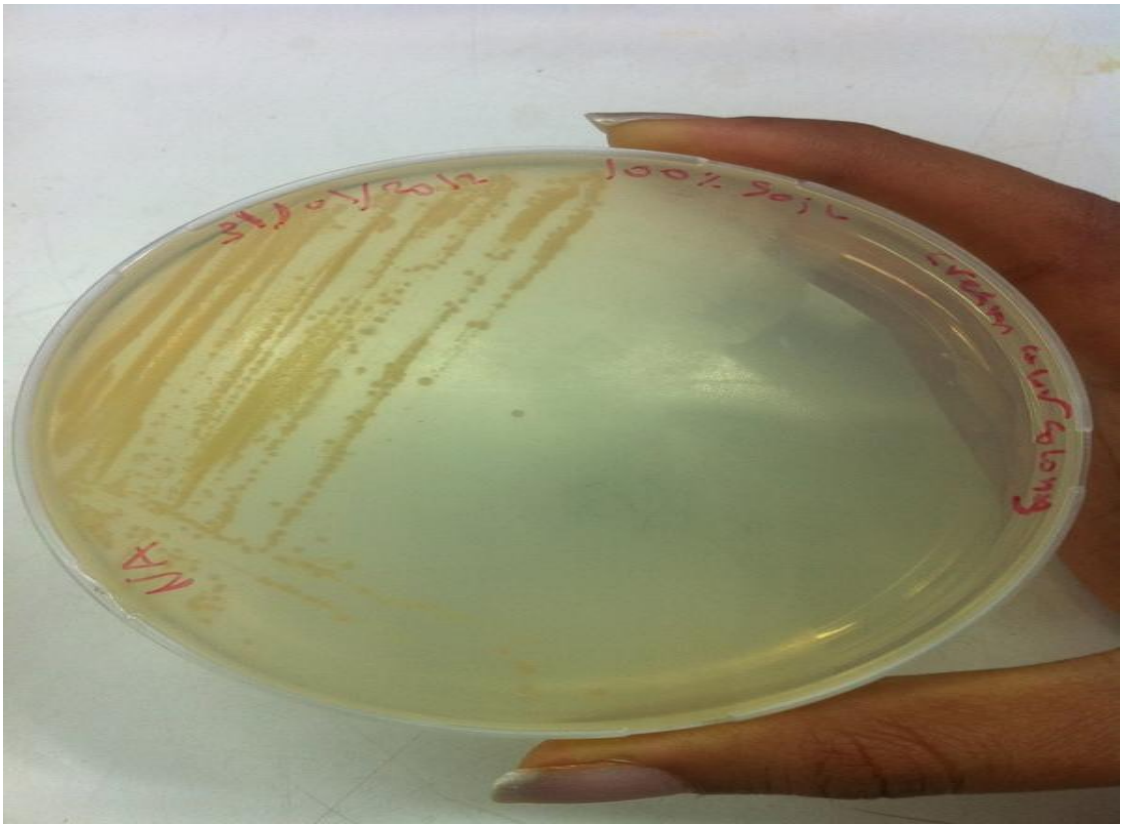
4-1: Watering *Haloxylon* planted into 100% oil-contaminated soil in the Block experiment where the watering is usually between 7-9 days apart.: water (300 ml) stays at the surface for a long time.

Due to lab resource limitation, a single plant specimen was taken at random from each of three treatments (100% oil contamination, 50% oil contamination and 50% clean; and 100% clean soil). For the cultivated *Haloxylon* the soil was utilized in the greenhouse experiments has a clean soil and perlite for aeration. The TPH concentration in each treatment was determined by Kuwait University Lab for bioremediation for microbial extraction (Fig. 4-2). In total 4 plants (3 from the treatments and 1 from the wild) were used for the microbial isolation, that means that the results might just give an idea about what might be there.

The isolation for the rhizosphere microorganisms process, was a standard technique used in Kuwait university lab for bioremediation, used nutrient agar with pH 7 on filter paper stained with crude oil to enhance and stimulate the growth of the carbon-lover bacterial/fungal. As the root system from the plants was very small, 9 ml of distilled sterile water was added to 1 g roots, the mixture was shaken in a shaker for 5 minutes and left to settle for 5 minutes. Next, 1 ml was taken from the top of the solution and mixed with an additional 9 ml of distilled sterile water to a concentration of (10^{-1}). The mixture was shaken in a shaker for 5 minutes, to make sure all the bacteria/fungi that attached to the root system were in the solution. The resulting solution was further diluted by mixing 1 ml from it with another 9 ml of distilled sterile water to a concentration of (10^{-2}). This stage was repeated for a 3rd time to reach a concentration of (10^{-3}). A spreader was used to spread 1 ml of the final solution on nutrient agar plates (PDA was used for fungal extraction). The culture was incubated for 5 days in 30 degrees after sealing with parafilm (Fig. 4-3, 4-4).



4-2: Amount of TPH in the soil from which bacteria and fungi were extracted (mV.sec) with one sample, which means no error bar are there.



4-3: Pure colonies of bacterial extraction of 100% oil contamination in nutrient agar after a few days in the incubator at 30 ºC, needed for identification purposes, following which they were grown on filter paper soaked with crude oil to make sure the bacteria which will grow in it are using and consuming the petroleum hydrocarbon.

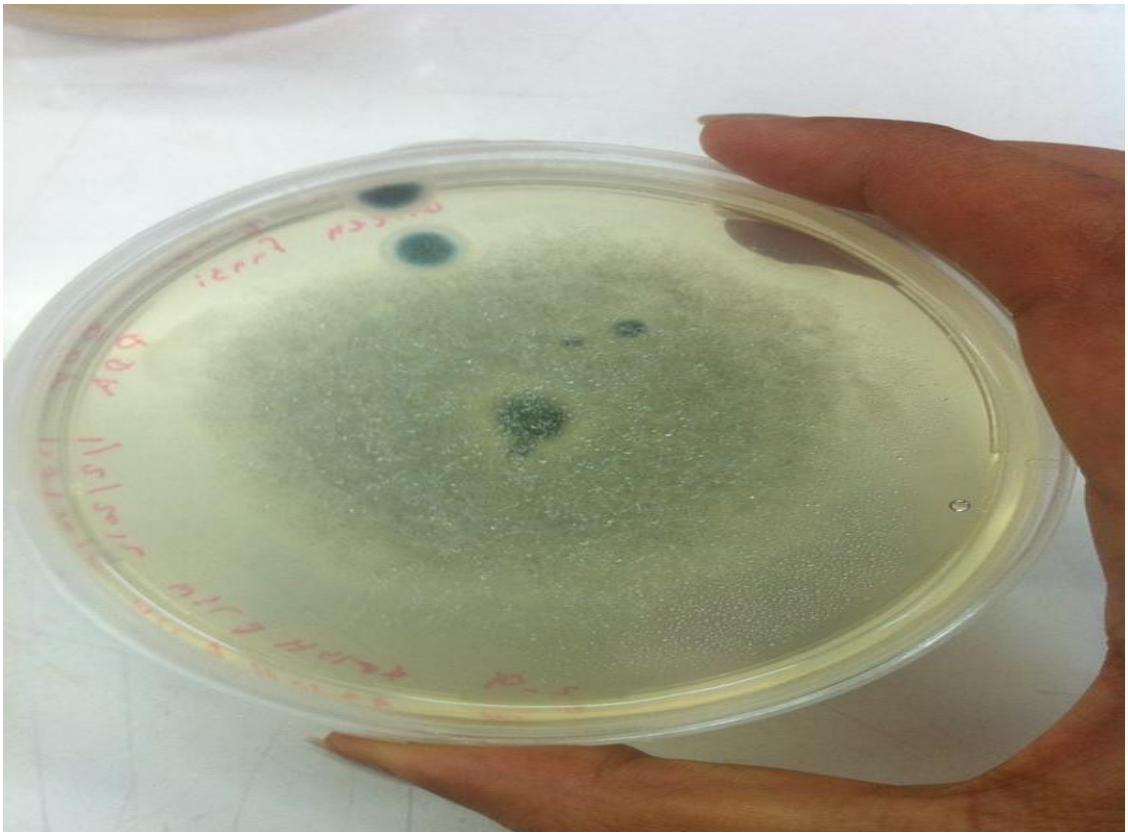


Figure 4-4: Pure fungal colonies of a wild *Haloxylon* in a PDA agar after incubation for 5 days in 30 °C, needed for identification purposes, following which they were grown on filter paper soaked with crude oil to make sure the fungi which will grow in it are using and consuming the petroleum hydrocarbon.

The same standard procedure was used for extracting bacteria from wild *Haloxylon salicornicum* plants. The plant tissues used in these extractions were from bigger (older plants). Accordingly, 90 ml distilled sterile water was added to 10g of roots, and the same previously mentioned procedure was made on to it for the mycorrhiza extraction. Furthermore, the colonies were counted manually using the colony counter for the extracted microbes. Finally, the pure samples were identified by gene sequencing then they've been sent to a specialist commercial company (James Hutton Institute, Aberdeen, Scotland). The identifications were made using a standard DNA sequencing procedure by comparing my culture sequence with numerous cultures sequencing in the international database called NCBI.

4.4 Results

4.4.1 Microbial and Fungal Identification

For the wild *Haloxylon* (10^{-2}) there were 178 and 138 colonies; for 100% weathered crude oil 271 colonies (10^{-3}), 50%/50% treatment 400 colonies (10^{-3}); and finally 564 colonies for the 100% clean soil (10^{-3}). Only 19 pure samples were collected (12 bacteria and 8 fungi). The results are presented in Table 4-1; see also Appendix 2 page 130**:**

Table 4-1: Bacterial identification from the DNA sequencing technique:

Treatment	Number of samples	Identification
Wild *Haloxylon salicornicum* from contaminated area	3 samples	**Streptomyces* spp. **Inquilinus* sp. *one unidentified
100% Clean soil (1)	1 sample	**Sphingopyxis* spp.
100% oil (1)	6 samples	**Rhodococcus manshanensis* **Agrobacterium tumefaciens* (mixed colony from one tube). **Nocardia cyriacigeorgica* ** Gordonia lacunae / Gordonia terrae* **Lysobacter* spp.
50% oil (1/2)	2 samples	**Gordonia lacunae / Gordonia terrae* **Agrobacterium tumefaciens*

Streptomycetes is a genus of Streptomycetaceae. It is an aerobic bacteria and is a widely distributed bacterium in soils of different types of environments, comprising the big part of the microbial population in marshes and hot deserts (Balachandran 2012), with grey colonies and an extensive branched substrate mycelium (Kämpfer 2006). Barabas (2001) and Radwan et al. (1998) cite several reports indicating that these organisms might play a significant role in hydrocarbon degradation. Radwan et al. (1995), after studying several annual desert plants most of them from the Asteraceae family in Kuwait like *Senecio glaucus* and a perennial *Cyperus conglomeratus*, emphasized that the bacteria are probably *Streptomycetes*, yet since they mixed the rhizosphere from various crops with that from native flora, we are not sure if the rhizosphere came from the native plant; yet it confirms its presence in the contaminated soil nonetheless. However, more recently Balachandran (2012) declared its ability to degrade petroleum and PAHs.

Inquilinus sp. belongs to the Rhodospirillaceae family (Web Site #5 2013). A number of the representatives of this family are known to exist in the rhizosphere habitat in general

(e.g. *Caenispirillum bisanense*, *Azospirillum amazonense*, *Azospirillum irakense*) (Khammas 1989; Magalhaes 1983; Yoon 2007). Based on this study both of these organisms are related to the rhizosphere of the wild *Haloxylon*.

Sphingopyxis sp. belongs to the family Sphingomonadaceae. It is an aerobic species with yellow pigmentation (Yabuuchi 2005). It is a widely spread species in nature, and can be found in corals, soils, plant surface and clinical samples (Balkwill 2006; Cavicchioli 1999; White 1996). Ward (2009) categorises *Sphingopyxis* and *Rhodococcus* with the biodegradable and biofiltrates of the volatile organic compounds (VOCs) bacteria, which emulsifies hydrocarbon by adherence, foaming argent, dispersion, soil flushing and detergent. *Rhodococcus* species can perform several actions such as de-emulsifying the oil emulsion and reducing its viscosity. Radwan et al. (1995) found a number of genera like *Rhodococcus*, *Bacillus* and *Pseudomonas* in the polluted non-rhizospheric soil. *Sphingopyxis* was found in the clean soil as well. A few studies suggested that the dry environment may develop some kind of resistance to radiation in bacteria, which can be useful in the removal of pollutants from the soil as documented for species found in mine sites (Fredrickson 2008; Liu 2011; Mattimore 1996; Rainey 2005). The same resistance may develop in arid species which might have the same effect on these bacteria to be available to clean the soil from oil contamination in the desert.

Rhodococcus, also used in biodenitrogenation, is reported to be found in the 100% weathered contaminated soil as an aerobically grown strain (Jonathan 2003). It is transforming and removing organic nitrogen from petroleum hydrocarbon molecules. *Rhodococcus* can be used in biorefining and bioprocessing (biodesulfurization) of oil, through transforming organic sulphur compounds biologically, and refining petroleum products (Ward. 2009). Radwan et al. (1990) found that the most abundant microbes in oil contamination was *Rhodococcus*, which is very efficient in consuming the aliphatic hydrocarbon. *Rhodococcus* can be found in different habitats like soil, or seawater (Larkin 1998).

Norcadia is a bacterium found in the soil. It is partially branched, and can produce a dry white colony (Palaniappan 1995). Like the *Rhodococcus*, *Norcadia* is an aerobic species belonging to the Nocardiaceae family (Brown-Elliott 2006; Palaniappan 1995), and it is also one of the species that can be used for biodesulfurization (Ward. 2009). Radwan et al. (1990) found it to be one of those species capable of consuming aliphatic hydrocarbons.

Agrobacterium (found in 100% and 50% weathered crude oil) is one of those species which belongs to pathogenic plant bacteria and it can transfer its genes to eukaryotic organisms through horizontal transfer (which also means transfer of the genes that are capable of degrading the hydrocarbon; on the other hand, the American Type Culture Collection (ATCC) considered *Agrobacterium* to be safe which means that there is no evidence that they can harm healthy adult human beings (Web site #9 2013). That means that this species can be used for soil remediation. The species might be using the carbon in the hydrocarbon as a sole resource for its survival; also it can grow in anaerobic conditions, which makes it perfect for bioremediation purposes (Steliga 2012). However, its existence in Kuwaiti contaminated soil had not been reported previously as far as I know.

The bacterial genus ***Gordonia*** (*Gordona* synonym) (found in 100% and 50% weathered crude oil) (Web site #6 2013) belongs to the Gordoniaceae family (Web site #6 2013). These bacteria can be found in environmental sources like water and soil, and many of them are able to degrade environmental pollutants (Lai 2009). The shape of the *Gordonia* colonies can be smooth or glossy or slimy, of irregular and rough shapes; sometimes even in the same medium they might change their shape (Arensko¨tter 2004). *Gordonia* are known for their abilities to modify and degrade aromatic, aliphatic hydrocarbons, benzothiophene, polyisoprene, halogenated aromatic compounds, nitrite, and last but not least integrated with the long aliphatic chains of the mycolic acids located in the cell walls associated with surface adhesion and hydrophobicity pollutants (Bendinger 1993). *Gordonia* have amphiphilic compounds (biosurfactants) usually associated with the excretion of biogenic surface-active, which enable the degradation of water insoluble pollutants or hydrophobic ones (Banat 2000). Al-Awadhi et al (2012) found *Gordonia* on the leaves of the plant *Chenopodium murale*, another native chenopod that can utilize the gaseous volatile hydrocarbon for bioremediation, which means that this species living in the soil (in the plant rhizosphere in our case) and on the leaves of chenopods can be useful in remediation.

Lysobacter is a rod-shaped aerobic bacterium, most of which do not have flagellae (Romanenko 2008). The genus *Lysobacter* is in the Xanthomonadaceae family, usually found in different environmental and geographical habitats, in soil, water and in agricultural soil that is more fertile because it contains sufficient nutrient, organic matter and aeration for crops or ornamental plants to grow into (Christensen 1978; Lee 2006; Webster 2007; Weon 2006). In general, the colony shapes for most strains are similar, with

smooth entire margins and the colour ranges between pale yellow to cream (Christensen 1978); yet there is no previous record of its existence in Kuwait.

> Labbé (2007) reported a group of bacteria which have been classified together because they have the same features, and one of those groups included *Lysobacter*. He found that they can "... grow rapidly in nutrient-rich environments and are r-strategists, consequently predominating in the contaminated soils where the nutrient source was represented by petroleum hydrocarbons."

The fungal identification revealed the existence of ***Trichoderma*** spp. (See Table 4-1; Appendix 2). They belong to the fungal family Hypocreaceae. *Trichoderma* are filamentous fungi, widely found in decaying vegetation and are abundant in soil. The fungi can grow very fast, and are characterised by a white colour and cotton-like texture that will turn to a yellowish-green to dark green compact clump (Burnett 1972; Web site #3 2013; Web site #7 2013; Web site #8 2013). Radwan et al. (1995) found it in the polluted non-rhizospheric soil in Kuwait.

Table 4-2: Fungal Identification from the DNA sequencing technique:

Treatment	Number of Samples	Identification
Wild *Haloxylon salicornicum* from contaminated area	3 samples	**Penicillium commune* **Penicillium* spp. *one unidentified
100% (1) Clean soil	2 samples	**Trichoderma asperellum* **Penicillium simplicissimum*
100% (1) oil	1 samples	**Penicillium simplicissimum*
50% (1/2) oil	1 sample	**Penicillium commune*

Penicillium spp. belongs to the fungal family Trichomaceae, comprised of almost 250 species. The complex of species exhibit little variation between each other (Guarro 1999). They are very abundant in soil and decaying vegetation. The colonies usually start white at first then become grey green, blue green, or pinkish or yellow (Web site #3 2013; Web site #7 2013; Web site #8 2013). Fedorak (1984) reported the presence of *Penicillium* in more than 70% of the fungus in oil contamination, while Oudot (1987) emphasized that

Penicillium is a very active species in oil degradation. Radwan et al. (1998; 1995) found *Penicillium* in the rhizosphere soil of Kuwaiti natives like *Cyperus conglomeratus, Launaea mucronata, Picris babylonica, Salsola imbricate*, as we did and the crops for the fungus isolation that they made for the microorganisms were associated with the petroleum hydrocarbon degradation.

4.5 Discussion and Conclusion

Studies previous to this work stated that *Streptomyces* is a bacterium related to PAH degradation and lives in both man-made environments and in nature (Balachandran 2012). In a study by Barabas (2001), three species of *Streptomyces* (*S. griseoflavus, S. parvus, S. plicatus*) were discovered in Burgan oil field in Kuwait. The three species have the ability to consume n-octadecane, kerosene, n-hexadecane and crude oil as a sole carbon source. *Inquilinus* sp. is also a bacterium known to be involved in petroleum degradation (Tuan 2011). In this study, both bacteria were found present with the wild *Haloxylon* growing in contaminated soils. The species *Sphingopyxis* sp. and *Gordonia lacunae/Gordonia terrae* (Nolvak 2012), *Rhodococcus* (Auffret 2009), *Agrobacterium tumefaciens, Nocardia cyriacigeorgica,* (Steliga 2012), are also all related to oil degradation. In addition, based on Steliga (2012), *Rhodococcus* has several representative bacterial species that can use aromatic and aliphatic hydrocarbons, and could be useful for oil bioremediation.

According to Steliga (2012) *Penicillium* species and *Trichoderma asperellum* would be useful for preparation of bioremediation strategies that will enhance the result of cleaning up contaminants. The results, presented by this study, for the fungi (*Penicillium simplicissimum*) demonstrate that they, in general with the limited number of samples used, it is abundant in both clean and contaminated soils. While Steliga (2012) reported that *Trichoderma asperellum* exhibit similar distribution to *Penicillium* and that both species are related to oil degradation, in the current work *Trichoderma asperellum* was detected only in clean soil, and not in contaminated soil sample. This could be because I simply failed to isolate it, or that the species does not tolerate the degree of petroleum hydrocarbon contamination in the tested soil.

Sphingopyxis sp. was found only in the clean soil but not in the contaminated sample, suggesting that this species might be in need of specific conditions, such as those in clean soil. Also, I cannot rule out the possibility that through some technicality I missed this species while I was isolating the bacteria.

Given the small number of samples, limited location, and the probability that I have missed some of the species, further studies are in order to know more about the relationship between these species and other species that might be associated with the *Haloxylon* rhizosphere from other contaminated areas or under different conditions.

The richness of bacteria and fungi in the analysed samples is quite evident, considering that the chenopods do not encourage their association with the fungi in the first place. This is a good indication of how beneficial *Haloxylon* can be in have a diversified soil microorganism community. A previous study by Yateem (2000), was undertaken on some crops as well as several native plants in Kuwait, of their microflora and their bioremediation abilities of the oil contaminated soil, in which *Cyperus conglomeratus* was one of the selected species. However, the authors immediately excluded the native species, as they believed that using native plants would need extreme conditions to support their growth. My work suggests the contrary, as it is possible to grow these plants in nurseries.

Radwan et al. (1995) argue that annual native plants are able to survive in a shallow or small amount of petroleum hydrocarbon, while still having clean white roots, because oil consuming microorganisms are detoxifying and bioremediating the soil very quickly from the aliphatic and aromatic hydrocarbon, especially at that time (only 4 years after the incident) where the contamination was still fresh. But they believe that in order for the plants to tolerate the heavy oil contamination (which they didn't believe the plants could handle on their own), the soil should be mixed with clean soil to dilute the levels of contamination. Lin (1998) also emphasised that a transplantation can be successful only when the oil contamination toxicity is reduced. This might be one of the reasons why *Cyperus* and *Haloxylon* monitored in this study, some 20 years after the pollution incident, exhibited notable tolerance to oil contamination, as the levels of toxicity have dropped due to natural attenuation. Furthermore, Radwan et al. (1998) studied plants from the south of Kuwait, which is dominated by *Cyperus*, hence they did not compare *Haloxylon* to *Cyperus*. The results of my project, 23 years from the pollution incident, confirm that the *Haloxylon* actually can tolerate weathered oil contamination, and do have a set of several micoorganisms around its root system that is related to the oil contamination degradation.

5 GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

Several studies have been undertaken to examine the severity of land degradation in Kuwait in the last few decades (Brown 1997 ; Brown 1998 ; Khalaf 1989; Omar 1991; Zaman 1997). Major anthropogenic effects are caused by camping and off road driving, as well as the impacts of quarrying (Khalaf 1989), and overgrazing. However, undoubtedly one of the major causes of damage occurred as a consequence of the Iraqi invasion and the aftermath of the First Gulf War (Brown 2003).

Land degradation in dryland ecosystems (some of the literature considers Kuwait to be arid (e.g. (Abdal 2009)) and others consider it to be semi-arid (e.g. (Marcella 2008)) is usually associated with extreme decrease and simplification of perennial vegetation structure. This is coupled with other problems like various impacts on water relations, soil structure, productivity, and microclimate (Le HoueHrou 1996). According to Warren (1996), semiarid environments support probably the most fragile and susceptible ecosystems in the world.

Both chronic and acute damage to dryland vegetation can be caused by petroleum hydrocarbon spillage, burning and penetration into the soil profile, as well as the impacts of measures used to clean up pollution and extinguish oil fires (e.g. use of seawater). The oil adsorbed in the soil can reach the groundwater where it can either float on the surface or disperse into it, or sink to the bottom of the water aquifer (Balba 2003). Subsequent uptake of such contaminated water by plant roots can cause reduction of both stem density and plant height as well as reduce shoot biomass, even if complete plant mortality does not occur (Alexander 1987; Ferrell 1984; Krebs 1981; Lin 1996).

Physical and chemical methods of clean-up of groundwater and soil (including pumping, excavation and vapour extraction, are both costly and inefficient, and it is here where biological treatment can come in as a very promising technique. So in order to re-establish and rehabilitate the heavily impacted dryland ecosystems of Kuwait, Le HoueHrou (1995) suggested artificial re-establishment of same native plants, which used to populate the impacted areas, to assist in the rehabilitation of their proper ecosystem. An obvious practical difficulty facing the re-establishment of native plants is the unpredictability of rainfall to support the establishment and growth of bioremediator plants, especially during the vulnerable establishment phase (Radwan 2000).

5.1 Advantages of Native Flora Communities

According to Gawronski (2007) the inspiration behind using plants as phytoremediators came from the discovery of what have been called “hyperaccumulators”, in the families Papilionaceae, Brassicaceae, Poaceae, Asteraceae, and Caryophyllaceae, with the original usage being to help clean up heavy metal polluted soils. An experiment by Lin (1998) to transplant the native plants (*Spartina alterniflora* and *Spartina patens*) in salt marshes, where an oil spill had occurred, proved that this could be a useful technique for the area’s recovery, by assisting the oil degradation process. In this case fertilizer application was used to enhance vegetation growth. Teas (1989) describes a similar successful use of transplanted mangrove plants into a mangrove swamp impacted by oil spillage.

Given that the Native Plants Conservation supervision — the Public Authority of Agriculture (PAAF) — already has the means to produce and grow several native plant species, and has established several mother plant communities to produce seeds, implementing such an approach in Kuwait is highly feasible. The advantage of using phytoremediation to treat the soil is that in the long run it will provide further ecological services. The reintroduced populations will help in maintaining the soil’s natural structure and texture, stabilising the desert surface, and ultimately help in reducing the frequency and intensity of dust and sandstorms. In addition, such techniques could increase the microbial biomass to high levels, which means higher fertility in the soil (Huang 2003), and the established communities would provide a microhabitat for different associated flora and fauna, as well as a fodder source for grazing. Furthermore, in comparison to alternative approaches which require heavy machinery and expensive mobilization, phytoremediation may very well be cheaper. However, we did not test the cost effectiveness per m^2. Follow up studies on cost effectiveness of using this technique are warranted.

To find out how damaging petroleum hydrocarbon pollution can be, it is important to know that the amount of petroleum hydrocarbon affecting the environment usually depends both on its concentration in the soil and the volume of the original spillage (Alexander 1987; Lin 1998). It is also important to realise that the degree to which the oil has weathered (and consequently reduced in toxicity) can be crucial to the success or failure of a transplantation programme (Lin 1998). Re-introduce phytoremediator vegetation too early and they may die (Cowell 1969). The processes involved in oil weathering are usually dissolution, evaporation, oxidation, and biodegradation (Boesch 1974). Rates of weathering mainly depend on the soil pH, fertility, acclimated microbes, the amount of

organic pollutant (Mahaffey 1991; Sims 1986), the season in which the spillage occurred, type of weather at the time of (and subsequent to) the pollution incident, and the soil composition (Alexander 1987; Harshner 1977; Lin 2002; Lin 1996; Lin 1998; Mendelssohn 1990; Pezeshki 2000).

Plant species have different responses to oil contamination, which means plant communities may be changed by the contamination. For example, if there is a mixture of two plants in one area, the one that is more oil contamination tolerant will come to dominate the area (Lin 1998). Other features of individual species can also be important in influencing their value as phytoremediators. For example, *Haloxylon salicornicum* can be a very good 'sand-trap' as it can grow to about 0.8 m - 1 m high, big enough to allow asymmetric "micro-nebkha" sand piles to form around them by aeolian deposit (Brown 2003). Charley (1975) stated that the formation of micro-nebkhas improves the amount of nutrients in the desert soil, and in oil-damaged soils they can also provide a relatively undamaged microhabitat (described as 'fertile islands' by Brown (2003)) for plant colonization, above the oil-damaged layers.

According to Le HoueHrou (1987) and Nickling (1994), the *Haloxylon* cover pre-oil damage in Kuwait used to be between 2 - 10% of the desert surface. During my fieldwork I found the same plant species associated with the *Haloxylon* as reported by Brown and (1998): namely *Plantago boissieri*, *Filago pyramidata,* and *Gypsophila capillaris*. In addition to maintaining the open vegetation structure and flora diversity in the area, the sand transportation and deposition around *Haloxylon* is of considerable importance in providing niches for several animal species.

Along with many other members of the Amaranthaceae, *Haloxylon salicornicum* is strongly adapted to tolerate environmental stress, allowing it to thrive in a very harsh and hostile environment, such as the conditions found in a dry oil lake. According to Harms (2003), many of the chenopods (now allocated to the family Amaranthaceae, but formerly Chenopodiaceae) are very tolerant of salinity and can grow in salt polluted areas. Considering that the conditions of the oil lakes are further complicated by high levels of salt, as sea water was used to extinguish the fires and saline residues were deposited all over the contaminated area, this additional complication further supports re-introducing *Haloxylon* as a main phytoremediator in this situation. *Haloxylon* have proven to be strong plants because they can absorb radionuclides (Harms 2003), and more relevantly, in the present instance, they can degrade small PAH molecules.

Haloxylon is a preferred species for camel grazing (Batanouny 1990 ; Brown 2003; Halwagy 1974; Halwagy 1982; Thalen 1979), and this is something that I personally noticed firsthand while conducting my field experiments in Kuwait. Clearly, protection from overgrazing, at least during the establishment period, will be necessary for the success of a planting programme for phytoremediation purposes. Subsequently, the re-established communities could be managed for controlled grazing. and more importantly it is been proven in this study so far that it can be a strong candidate to be a phytoremediator since it can tolerate and live on top of the oil contamination after experimenting it inside a greenhouse with different amount of oil contamination and in the filed on top of dry oil lake, plus finding PAHs in the *Haloxylon* tissues.

5.2 The Role of Mycorrhizal Association

Halocnemum strobilaceum is another chenopod native to Kuwait. Like *Haloxylon*, it is a perennial living in saline areas (Al-Mailem 2010). Yateem (2000) gave up testing the native plants due to the hard time that those plants gave the researcher in growing them in the first place, and it was suggested to use legumes or ornamental trees to undertake the rhizoremediation study. Nonetheless, Yateem (2000) found species of several microbes in the root system of the study plants (*Halobacterium* sp., *Halococcus* sp., *Brevibacillus borstelensis, Pseudoalteromonas ruthenica*, and *Halomonas sinaensis*). Those bacterial species are associated with hydrocarbon degradation for removing the aliphatic and aromatic hydrocarbons as a sole source of carbon and also as a powerful attenuator in nitrogen-free media with a broad range of salinity.

For everything mentioned before and from personal observation it is clearly important to focus on the perennials for phytoremediation, because in my opinion using annuals is not ideal for cleaning up the contaminated soil, since their life span is short and they tend to have only a very shallow root system, which means they can’t reach deep down where the oil contamination might seep into the soil surface which go very deep under the soil surface.

A study by Radwan (1998; 1995), examined the types of microbes associated with petroleum hydrocarbon in some native plants, mostly annuals from the Asteraceae family, *Cyperus* from the Cyperaceae family, and also *Salsola imbricate* (Syn. *Salsola foetida*) a chenopod from the Amaranthaceae family. Similar to my results, he found a few fungi (*Penicillium* sp. was one of them) as well as bacteria for example *Streptomyces* spp. (like our results from wild *Haloxylon*) and *Rhodococcus erythropolis* (which is the same genus

but different species to the *Rhodococcus manshanensis* which I found in 100% oil contaminated soil). He concluded that those microorganisms improve the hydrocarbon utilization in the rhizosphere, yet again he didn't take this study further to discover how efficient those species can be; therefore, further studies are needed.

Some chenopods have the capability to support fungal mycorrhiza (Gawronski 2007). In my study I found a small number of fungi on the rhizosphere of the *Haloxylon*, the most dominant and common fungus being *Penicillium*, which is also a very common species in the environment, but it is a strong participant in the degradation of the oil contaminants. It is also a species that has been discovered before by Radwan (1995) in oil contamination.

Fletcher (1995) reported degradation process stimulation by flavonoids (a molecule with six carbon rings) released into the rhizosphere of some plants, where those flavonoids would encourage the right microorganisms to grow. It has been suggested that the chemical structure similarity between those flavonoids and the PAHs contributes to the contaminant degradation by the microbes. This could be the same process for *Haloxylon*, since I found microbes/fungi bioremediators in the rhizosphere of this plant: again there is a clear need for further work here.

Considering the second (*Cyperus conglomeratus*) and the third (*Rhanterium epapposum*) species that I believe to be the best potential plant phytoremediators for use in oil pollution clean-up in Kuwait, the former is far more resistant to grazing than the latter. Accordingly, in Kuwait it did not suffer as much and its communities were not eroded the same way as the *Rhanterium* did in the north of Kuwait due to overgrazing by camels, goats and sheep (Brown 2003). According to (Gawronski 2007), the Cyperaceae also contain a number of strong, stress-tolerant species that can tolerate different types of soil pollution, and this supports my observations of *Cyperus conglomeratus* being the second strongest species after *Haloxylon* to live in soil overlying oil contamination. This was also suggested by Radwan (1998; 1995). Yet he found that the amount of nitrogen fixation in the legumes was higher than those in the wild desert plants he tested (*Cyperus conglomeratus* and *Salsola imbricata* as a perennial, and *Picris babylonica*, *Launaea mucronata* as annuals). The third species (*Rhanterium epapposum*) on my list of potential phytoremediators is a member of the Asteraceae family. According to Gawronski (2007), some species of this family have proved to be good phytoremediators for radionuclide pollutant clean-up. We can argue here that it needs to be tested for the hydrocarbon contaminants as well, since I

found that it was a strong species that could live in oil contaminated soils— at least in some of the polluted areas of Kuwait.

Based on several reports (Cunningham 1996; McIntire 1997; Raskin 1997), it is important to accelerate PAHs (or TPH) degradation and removal from the soil ecosystem. In order to do that, the biomass must be increased on top of the contaminated soil. A problem in using plants to clean up the soil is that, especially in desert conditions, biomass may only accumulate slowly. Also the amount of microbial biomass present might not, in such conditions, be optimal either for degradation purposes, or to assist plant growth (Balwin 1922; Glick 1995; Siciliano 1997).

Several studies have shown that using nitrogen fertilizers can be a very good addition to increase the rate of microbial degradation of pollutants in soil (Lehmann 1998; Prince 1993; Radwan 1995) as well as the biomass of the plants. Dashti (2009) showed that the root nodules for the *Vicia faba* (crop legume, which means it can help in nitrogen-fixation very efficiently) has a hydrocarbon-utilizing bacteria on its surface. This means it will contribute with the hydrocarbon utilizing bacteria on the surface of the rhizosphere to clean up the oil contamination from the soil. In the above mentioned study, it removed 58% of 0.5% weathered oil contamination concentration when mixed with desert soil.

Another way can be by using Plant Growth-Promoting Rhizobacteria (PGPR), where a careful and well thought-out usage of microbes can facilitate phytoremediation and that can be achieved by using rhizobacteria from. In addition, there is a need to use one or more specific types of bacteria that can degrade the contaminant (Ajithkumar 1998; Burd 1998; Siciliano 1997). I noticed in the green house experiment that the amount of TPH decreased drastically for the 25% and 50% treatments, which can indicates degradation.

Yi (2007) tested 43 different plant species in the lab. Of these, only 4 crop species reduced the amount of Pyrene (the type of PAHs tested) significantly. However, the study found that the PAH uptake was insignificant, as it was less than 0.01 mg/kg detected in the shoot and roots for the rest of them. In my greenhouse experiment, we saw that the presence of *Haloxylon* plants was associated with significant decrease in the amount of TPH in the soil in the 25-50% oil contamination treatments. The idea of contaminant translocation through the roots to the shoots is controversial, but Slaski (2000) emphasised that the mechanism might be dependent on the species and substrate in question.

Phytoremediation also has been shown to be successful in removing the large PAH molecules like Dibenzo (a, h) pyrene (DBP), benzo[ghi]perylene (BGP), Indo (123-cd) pyrene (IPY), which are very resistant to breakdown by other methods in landfarming or bioremediation (Huang 2003). The most important usage for the bioremediation is breaking down toxic organic molecules to carbon and water as a by-product, when the microorganisms use the carbon in the oil as an energy source, and once the source is consumed completely the microbes will die or go dormant (Balba 2003).

Inoculation of some specific types of microorganisms, known as bioaugmentation, enhances the metabolic capacity of the indigenous population of microbes (Gentry 2004). According to Bogan (2003), a microbe like *Mycobacterium* species can uptake hydrophobic contaminants, as it has a strong lipophilic surface, which means they can remove PAHs very easily. Based on Mueller (1989) this treatment can be used on aged contaminated soils which contain mainly high molecular weight (HMW) PAHs, because those molecules tend to accumulate during the remediation process, thus hindering it, or on a very limited amount of microbial population numbers with a limitation in adaptation, therefore increasing the effectiveness. There are numerous reports suggesting that bioaugmentation can be useful in improving the bioremediation of contaminated soil (Lendvay 2003; Silva 2004). On the other hand, the usage of this technique has often exhibited only limited success when applied on a field scale. Alexander (1999), and others, suggest that this procedure did not significantly improve the biodegradation (Bouchez 2000) . However, in our case it may be useful to test this technique in future studies to stimulate microbial growth, and therefore potentially provide more degradation, as an alternative to just depending on the microbes associated (if that is the case) with *Haloxylon* and its rhizosphere.

In a study by Huang (2003), landfarming, bioremediation and inoculation of plants with PGPR were used with phytoremediation to increase its efficiency. This approach proved able to remove most (78-88%) of the large hydrophobic PAHs under lab conditions. Several studies showed that using PGPR in landfarmed soil will help the plants germinate and grow, and more importantly will increase the amount of the root biomass. It is thought that PGPR alleviates the stress on the plants, which will help them to grow faster and gain higher root biomass (Glick 1998; Huang 2004; Huang 2001; Siciliano 1997). Fluoranthene and Pyrene can be removed by soil inoculation with PAH-degrading bacteria (Huang 2003).

The accumulation of PAH in plants can be affected by several factors which can be either biotic or abiotic, including the total leaf surface area (green stems in our case, or fruits in other cases), surrounding temperature, and the concentration of the lipid in the plant tissue. In the case of PAH compounds with intermediate volatility, they are usually subjected to dry gaseous deposition. On the other hand nonvolatile substances (with six or more rings) basically will be accumulated as dry, particle-bound deposits on the plant surface; the temperature can play a role in partitioning the PAHs on the vegetation and the atmosphere. For example, in the winter most of the conifers and evergreen plants will be scavenging for the emitted PAHs in the atmosphere since it is considered as an important sink and also important for the PAHs annual cycle, while in the summer the low molecular weight compounds of PAHs can revolatilize (Slaski 2000). This study concluded that the plants do uptake PAH directly from air.

Accordingly, the total leaf area can affect the accumulation and interception rate of airborne PAHs, where the volatile PAHs entered the plant via gaseous diffusion through the stomata. At the same time the waxy leaf surface (which intercepts the vapour and particulate contaminants) will contain some of the PAH making up the total amount of the PAH in the plant (Slaski 2000).

Further physiological studies are needed to see in better detail how plants process these compounds. Also, there is a need to see if any plants actually have a blocking system in their root system that would prevent specific contaminants from getting to the plants' tissues, so anatomical studies are in order too.

We need to understand more about the amount of lipid (cutin) concentration that might help in getting more of the PAH inside the plant by knowing the time of year to take those samples from the plants and the age of the plant, and the plant's condition; because the more lipid-like wax on the plant tissue the more it will trap the PAHs when exposed to the same amount of PAH (Slaski 2000). Lodovici (1998) said that the lipophilic compartment of leaves is powerfully bound to the PAHs; in this study they tried to wash away the foliar surface of *Laurus* with controlled water rinsing but it did not alter the PAH level in the leaves. Because my samples were not washed before testing the amount of PAHs in our specimens, we might assume that the plants could tolerate high levels of PAH whether the source is from the soil or from the air. Still that would just mean that we need to conduct more sample analysis, one where we wash the samples to see the amount of PAHs inside and the other test will be without washing; and we also need to measure the amount of

PAHs on the samples (leaf and stem surface) and inside (xylem, phloem) them to see exactly where most of the PAHs are actually trapped; if we found out that the amount of PAHs inside the samples is negligible, we can still enhance the results with the other techniques as mentioned before to make the *Haloxylon* or the other two species a better phytoremediator.

A lot of plants can be considered as markers when exposed to emissions as accumulators for the organic compounds and many studies have used vegetation as a marker of PAH exposure. Because the plants can be a cheap and effective way to act as an "air sampler" to accumulate PAH over time, this technique will be very useful especially in the remote areas to collect the plant tissue samples and analyse them; so those samples can be easily frozen and stored for analysis without any major loss of the PAH (Ignesti 1992; Lodovici 1994 ; Thomas 1985). In order to use biomonitoring, there are basic strategies to do that. The first one is called active monitoring and it can be defined as the

> "process in which plants have been grown in an artificial environment, without previous contact with a pollutant of interest" (Slaski 2000).

Where it is easy to recognize the time-dependent factor (the time from planting the plant till the day of collecting samples for analysis, thus knowing exactly the amount of PAHs it accumulated) and also can be standardized, for the passive monitoring it will be easy to compare between sites that don't have common and useful phytomonitors. Passive monitoring will depend on analysis of the native plant samples taken from their natural habitat (Slaski 2000). Combining several of these techniques is probably the best solution to the problem (Lin 1998).

The most important aim of my research was to help find a native plant phytoremediator that might be an ecologically viable option and hopefully a low cost way to clean up the Kuwaiti environment: low cost means hopefully less labour, less lab work, and less heavy machinery cost. So with all the techniques mentioned above, if they are going to cost more or equal or even higher but with better ecological results in the long run then we shouldn't work with them or try to find other ways or compromise to find the best solution.

In final summary the recommendations arising from my study are:

- Since the *Haloxylon salicornicum* has proven to be of great value for the phytoremediation process, I would recommend using it on the oil-polluted soil in

Kuwait, especially inside the oil KOC where the oil production company is, with the need to conduct more studies to have a full evaluation of its abilities.

- I would recommend that *Haloxylon* be propagated and planted on a large scale, primarily in polluted areas, but in clean areas also, so it will enhance the wildlife in the area.

- Genetic studies are required for *Haloxylon* to find the stress-tolerance gene(s) that is/are responsible for its ability to withstand that level of pollution without dying, hence using it for genetic engineering to strengthen the plants in case of another environmental catastrophe of this kind; therefore, we would have plants ready to be used, to clean it up.

- Further physiological studies are needed to know how *Haloxylon* deals with the contamination inside its system, or how it neutralizes its toxic ability.

- Anatomical studies are also needed to see the effect of the PAHs on the cells of *Haloxylon* plants.

- More studies about the association of those microbes (if there is any) with the *Haloxylon salicornicum, Cyperus conglomeratus,* and *Rhanterium epapposum* rhizosphere, to know if the plant actually can stimulate or help them to grow, therefore a better oil degradation.

- Further studies are due for *Cyperus conglomeratus*, and *Rhanterium epapposum* as well with everything that I've made here before, or as much as all those three species confirms their abilities to clean up oil contamination.

Appendix 1:

- Kruskal-Wallis Test: Plant height versus Area (Bahra (1), Sabah AL-Ahmad Protected area (2), Burgan (3), Um Alaish (4), Um Al-Rros (5), Sabriya (6), Um-Ghadair (7)).

Kruskal-Wallis Test on Plant height

Area	N	Median	Ave Rank	Z
1	30	6.500	70.9	-3.04
2	45	7.000	90.3	-1.35
3	30	10.600	106.1	0.57
4	35	17.800	131.6	3.50
5	10	1.000	20.1	-4.51
6	30	13.400	131.5	3.18
7	20	10.100	98.8	-0.14
Overall	200		100.5	

H = 47.56 DF = 6 P = 0.000
H = 47.57 DF = 6 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: Plant height versus Direction (North (1), South (2))

Kruskal-Wallis Test on Plant height

Direction	N	Median	Ave Rank	Z
1	150	9.200	99.6	-0.38
2	50	10.300	103.2	0.38
Overall	200		100.5	

H = 0.14 DF = 1 P = 0.707
H = 0.14 DF = 1 P = 0.707 (adjusted for ties)

-Kruskal-Wallis Test: Species Diversity versus Area (Bahra (1), Sabah AL-Ahmad Protected area (2), Burgan (3), Um Alaish (4), Um Al-Rros (5), Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on Species Diversity

Area	N	Median	Ave Rank	Z
1	30	1.000	84.0	-1.69
2	45	2.000	117.0	2.17
3	30	1.000	76.4	-2.47
4	35	3.000	131.9	3.53
5	10	1.000	48.5	-2.92
6	30	2.000	119.4	1.94
7	20	1.000	67.0	-2.73
Overall	200		100.5	

H = 39.59 DF = 6 P = 0.000
H = 42.85 DF = 6 P = 0.000 (adjusted for ties)

-Kruskal-Wallis Test: Species Diversity versus Direction (North (1), South (2))

Kruskal-Wallis Test on Sp. Diversity

Direction	N	Median	Ave Rank	Z

1	150	2.000	109.8	3.93
2	50	1.000	72.6	-3.93
Overall	200		100.5	

H = 15.46 DF = 1 P = 0.000
H = 16.73 DF = 1 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: *Haloxylon salicornicum* versus Area (Bahra (1), Sabah AL-Ahmad Protected area (2), Um Alaish (4))

Kruskal-Wallis Test on *Haloxylon salicornicum*

Area	N	Median	Ave Rank	Z
1	30	5.30000E+01	63.0	1.52
2	45	0.000000000	29.5	-7.10
4	35	1.60000E+02	82.4	6.05
Overall	110		55.5	

H = 56.42 DF = 2 P = 0.000
H = 60.87 DF = 2 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: *Cyperus conglomeratus* versus Area (Bahra (1), Sabah AL-Ahmad Protected area (2), Burgan (3), Sabriya (6), Um-Ghadair (7)).

Kruskal-Wallis Test on *Cyperus conglomeratus*

Area	N	Median	Ave Rank	Z
1	30	0.000000000	53.2	-3.37
2	45	0.000000000	51.9	-4.63
3	30	2.55000E+01	90.8	1.74
6	30	5.40000E+01	104.1	3.55
7	20	7.75000E+01	115.5	4.00
Overall	155		78.0	

H = 50.92 DF = 4 P = 0.000
H = 66.79 DF = 4 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: *Cyperus conglomeratus* versus Direction (North (1), South (2))

Kruskal-Wallis Test on *Cyperus conglomeratus*

Direction	N	Median	Ave Rank	Z
1	105	0.000000000	67.2	-4.34
2	50	3.60000E+01	100.7	4.34
Overall	155		78.0	

H = 18.84 DF = 1 P = 0.000
H = 24.71 DF = 1 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: *Rhanterium epapposum* versus Area (Sabah AL-Ahmad Protected area (2), Um Alaish (4), Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on *Rhanterium epapposum*

Area	N	Median	Ave Rank	Z
2	45	0.000000000	57.4	-1.79
4	35	0.000000000	53.5	-2.21
6	30	2.55000E+01	90.0	4.07

7	20	0.000000000	68.0	0.33
Overall	130		65.5	

H = 18.48 DF = 3 P = 0.000
H = 36.76 DF = 3 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: *Rhanterium epapposum* versus Direction (North (1), South (2))

Kruskal-Wallis Test on *Rhanterium epapposum*

Direction	N	Median	Ave Rank	Z
1	110	0.000000000	65.0	-0.33
2	20	0.000000000	68.0	0.33
Overall	130		65.5	

H = 0.11 DF = 1 P = 0.742
H = 0.22 DF = 1 P = 0.643 (adjusted for ties)

-Oil Damage Score between seven areas (Bahra (1), Sabah AL-Ahmad Protected area (2), Burgan (3), Um Alaish (4), Um Al-Rros (5), Sabriya (6), Um-Ghadair (7)) using Kruskal – Wallis test

Kruskal-Wallis Test on Oil Damages

Area	N	Median	Ave Rank	Z
1	30	1.000	80.0	-2.10
2	45	1.000	49.5	-6.71
3	30	3.000	120.2	2.02
4	35	3.000	125.2	2.78
5	10	1.000	49.5	-2.86
6	30	3.000	155.5	5.65
7	20	2.500	116.3	1.28
Overall	200		100.5	

H = 84.88 DF = 6 P = 0.000
H = 107.31 DF = 6 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test on Oil Damage Score versus Direction (North (1), South (2))

Direction	N	Median	Ave Rank	Z
1	150	1.000	94.5	-2.55
2	50	3.000	118.6	2.55
Overall	200		100.5	

H = 6.52 DF = 1 P = 0.011
H = 8.24 DF = 1 P = 0.004 (adjusted for ties)

- Kruskal-Wallis Test: Chrysene (CH) versus Area (Bahra (1), Burgan (3), Um Alaish (4), Sabriya (6), Um-Ghadair (7)).

Kruskal-Wallis Test on Chrysene (CH)

Area	N	Median	Ave Rank	Z
1	26	0.000000000	62.6	-0.58
3	21	0.000000000	48.0	-2.41
4	35	0.000000000	64.7	-0.33
6	30	4.535000000	95.2	4.67
7	20	0.000000000	51.1	-1.95
Overall	132		66.5	

H = 25.33 DF = 4 P = 0.000
H = 31.91 DF = 4 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: Phenanthrene (PH) versus Area (Bahra (1), Sabah AL-Ahmad Protected area (2), Burgan (3), Um Alaish (4), Sabriya (6), Um-Ghadair (7)).

Kruskal-Wallis Test on Phenanthrene (PH)

Area	N	Median	Ave Rank	Z
1	26	0.425500000	104.7	1.69
2	45	0.000000000	64.3	-3.74
3	21	0.000000000	74.2	-1.41
4	35	0.000000000	84.8	-0.54
6	30	1.425000000	137.6	5.70
7	20	0.000000000	74.0	-1.39
Overall	177		89.0	

H = 43.56 DF = 5 P = 0.000
H = 60.02 DF = 5 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: Pyrene (PY) versus Area (Bahra (1), Burgan (3), Um Alaish (4), Sabriya (6)).

Kruskal-Wallis Test on Pyrene (PY)

Area	N	Median	Ave Rank	Z
1	26	0.000000000	57.0	0.09
3	21	0.000000000	44.0	-1.96
4	35	0.000000000	49.3	-1.58
6	30	0.807500000	73.2	3.29
Overall	112		56.5	

H = 12.76 DF = 3 P = 0.005
H = 18.91 DF = 3 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: Anthracene (ANTH) versus Area (Bahra (1), Burgan (3), Sabriya (6))

Kruskal-Wallis Test on Anthracene (ANTH)

Area	N	Median	Ave Rank	Z
1	26	0.000000000	39.3	0.10
3	21	0.000000000	38.4	-0.15
6	30	0.000000000	39.1	0.04
Overall	77		39.0	

H = 0.02 DF = 2 P = 0.988
H = 0.13 DF = 2 P = 0.938 (adjusted for ties)

- Kruskal-Wallis Test: Fluoranthene (FLAN) versus Area (Bahra (1), Burgan (3), Um Alaish (4), Sabriya (6))

Kruskal-Wallis Test on Fluoranthene (FLAN)

Area	N	Median	Ave Rank	Z
1	26	0.000000000	57.6	0.20
3	21	0.000000000	48.9	-1.19
4	35	0.000000000	49.2	-1.61
6	30	0.000000000	69.4	2.54

```
Overall  112                    56.5

H = 7.70  DF = 3  P = 0.053
H = 16.60  DF = 3  P = 0.001  (adjusted for ties)
```

- One-way ANOVA: log P.T.A. Phenanthrene (PH) versus Area (Um Alaish (4), Sabriya (6), Um-Ghadair (7))

```
Source  DF      SS      MS     F      P
Area     2  0.8088  0.4044  5.12  0.009
Error   63  4.9797  0.0790
Total   65  5.7885

S = 0.2811   R-Sq = 13.97%   R-Sq(adj) = 11.24%


                           Individual 95% CIs For Mean Based on
                           Pooled StDev
Level   N    Mean   StDev   -+---------+---------+---------+--------
4      35  4.5902  0.1959      (--*-)
6      30  4.4442  0.3560   (-*--)
7       1  5.2095       *          (-------------*-------------)
                            -+---------+---------+---------+--------
                           4.40      4.80      5.20      5.60

Pooled StDev = 0.2811


Grouping Information Using Tukey Method

Area   N    Mean  Grouping
7      1  5.2095  A
4     35  4.5902  A B
6     30  4.4442    B

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Area

Individual confidence level = 98.05%
Area = 4 subtracted from:

Area    Lower   Center   Upper  --------+---------+---------+---------+-
6     -0.3137  -0.1460  0.0217                (-*-)
7     -0.0642   0.6193  1.3028                   (--------*-------)
                                --------+---------+---------+---------+-
                                     -0.80      0.00      0.80      1.60


Area = 6 subtracted from:

Area   Lower  Center   Upper  --------+---------+---------+---------+-
7     0.0803  0.7653  1.4504                      (--------*-------)
                             --------+---------+---------+---------+-
                                  -0.80      0.00      0.80      1.60
```

- One-way ANOVA: log P.T.A. Benzo(a)anthracene (B) versus Area (Um Alaish (4), Sabriya (6), Um-Ghadair (7)).

Source	DF	SS	MS	F	P
Area	2	24.10	12.05	10.43	0.000
Error	63	72.75	1.15		
Total	65	96.84			

S = 1.075 R-Sq = 24.88% R-Sq(adj) = 22.50%

			Individual 95% CIs For Mean Based on Pooled StDev
Level	N	Mean	StDev
4	35	0.135	0.447
6	30	1.344	1.508
7	1	0.000	*

-1.2 0.0 1.2 2.4

Pooled StDev = 1.075

Grouping Information Using Tukey Method

Area	N	Mean	Grouping
6	30	1.344	A
4	35	0.135	B
7	1	0.000	A B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Area

Individual confidence level = 98.05%

Area = 4 subtracted from:

Area	Lower	Center	Upper
6	0.568	1.209	1.850
7	-2.747	-0.135	2.478

-4.0 -2.0 0.0 2.0

Area = 6 subtracted from:

Area	Lower	Center	Upper
7	-3.963	-1.344	1.274

-4.0 -2.0 0.0 2.0

- One-way ANOVA: log P.T.A. Fluoranthene (FLAN) versus Area (Um Alaish (4), Sabriya (6), Um-Ghadair (7)).

Source	DF	SS	MS	F	P
Area	2	0.7810	0.3905	12.38	0.000
Error	63	1.9874	0.0315		
Total	65	2.7684			

S = 0.1776 R-Sq = 28.21% R-Sq(adj) = 25.93%

```
                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level   N    Mean   StDev  -----+---------+---------+---------+----
4      35  3.6533  0.1517       (--*--)
6      30  3.8716  0.2038                 (---*--)
7       1  3.8607       *  (-----------------*-----------------)
                           -----+---------+---------+---------+----
                              3.60      3.80      4.00      4.20

Pooled StDev = 0.1776


Grouping Information Using Tukey Method

Area   N    Mean  Grouping
6     30  3.8716  A
7      1  3.8607  A B
4     35  3.6533    B

Means that do not share a letter are significantly different.


Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Area

Individual confidence level = 98.05%


Area = 4 subtracted from:

Area    Lower  Center   Upper  -------+---------+---------+---------+--
6      0.1124  0.2183  0.3242                       (--*--)
7     -0.2243  0.2075  0.6393            (-----------*-----------)
                          -------+---------+---------+---------+--
                              -0.35      0.00      0.35      0.70


Area = 6 subtracted from:

Area    Lower   Center   Upper  -------+---------+---------+---------+--
7     -0.4436  -0.0108  0.4220      (-----------*-----------)
                          -------+---------+---------+---------+--
                              -0.35      0.00      0.35      0.70
```

- One-way ANOVA: log P.T.A. Pyrene (PY) versus Area Um Alaish (4), Sabriya (6), Um-Ghadair (7))

```
Source  DF      SS      MS     F      P
Area     2  0.4724  0.2362  6.16  0.004
Error   63  2.4142  0.0383
Total   65  2.8866

S = 0.1958   R-Sq = 16.36%   R-Sq(adj) = 13.71%


                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level   N    Mean   StDev  ---+---------+---------+---------+------
4      35  3.0765  0.1654               (--*--)
6      30  3.2474  0.2262                        (--*---)
7       1  3.1311       *  (------------------*------------------)
```

```
                                  ---+---------+---------+---------+------
                                   2.80      3.00      3.20      3.40
```

Pooled StDev = 0.1958

Grouping Information Using Tukey Method

Area	N	Mean	Grouping
6	30	3.2474	A
7	1	3.1311	A B
4	35	3.0765	B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons among Levels of Area

Individual confidence level = 98.05%

Area = 4 subtracted from:

```
Area    Lower  Center   Upper  ---------+---------+---------+---------+
6      0.0541  0.1709  0.2877                    (--*--)
7     -0.4213  0.0546  0.5305          (-------------*------------)
                              ---------+---------+---------+---------+
                                    -0.35      0.00      0.35      0.70
```

Area = 6 subtracted from:

```
Area    Lower   Center   Upper  ---------+---------+---------+---------+
7     -0.5933  -0.1163  0.3607     (-------------*------------)
                              ---------+---------+---------+---------+
                                    -0.35      0.00      0.35      0.70
```

- Kruskal-Wallis Test: P.T.A. Chrysene (CH) versus Area (Um Alaish (4), Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on P.T.A. Chrysene (CH)

Area	N	Median	Ave Rank	Z
4	35	5.510000000	29.6	-1.74
6	30	6.245000000	39.0	2.11
7	1	0.000000000	5.0	-1.50
Overall	66		33.5	

H = 6.06 DF = 2 P = 0.048
H = 6.08 DF = 2 P = 0.048 (adjusted for ties)

* NOTE * One or more small samples

- Kruskal-Wallis Test: P.T.A. Naphthalene (NA) versus Area (Um Alaish (4), Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on P.T.A. Naphthalene(NA)

Area	N	Median	Ave Rank	Z
4	35	20.60	23.9	-4.34
6	30	29.15	44.9	4.40
7	1	21.10	29.5	-0.21
Overall	66		33.5	

H = 19.42 DF = 2 P = 0.000
H = 19.43 DF = 2 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

- Kruskal-Wallis Test: P.T.A Anthracene (ANTH) versus Area (Um Alaish (4), Um Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on P.T.A Anthracene (ANTH)

Area	N	Median	Ave Rank	Z
4	35	0.000000000	26.8	-3.03
6	30	4.795000000	40.5	2.72
7	1	6.590000000	58.0	1.29
Overall	66		33.5	

H = 9.99 DF = 2 P = 0.007
H = 11.28 DF = 2 P = 0.004 (adjusted for ties)

- Kruskal-Wallis Test: P.T.A. Fluorene (FL) versus Area (Um Alaish (4), Um Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on P.T.A. Fluorene (FL)

Area	N	Median	Ave Rank	Z
4	35	10.200	26.0	-3.39
6	30	24.500	42.6	3.52
7	1	8.440	24.0	-0.50
Overall	66		33.5	

H = 12.42 DF = 2 P = 0.002
H = 12.42 DF = 2 P = 0.002 (adjusted for ties)

* NOTE * One or more small samples

- Kruskal-Wallis Test: P.T.A. P-moist versus Area (Um Alaish (4), Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on P.T.A. P-moist

Area	N	Median	Ave Rank	Z
4	35	54.90	21.0	-5.63
6	30	69.75	48.4	5.76
7	1	59.50	25.0	-0.45
Overall	66		33.5	

H = 33.18 DF = 2 P = 0.000
H = 33.18 DF = 2 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

- Kruskal-Wallis Test: P-moist versus Area (Bahra (1), Sabah AL-Ahmad Protected area (2), Burgan (3), Um Alaish (4), Um Al-Rros (5), Sabriya (6), Um-Ghadair (7))

Kruskal-Wallis Test on P-moist

Area	N	Median	Ave Rank	Z
1	26	1.4700	69.6	-2.37
2	45	2.9600	97.5	0.72
3	21	0.6960	57.3	-3.22
4	35	5.6700	133.6	5.08
5	7	3.8000	111.4	0.96
6	30	3.2100	101.9	1.05
7	20	0.8285	55.3	-3.31
Overall	184		92.5	

H = 46.82 DF = 6 P = 0.000
H = 46.82 DF = 6 P = 0.000 (adjusted for ties)

***Frequency dataset: May11**

TWINSPAN end-groups membership.

Letter codes in sample names are as follows: Bahra oil field BA; Sabah AL-Ahmad **Protected area** SA; Burgan oil field BU;, Um Alaish oil field UM; Um Al-Rros military base UA; Sabriya oil field SB; Um-Ghadair UG.

A Items in NEGATIVE group 8 (N= 6) i.e. group *000

25SA0551 26SA0561 36SA0661 37SA0671 38SA0681 40SA0701

B Items in POSITIVE group 9 (N= 75) i.e. group *001

01BA0013 02BA0022 03BA0031 04BA0042 05BA0051 06BA0062 07BA0071 08BA0081 09BA0091 10BA0101 11BA0112 14BA0141 15BA0151 16BA0163 17BA0172 18BA0182 19BA0192 20BA0201 21BA0211 22BA0221 23BA0231 24BA0243 25BA0251 26BA0261 27BA0271 28BA0281 29BA0292 30BA0301 21SA0511 27SA0571 28SA0581 29SA0591 30SA0601 31SA0611 43SA0731 44SA0741 01UM1063 02UM1073 03UM1083 04UM1093 05UM1103 06UM1113 07UM1123 08UM1133 09UM1143 10UM1153 11UM1162 12UM1172 13UM1182 14UM1192 15UM1202 16UM1211 17UM1221 18UM1231 19UM1241 20UM1251 02UG1573 03UG1583 07UG1623 08UG1633 09UG1643 13UG1682 23UM1783 24UM1793 25UM1803 26UM1813 27UM1823 28UM1833 29UM1843 30UM1853 31SB1961 32SB1971 33SB1981 34SB1991 35SB2001

Eigenvalue 0.790 at iteration 5

INDICATORS, together with their SIGN: Hasa (*Haloxylon salicornicum*) 1(+) Coce (*Convolvulus cephalopod*) 1(-) Trst (*Trigonella stellata*) 1(-) Gysi (*Gynandriris sisyrinchium*) 1(-) Puun (*Pulicaria undulata*) 5(-) Stpl (*Stipagrostis plumose*) 1(+)

C Items in NEGATIVE group 10 (N= 66) i.e. group *010

12BA0123 34SA0641 41SA0711 42SA0721 45SA0751 01BU0763 02BU0773 03BU0783 04BU0793 05BU0803 06BU0813 07BU0823 08BU0833 09BU0843 11BU0863 12BU0873 13BU0883 14BU0893 15BU0903 16BU0913 20BU0953 23BU0981 01SB1363 02SB1373 03SB1383 04SB1393 05SB1403 06SB1413

07SB1423 08SB1433 09SB1443 10SB1453 11SB1463 12SB1473 13SB1483 14SB1493 15SB1503 16SB1513 17SB1523 18SB1533 19SB1543 20SB1553 01UG1563 04UG1593 05UG1603 06UG1613 10UG1653 11UG1662 12UG1672 14UG1692 15UG1702 16UG1711 17UG1721 18UG1731 19UG1741 20UG1751 21SB1863 22SB1873 23SB1883 24SB1893 25SB1903 26SB1913 27SB1923 28SB1933 29SB1943 30SB1953

D Items in POSITIVE group 11 (N= 12) i.e. group *011

18BU0933 19BU0943 21BU0961 22BU0971 24BU0991 25BU1001 26BU1011 27BU1021 28BU1031 30BU1051 21UM1763 22UM1773

Eigenvalue 0.828 at iteration 6

INDICATORS, together with their SIGN: Zyqa (*Zygophyllum qatarense*) 1(+) Cyco (*Cyperus conglomeratus*) 3(-) Saim (*Salsola imprecata*) 3(+) Rhep (*Rhanterium epapposum*) 1(-)

E Items in NEGATIVE group 12 (N= 23) i.e. group *100

01SA0311 02SA0321 03SA0331 04SA0341 05SA0351 06SA0361 07SA0371 08SA0381 09SA0391 10SA0401 11SA0411 12SA0421 14SA0441 01UA1261 02UA1271 03UA1281 04UA1291 05UA1301 06UA1311 07UA1321 08UA1331 09UA1341 10UA1351

F Items in POSITIVE group 13 (N= 9) i.e. group *101

13SA0431 15SA0451 16SA0461 17SA0471 18SA0481 19SA0491 20SA0501 33SA0631 10BU0853

Eigenvalue 0.583 at iteration 5

INDICATORS, together with their SIGN: Pedi (*Pennisetum divisum*) 1(+) Fabr (*Fagonia bruguieri*) 1(-)

G Items in POSITIVE group 7 (N= 4) i.e. group *11

32SA0621 35SA0651 39SA0691 29BU1041

Eigenvalue 0.716 at iteration 6

INDICATORS, together with their SIGN: Hebe (*Heliotropium bacciferum*) 1(+) Cico (*Cistanche tubulosa*) 1(+)

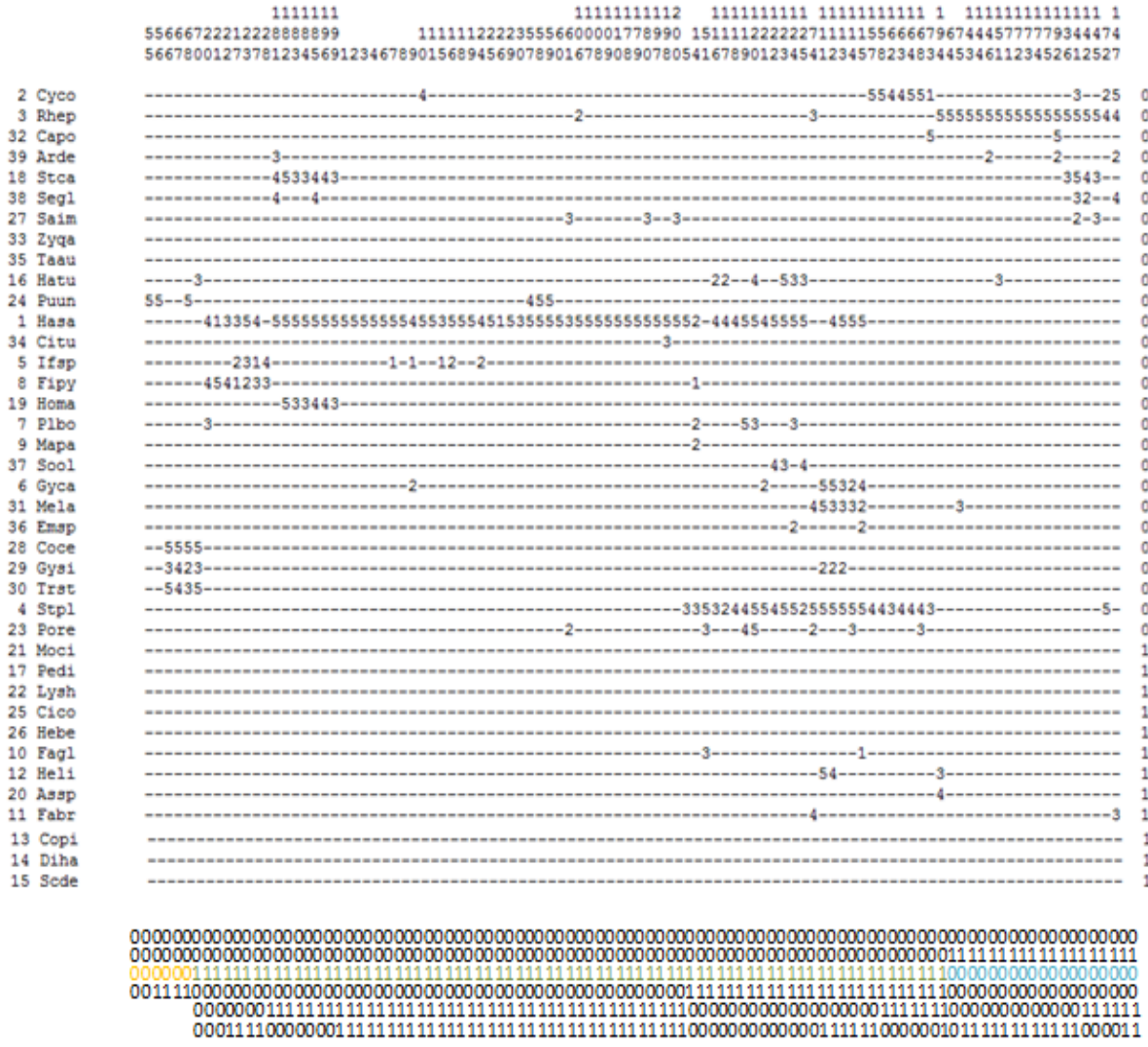
H Samples with no vegetation form this group

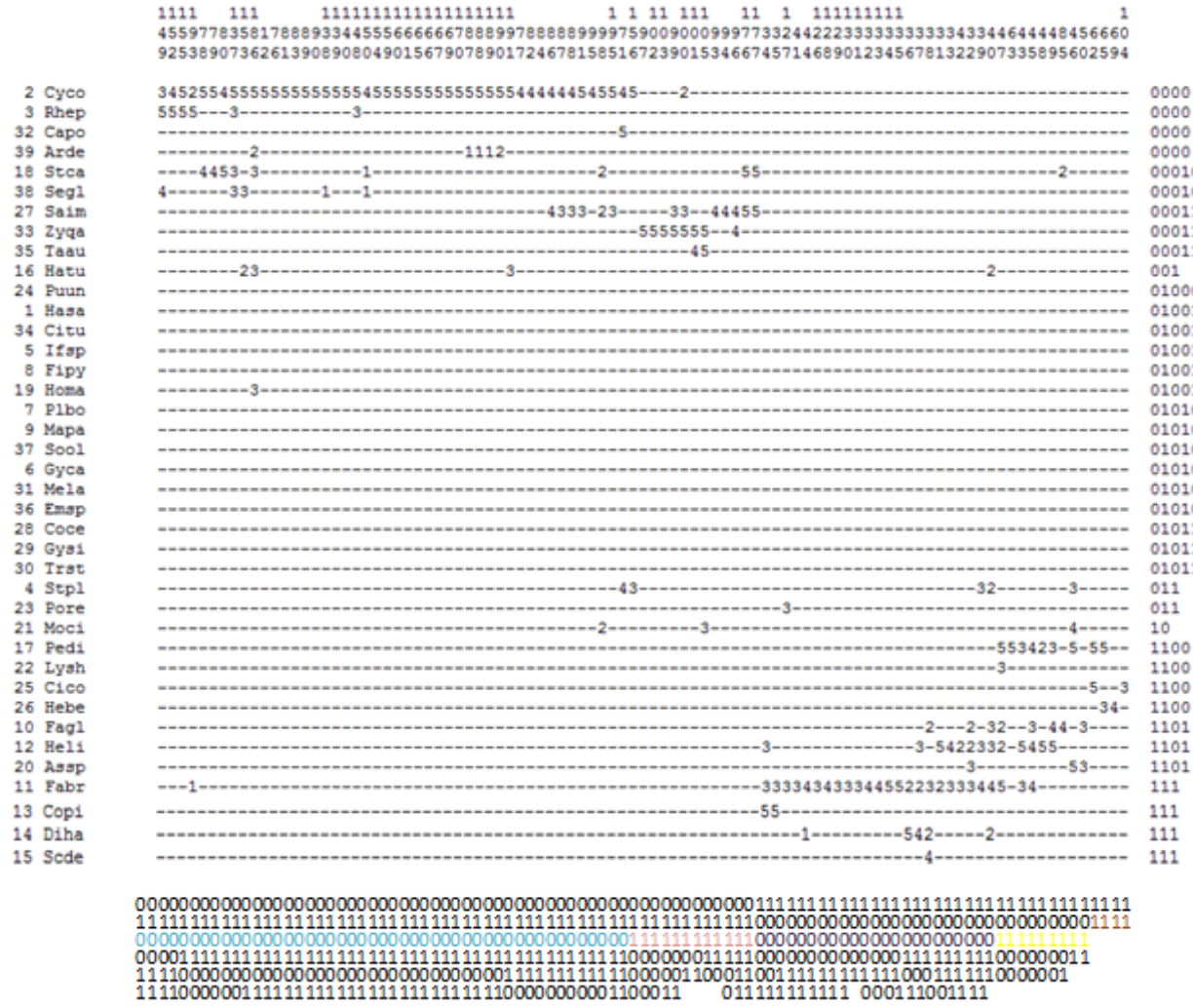
13BA0133 22SA0521 23SA0531 24SA0541 17BU0923

-DCA eigenvalues for Axis 1 – 4 of the ordination:

Axes	1	2	3	4	Total inertia
Eigenvalues	0.972	0.903	0.797	0.563	16.029
Lengths of	10.696	8.309	3.840	3.322	

gradient					
Cumulative percentage variance of species data	6.1	11.7	16.7	20.2	
Sum of all eigenvalues					16.029





Key: Group A, Group B, Group C, Group D, Group E, Group F, Group G

- Plants Heights Kruskal-Wallis Test: TWINSPAN Groups versus Area collection (1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H))

Kruskal-Wallis Test on TWINSPAN Groups

Area collection	N	Median	Ave Rank	Z
1	6	6.000	51.0	-2.07
2	75	10.200	103.0	0.99
3	66	11.300	110.7	2.25
4	12	22.700	148.5	3.20
5	23	2.000	29.8	-6.17
6	9	8.000	96.6	-0.08
7	4	9.500	107.9	0.35
Overall	195		98.0	

H = 51.40 DF = 6 P = 0.000
H = 51.41 DF = 6 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

- Species Diversity One-way ANOVA: Log A, Log B, Log C, Log D, Log E, Log F, Log G

Source	DF	SS	MS	F	P
Factor	6	2.083	0.347	2.66	0.017
Error	188	24.568	0.131		
Total	194	26.651			

S = 0.3615 R-Sq = 7.81% R-Sq(adj) = 4.87%

				Individual 95% CIs For Mean Based on Pooled StDev
Level	N	Mean	StDev	--------+---------+---------+---------+-
Log A	6	1.0769	0.6128	(-----------*-----------)
Log B	75	1.1873	0.3872	(--*---)
Log C	66	1.0003	0.3175	(--*---)
Log D	12	0.9776	0.2717	(-------*-------)
Log E	23	0.9940	0.3475	(-----*-----)
Log F	9	1.2832	0.4197	(--------*---------)
Log G	4	0.8959	0.2341	(-------------*-------------)
				--------+---------+---------+---------+-
				0.75 1.00 1.25 1.50

Pooled StDev = 0.3615

Grouping Information Using Tukey Method

	N	Mean	Grouping
Log F	9	1.2832	A B
Log B	75	1.1873	A
Log A	6	1.0769	A B
Log C	66	1.0003	B
Log E	23	0.9940	A B
Log D	12	0.9776	A B
Log G	4	0.8959	A B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 99.68%

Log A subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
Log B	-0.3473	0.1103	0.5680	(-------*------)
Log C	-0.5365	-0.0766	0.3834	(-------*------)
Log D	-0.6387	-0.0993	0.4401	(--------*--------)
Log E	-0.5774	-0.0829	0.4116	(--------*-------)
Log F	-0.3622	0.2063	0.7749	(--------*---------)
Log G	-0.8773	-0.1810	0.5153	(-----------*-----------)
				-------+---------+---------+---------+--
				-0.60 0.00 0.60 1.20

Log B subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
Log C	-0.3690	-0.1869	-0.0049	(--*--)
Log D	-0.5450	-0.2096	0.1258	(-----*----)
Log E	-0.4503	-0.1932	0.0639	(----*---)
Log F	-0.2846	0.0960	0.4765	(------*-----)
Log G	-0.8449	-0.2914	0.2622	(--------*--------)
				-------+---------+---------+---------+--
				-0.60 0.00 0.60 1.20

Log C subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
Log D	-0.3612	-0.0227	0.3158	(-----*----)
Log E	-0.2675	-0.0063	0.2549	(---*---)
Log F	-0.1004	0.2829	0.6662	(------*-----)
Log G	-0.6599	-0.1045	0.4510	(--------*---------)
				-------+---------+---------+---------+--
				-0.60 0.00 0.60 1.20

Log D subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
Log E	-0.3677	0.0164	0.4005	(-----*------)
Log F	-0.1701	0.3056	0.7813	(-------*-------)
Log G	-0.7045	-0.0817	0.5411	(----------*---------)
				-------+---------+---------+---------+--
				-0.60 0.00 0.60 1.20

Log E subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
Log F	-0.1349	0.2892	0.7133	(------*------)
Log G	-0.6825	-0.0981	0.4862	(--------*---------)
				-------+---------+---------+---------+--
				-0.60 0.00 0.60 1.20

Log F subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
Log G	-1.0356	-0.3873	0.2609	(----------*---------)
				-------+---------+---------+---------+--
				-0.60 0.00 0.60 1.20

- *Haloxylon salicornicum* Kruskal-Wallis Test: TWINSPAN Groups versus Area collection (2 (B), 7(G))

Kruskal-Wallis Test on B, G collec after division by 4

Area collection nozeroes	N	Median	Ave Rank	Z
2	75	1.87500E+01	41.7	2.77
7	4	0.000000000	9.0	-2.77
Overall	79		40.0	

H = 7.69 DF = 1 P = 0.006
H = 7.72 DF = 1 P = 0.005 (adjusted for ties)

* NOTE * One or more small samples

- *Cyperus conglomeratus* Kruskal-Wallis Test: Groups B, C collected versus Area collection (2 (B), 3 (C))

Kruskal-Wallis Test on Groups B, C collected

Area collection	N	Median	Ave Rank	Z
2	62	0.000000000	43.6	-6.16
3	66	5.30000E+01	84.1	6.16
Overall	128		64.5	

H = 38.01 DF = 1 P = 0.000
H = 45.44 DF = 1 P = 0.000 (adjusted for ties)

- *Rhanterium epapposum* Kruskal-Wallis Test: Groups B, C, D versus Area collection (2 (B), C (3), D (4))

Kruskal-Wallis Test on Groups B, C, D

Area collection	N	Median	Ave Rank	Z
2	75	0.000000000	60.0	-4.64
3	66	5.30000E+01	101.2	5.88
4	12	0.000000000	50.0	-2.20
Overall	153		77.0	

H = 35.10 DF = 2 P = 0.000
H = 44.45 DF = 2 P = 0.000 (adjusted for ties)

- Kruskal-Wallis Test: TWINSPAN Groups versus Oil Damage Score of TWINSPAN Groups versus (1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H))

Kruskal-Wallis Test on TWINSPAN Groups

Area Collection	N	Median	Ave Rank	Z
1	6	1.000	49.0	-2.21
2	75	2.000	97.5	-0.56
3	66	3.000	138.4	6.49
4	12	1.000	85.3	-0.94
5	23	1.000	49.0	-4.54

6	9	1.000	61.1	-2.09
7	4	1.000	49.0	-1.80
8	5	1.000	92.6	-0.31
Overall	200		100.5	

H = 59.66 DF = 7 P = 0.000
H = 73.79 DF = 7 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

- P-moist Kruskal-Wallis Test: TWINSPAN group right versus Area Collection (1 (A), 2 (B), 3 (C), 4 (D), 5 (E), 6 (F), 7 (G), 8 (H))

Kruskal-Wallis Test on TWINSPAN group

Area Collection	N	Median	Ave Rank	Z
1	6	3.7700	119.2	1.47
2	71	2.6400	92.7	0.80
3	59	1.9600	72.2	-3.08
4	11	5.1400	112.1	1.55
5	13	6.5300	143.2	3.96
6	9	1.8500	75.8	-0.79
7	4	0.3735	39.9	-1.94
8	4	1.4950	63.4	-1.01
Overall	177		89.0	

H = 30.85 DF = 7 P = 0.000
H = 30.85 DF = 7 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

- Kruskal-Wallis Test: B, C, D TWINSPAN groups versus Area collection Chrysene (CH) (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on B, C, D TWINSPAN groups

Area collection	N	Median	Ave Rank	Z
2	71	0.000000000	65.3	-1.68
3	59	0.000000000	78.3	1.80
4	11	0.000000000	68.9	-0.18
Overall	141		71.0	

H = 3.31 DF = 2 P = 0.192
H = 4.42 DF = 2 P = 0.110 (adjusted for ties)

- Fluoranthene (FLAN) Kruskal-Wallis Test: TWINSPAN Groups B, C versus Area collection (2 (B), 3 (C))

Kruskal-Wallis Test on TWINSPAN Groups B, C

Area collection	N	Median	Ave Rank	Z
2	71	0.000000000	61.8	-1.22
3	59	0.000000000	69.9	1.22
Overall	130		65.5	

H = 1.48 DF = 1 P = 0.223
H = 3.61 DF = 1 P = 0.057 (adjusted for ties)

- Pyrene (PY) Kruskal-Wallis Test: B, C, D TWINSPAN versus Area collection (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on B, C, D TWINSPAN

Area collection	N	Median	Ave Rank	Z
2	71	0.000000000	66.3	-1.36
3	59	0.000000000	77.4	1.58
4	11	0.000000000	66.7	-0.37
Overall	141		71.0	

H = 2.50 DF = 2 P = 0.287
H = 4.34 DF = 2 P = 0.114 (adjusted for ties)

- Phenanthrene (PH) Kruskal-Wallis Test: B, C, D, E, H TWINSPAN Groups versus Area collection (2 (B), 3 (C), 4 (D), 5 (E), 8 (H))

Kruskal-Wallis Test on B, C, D, E, H TWINSPAN GROUPS

Area collection	N	Median	Ave Rank	Z
2	71	0.000000000	79.8	0.08
3	59	0.000000000	84.7	1.11
4	11	0.000000000	68.8	-0.80
5	13	0.000000000	67.2	-1.02
8	4	0.000000000	66.3	-0.59
Overall	158		79.5	

H = 2.66 DF = 4 P = 0.617
H = 3.67 DF = 4 P = 0.452 (adjusted for ties)

* NOTE * One or more small samples

- Anthracene (ANTH) Kruskal-Wallis Test: B, C, D TWINSPAN Groups versus Area collection (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on B, C, D TWINSPAN Groups

Area collection	N	Median	Ave Rank	Z
2	71	0.000000000	70.4	-0.16
3	59	0.000000000	70.9	-0.02
4	11	0.000000000	75.0	0.34
Overall	141		71.0	

H = 0.12 DF = 2 P = 0.942
H = 1.16 DF = 2 P = 0.561 (adjusted for ties)

- P.T.A Anthracene (ANTH) Kruskal-Wallis Test: B, C, D TWINSPAN Group versus Area collection (2 (B), 3 (C), 4 (D))

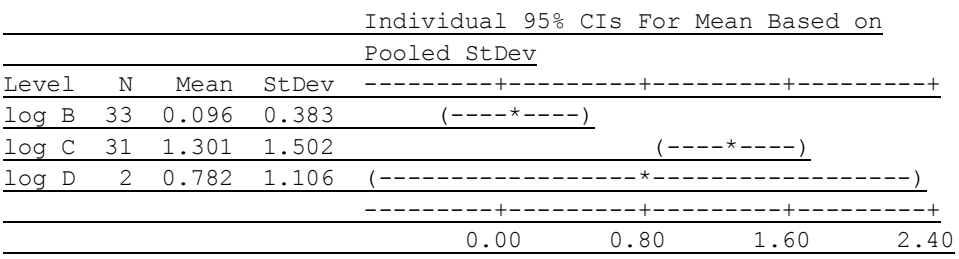
Kruskal-Wallis Test on B, C, D TWINSPAN Group

Area collection	N	Median	Ave Rank	Z
2	33	0.000000000	26.3	-3.03
3	31	5.010000000	41.1	3.03
4	2	3.320000000	33.5	0.00

```
Overall     66                    33.5

H = 9.46  DF = 2  P = 0.009
H = 10.67  DF = 2  P = 0.005  (adjusted for ties)

* NOTE * One or more small samples
```

- P.T.A Benzo(a)anthracene (B[a]A) One-way ANOVA: log B, log C, log D

```
Source  DF     SS     MS     F      P
Factor   2  23.24  11.62  9.94  0.000
Error   63  73.61   1.17
Total   65  96.84

S = 1.081   R-Sq = 23.99%   R-Sq(adj) = 21.58%
```




```
Pooled StDev = 1.081

Grouping Information Using Tukey Method

        N   Mean  Grouping
log C  31  1.301  A
log D   2  0.782  A B
log B  33  0.096    B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 98.05%

log B subtracted from:

        Lower  Center  Upper
log C   0.557   1.205  1.853
log D  -1.200   0.687  2.573
                    -1.5       0.0       1.5       3.0

log C subtracted from:

        Lower  Center  Upper
log D  -2.409  -0.519  1.372
```

```
                                    -1.5        0.0        1.5        3.0
```

- P.T.A Fluoranthene (FLAN) One-way ANOVA: log B, log C, log D

Source	DF	SS	MS	F	P
Factor	2	0.7845	0.3923	12.46	0.000
Error	63	1.9839	0.0315		
Total	65	2.7684			

S = 0.1775 R-Sq = 28.34% R-Sq(adj) = 26.06%

```
                                Individual 95% CIs For Mean Based on
                                Pooled StDev
Level    N    Mean   StDev  ------+---------+---------+---------+---
log B   33  3.6558  0.1542                  (---*---)
log C   31  3.8712  0.2004                            (---*---)
log D    2  3.6119  0.1335  (----------------*----------------)
                           ------+---------+---------+---------+---
                              3.45      3.60      3.75      3.90
```

Pooled StDev = 0.1775

Grouping Information Using Tukey Method

	N	Mean	Grouping
log C	31	3.8712	A
log B	33	3.6558	B
log D	2	3.6119	A B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 98.05%

log B subtracted from:

```
         Lower   Center   Upper  ---------+---------+---------+---------+
log C   0.1090   0.2154  0.3218                        (--*---)
log D  -0.3537  -0.0439  0.2659        (----------*---------)
                          ---------+---------+---------+---------+
                             -0.30      0.00      0.30      0.60
```

log C subtracted from:

```
         Lower   Center   Upper  ---------+---------+---------+---------+
log D  -0.5697  -0.2594  0.0510  (---------*----------)
                          ---------+---------+---------+---------+
                             -0.30      0.00      0.30      0.60
```

- P.T.A Fluorene (FL) Kruskal-Wallis Test: TWINSPAN Group versus Area collection (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on TWINSPAN Group

Area

collection	N	Median	Ave Rank	Z
2	33	10.600	26.9	-2.78
3	31	24.500	42.0	3.39
4	2	5.935	10.0	-1.76
Overall	66		33.5	

H = 12.97 DF = 2 P = 0.002
H = 12.97 DF = 2 P = 0.002 (adjusted for ties)

* NOTE * One or more small samples

- P.T.A Naphthalene (NA) Kruskal-Wallis Test: TWINSPAN Groups versus Area collection (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on TWINSPAN Groups

Area

collection	N	Median	Ave Rank	Z
2	33	20.60	24.4	-3.87
3	31	28.90	44.4	4.34
4	2	18.75	15.5	-1.35
Overall	66		33.5	

H = 19.20 DF = 2 P = 0.000
H = 19.21 DF = 2 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

- P.T.A Phenanthrene (PH) One-way ANOVA: log B, log C, log D

Source	DF	SS	MS	F	P
Factor	2	0.2411	0.1206	1.40	0.254
Error	63	5.4219	0.0861		
Total	65	5.6630			

S = 0.2934 R-Sq = 4.26% R-Sq(adj) = 1.22%

				Individual 95% CIs For Mean Based on Pooled StDev
Level	N	Mean	StDev	+---------+---------+---------+---------
log B	33	4.5971	0.1987	(---*---)
log C	31	4.4810	0.3719	(---*---)
log D	2	4.6562	0.1007	(----------*----------)
				+---------+---------+---------+---------
				4.25 4.50 4.75 5.00

Pooled StDev = 0.2934

Grouping Information Using Tukey Method

	N	Mean	Grouping
log D	2	4.6562	A
log B	33	4.5971	A
log C	31	4.4810	A

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 98.05%

log B subtracted from:

	Lower	Center	Upper	--------+---------+---------+---------+-
log C	-0.2920	-0.1161	0.0598	(---*---)
log D	-0.4530	0.0591	0.5712	(-----------*-----------)
				--------+---------+---------+---------+-
				-0.40 0.00 0.40 0.80

log C subtracted from:

	Lower	Center	Upper	--------+---------+---------+---------+-
log D	-0.3378	0.1752	0.6882	(-----------*-----------)
				--------+---------+---------+---------+-
				-0.40 0.00 0.40 0.80

- P.T.A Pyrene (PY) One-way ANOVA: log B, log C, log D

Source	DF	SS	MS	F	P
Factor	2	0.4533	0.2267	6.47	0.003
Error	63	2.2076	0.0350		
Total	65	2.6609			

S = 0.1872 R-Sq = 17.04% R-Sq(adj) = 14.40%

				Individual 95% CIs For Mean Based on Pooled StDev
Level	N	Mean	StDev	---------+---------+---------+---------+
log B	33	3.1292	0.1588	(---*---)
log C	31	3.2828	0.2145	(---*---)
log D	2	3.0057	0.1398	(----------*----------)
				---------+---------+---------+---------+
				2.88 3.04 3.20 3.36

Pooled StDev = 0.1872

Grouping Information Using Tukey Method

	N	Mean	Grouping
log C	31	3.2828	A
log B	33	3.1292	B
log D	2	3.0057	A B

Means that do not share a letter are significantly different.

Tukey 95% Simultaneous Confidence Intervals
All Pairwise Comparisons

Individual confidence level = 98.05%

log B subtracted from:

	Lower	Center	Upper	-------+---------+---------+---------+--
log C	0.0414	0.1537	0.2659	(--*---)

```
log D  -0.4502  -0.1235  0.2033      (--------*---------)
                                  -------+---------+---------+---------+--
                                      -0.35      0.00      0.35      0.70
```

log C subtracted from:

```
          Lower   Center   Upper  -------+---------+---------+---------+--
log D  -0.6045  -0.2771  0.0503  (--------*--------)
                                  -------+---------+---------+---------+--
                                      -0.35      0.00      0.35      0.70
```

- P.T.A. Chrysene (CH) Kruskal-Wallis Test: TWINSPAN Groups versus Area collection (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on TWINSPAN Groups

Area collection	N	Median	Ave Rank	Z
2	33	5.520000000	31.1	-1.01
3	31	6.140000000	37.9	1.74
4	2	0.000000000	5.0	-2.13
Overall	66		33.5	

H = 6.52 DF = 2 P = 0.038
H = 6.54 DF = 2 P = 0.038 (adjusted for ties)

* NOTE * One or more small samples

- P.T.A P-moist Kruskal-Wallis Test: TWINSPAN Groups versus Area collection (2 (B), 3 (C), 4 (D))

Kruskal-Wallis Test on TWINSPAN Groups

Area collection	N	Median	Ave Rank	Z
2	33	54.70	18.5	-6.35
3	31	69.70	47.6	5.63
4	2	76.00	62.0	2.13
Overall	66		33.5	

H = 41.43 DF = 2 P = 0.000
H = 41.43 DF = 2 P = 0.000 (adjusted for ties)

* NOTE * One or more small samples

Appendix 2

Bacterial sequences

>Contig_**isolate_1a**, Rhodococcus maanshanensis FR750959 Coverage 100%, Identity 99% (two mismatches), 1257 bp

CGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCAAAGGCTGCAT
GGCTTTTGGTGGAAAGGTTTACTGGTGCAGGATGGGCCCGCGGCCTATCAGCT
TGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAG
GGTGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCA
GCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT
GAGGGATGAAGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGACGAAGCGCAA
GTGACGGTACCTGCAGAAGAAGCACCGGCTAACTACGTGCCAGCAGCCGCGG
TAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGTTCGTA
GGCGGTTTGTCGCGTCGTCTGTGAAAACCCATCGCTCAACGATGGGCCGGCAG
GCGATACGGGCAGACTTGAGTACTGCAGGGGAGACTGGAATTCCTGGTGTAGC
GGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTG
GGCAGTAACTGACGCTGAGGAACGAAAGCGTGGGTAGCAAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGGTGGGCGCTAGGTGTGGGTTCCTTCCAC
GGGATCTGTGCCGTAGCTAACGCATTAAGCGCCCCGCCTGGGGAGTACGGCCG
CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCAT
GTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATATACTG
GAAAGCTGCAGAGATGTAGCCCCCYTTGTGGCCGGTATACAGGTGGTGCATGG
CTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAA
CCCTTGTCTTATGTTGCCAGCACGTAATGGTGGGGACTCGTAAGAGACTGCCG
GGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGT
CCAGGGCTTCACACATGCTACAATGGCCAGTACAGAGGGCTGCGAGACCGTGA
GGTGGAGCGAATCCCTTAAAGCTGGTCTCAGTTCGGATCGGGGTCTGCAACTC
GACCCCGTGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGA
ATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAG

>Contig_**isolate_1b** Agrobacterium tumefaciens strain EU87 JF681291, Coverage 100%, Identity 100%, 1214 bp

TACCGCATACGCCCTACGGGGGAAAGATTTATCGGGGAAGGATTGGCCCGCGT
TGGATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTG
GTCTGAGAGGATGATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTAC
GGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCA
TGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAG
ATAATGACGGTAGTCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGC
GGTAATACGAAGGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACG
TAGGCGGATATTTAAGTCAGGGGTGAAATCCCGCAGCTCAACTGCGGAACTGC
CTTTGATACTGGGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTA
GAGGTGAAATTCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACT
GGTCCATTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTATAC
TGTTCGGTGGCGCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGC
AAGATTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCAACGCGCAGAACCTTACCAGCTCTTGACATTCGGGGT
ATGGGCATTGGAGACGATGTCCTTCAGTTAGGCTGGCCCCAGAACAGGTGCTG
CATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAG
CGCAACCCTCGCCCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGGGACTG
CCGGTGATAAGCCGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTT
ACGGGCTGGGCTACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGAC

AGCGATGTCGAGCTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCA
ACTCGAGTGCATGAAGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGG

>Contig_**isolate_2**, Nocardia cyriacigeorgica JQ638648, Coverage 100%, Identity 100%, 1305 bp
GGGTGAGTAACACGTGGGTGATCTGCCTCGCACTCTGGGATAAGCCTGGGAAA
CTGGGTCTAATACCGGATATGACCTTACATCGCATGGTGTTTGGTGGAAAGATT
TATCGGTGCGAGATGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCC
TACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTCGACAGGGACGAAGCGCAAGTGACGGTACCTGTAGAAG
AAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGC
GTTGTCCGGAATTACTGGGCGTAAAGAGCTTGTAGGCGGCTTGTCGCGTCGAT
CGTGAAAACTTGGGGCTCAACCCCAAGCTTGCGGTCGATACGGGCAGGCTTGA
GTACTTCAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATC
AGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGAAGTAACTGACGCTGAG
AAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCG
TAAACGGTGGGTACTAGGTGTGGGTTTCCTTCCACGGGATCCGTGCCGTAGCT
AACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGC
AACGCGAAGAACCTTACCTGGGTTTGACATACACCGGAAACCTGCAGAGATGT
AGGCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATCTTATGTTGCC
AGCGCGTAATGGCGGGGACTCGTGAGAGACTGCCGGGGTCAACTCGGAGGAA
GGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATG
CTACAATGGCCGGTACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTT
AAAGCCGGTCTCAGTTCGGATCGGGGTCTGCAACTCGACCCCGTGAAGTTGGA
GTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACCC

>Contig_**isolate_3**, Gordonia lacunae strain A4-20 JN627169, Coverage 100%, Identity 100%, 1273 bp: Gordonia terrae strain MRbS27, FJ959396, Coverage 100%, Identity 100%, 1273 bp
CTCTGGGATAAGCCTGGGAAACTGGGTCTAATACCGGATATGACCAACTGTCG
CATGGTGGTTGGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTATCA
GCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAG
AGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGG
CAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGC
GTGAGGGATGACGGCCTTCGGGTTGTGAACCTCTTTCACCAGGGACGAAGCGT
GAGTGACGGTACCTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGC
GGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCG
TAGGCGGTTTGTCGCGTCGTCTGTGAAATTCTGCAACTCAATTGCAGGCGTGCA
GGCGATACGGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAG
CGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCT
GGGTAGTAACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGA
TACCCTGGTAGTCCACGCCGTAAACGGTGGGTACTAGGTGTGGGTTCCTTTTCA
CGGGATCCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCC
GCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCA
TGTGGATTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACC
AGACGCGGCTAGAGATAGTCGTTCCCTTGTGGTTGGTGTACAGGTGGTGCATG
GCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA

ACCCTTGTCCTGTATTGCCAGCGGGTTATGCCGGGGACTTGCAGGAGACTGCC
GGGGTCAACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATG
TCCAGGGCTTCACACATGCTACAATGGCTGGTACAGAGGGCTGCGATACCGTG
AGGTGGAGCGAATCCCTTAAAGCCAGTCTCAGTTCGGATTGGGGTCTGCAACT
CGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTG
AATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAA
CACCC

>Contig_**isolate_4**, Gordonia lacunae strain A4-20 JN627169, Coverage 100%, Identity 99%, 1315 bp: Gordonia terrae strain MRbS27, FJ959396, Coverage 100%, Identity 99%, 1315 bp – (one mismatch)

GAGTGGCGAACGGGTGAGTAACACGTGGGTGATCTGCCCTGCACTCTGGGATA
AGCCTGGGAAACTGGGTCTAATACCGGATATGACCAACTGTCGCATGGTGGTT
GGTGGAAAGCTTTTGCGGTGTGGGATGGGCCCGCGGCCTATCAGCTTGTTGGT
GGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGACCTGAGAGGGTGATC
GGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGCGCAAGCCTGATGCAGCGACGCCGCGTGAGGGAT
GACGGCCTTCGGGTTGTAAACCTCTTTCACCAGGGACGAAGCGTGAGTGACGG
TACCTGGAGAAGAAGCACCGGCCAACTACGTGCCAGCAGCCGCGGTAATACG
TAGGGTGCGAGCGTTGTCCGGAATTACTGGGCGTAAAGAGCTCGTAGGCGGTT
TGTCGCGTCGTCTGTGAAATTCTGCAACTCAATTGCAGGCGTGCAGGCGATAC
GGGCAGACTTGAGTACTACAGGGGAGACTGGAATTCCTGGTGTAGCGGTGAA
ATGCGCAGATATCAGGAGGAACACCGGTGGCGAAGGCGGGTCTCTGGGTAGT
AACTGACGCTGAGGAGCGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTG
GTAGTCCMCGCCGTAAACGGTGGGTACTAGGTGTGGGTTCCTTTTCACGGGAT
CCGTGCCGTAGCTAACGCATTAAGTACCCCGCCTGGGGAGTACGGCCGCAAGG
CTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGA
TTAATTCGATGCAACGCGAAGAACCTTACCTGGGTTTGACATACACCAGACGC
GGCTAGAGATAGTCGTTCCCTTGTGGTTGGTGTACAGGTGGTGCATGGCTGTCG
TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTG
TCCTGTATTGCCAGCGGGTTATGCCGGGGACTTGCAGGAGACTGCCGGGGTCA
ACTCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGCCCCTTATGTCCAGGG
CTTCACACATGCTACAATGGCTGGTACAGAGGGCTGCGATACCGTGAGGTGGA
GCGAATCCCTTAAAGCCAGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCC
ATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCAACGCTGCGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACGTCATGAAAGTCGGTAACACC

Isolate 5- no sequence obtained

>Contig_**isolate_6,** Streptomyces sp. 210_66, GQ199768, Coverage 100%, Identity 99%, 1218 bp (4 mismatches)
CGGGGTCTAATACCGGATACGACCTGGGAAGGCATCTTCTCGGGTGGAAAGCT
CCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAACGGC
TCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCACACTGG
GACTGAGACACGGCCCASACTCCTACGGGAGGCAGCAGTGGGGAATATTGCAC
AATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGG
GTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAA
GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGCGCAAG
CGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGG
GTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATACGGGCAGGCTAG
AGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATAT
CAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGA

GGAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCCGCAGC
TAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAA
GGAATTGACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGC
AACGCGTAGAACCTTACCAAGGCTTGACATATACCGGAAACATCCAGAGATGG
GTGCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCC
AGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCG
GAGGAAGGTGGGGACCGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGC
ACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATGCCGCGAGGCGGAGCG
AATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCAYG
AAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCC
CGGGCC

>Contig_**isolate_7**, Lysobacter sp. Closest match JQ659518 Lysobacter soli, coverage 96%, identity 99%, (12 mismatches)
TCGAAAGGTGACTGTCTCCTCTCCATGCGCTTCGGATGGTAGCCATGGGGGCTT
GCTCCTTTGGGTGGCGAGTGGCGGACGGGGGAGGAATGCATCGGAATCTGCCT
ATTTGTGGGGGATAACGTAGGGAAACTTACGCTAATACCGCATACGTCCTACG
GGAGAAAGTGGGGGACCTTCGGGCCTCACGCAGATAGATGAGCCGATGCCGG
ATTAGCTAGTTGGCGGGGTAAAGGCCCACCAAGGCGACGATCCGTAGCTGGTC
TGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGG
GAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATG
CCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTTGTCCGGAAAGAA
AAGCGCTCGATTAATACTCGGGTGTCATGACGGTACCGGAAGAATAAGCACCG
GCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGG
AATTACTGGGCGTAAAGCGTGCGTAGGTGGTTTGTTAAGTCTGATGTGAAAGC
CCTGGGCTCAACCTGGGAACTGCATTGGATACTGGCTTACTAGAGTGCGGTAG
AGGGGTGTGGAATTCCCGGTGTAGCAGTGAAATGCGTAGATATCGGGAGGAA
CATCTGTGGCGAAGGCGACACCCTGGACCAGCACTGACACTGAGGCACGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCCTAAACGATG
CGAACTGGATGTTGGGGGCAACTTGGCCCTCAGTATCGAAAGCTAACGCGTTA
AGTTCGCCGCCTGGGAAAGTACGGTCGCAAAGACTGAAACTTCAAAGGAAATT
GACGGGGGCCCGCACMAGCGGTGGAGTATGTGGTTTAATTCGATGCAACGCG
AAGAACCCTTACCTGGCCTTGACATGCCACGGAACTTTCCAGAGATGGATTGG
TGCCTTCGGGAACCGTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCG
TGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTTAGTTGCCA
GCACGTAATGGTGGGAACTCTAAGGAGACCGCCGGTGACAAACCGGAGGAAG
GTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTAC
TACAATGGTGGGGACAGAGGGCTGCAAACCCGCGAGGGCAAGCCAATCCCAG
AAACCCCATCTCAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGA
ATCGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTT
GTACACACCGCCCGTCACACCATGGGA

>Contig_**isolate_8**, Inquilinus sp, closest match Inquilinus limosus strain AU1979 AY043375, coverage 99%, identity 99%, (9 mismatches)
AGGGGAGTGGCGCACGGGTGAGTAACACGTGGGAACCTACCTTCTGGTACGG
AACAACCGAGGGAAACTTCGGCTAATACCGTATACGACCTCCGGGTGAAAGAT
TGATCGCCGGAAGAGGGGCCCGCGTCCGATTAGGTAGTTGGTGGGGTAACGGC
CTACCAAGCCGACGATCGGTAGCTGGTCTGAGAGGATGACCAGCCACACTGGG
ACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGAC
AATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGTGATGAAGGCCTTCGGG
TTGTAAAGCTCTTTCACCCACGACGATGATGACGGTAGTGGGAGAAGAAGCCC

CGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCAAGCGTTGTTC
GGAATGACTGGGCGTAAAGGGCGCGTAGGCGGTTCGTTGCGTCAGATGTGAA
AGCCCCGGGCTCAACCTGGGAACTGCATTTGATACGGGCGGGCTTGAATCCAA
GAGAGGGTGGTGGAATTCCCAGTGTAGAGGTGAAATTCGTAGATATTGGGAAG
AACACCAGTGGCGAAGGCGGCCACCTGGCTTGGTATTGACGCTGAGGCGCGA
AAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACG
ATGTGTGCTAGCCGTCGGGCAGCTTGCTGTTCGGTGGCGCAGCTAACGCGATA
AGCACACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGAC
GGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGCAG
AACCTTACCAACCCTTGACATGGGGAGTGTGGGCCCGGGAGATCGGGTCCTTC
AGTTCGGCTGGCTCCCACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTG
AGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCCTCTTCAGTTGCCAGC
ATTGAGTTGGGCACTCTGGAGATACTGCCGGTGACAAGCCGGAGGAAGGCGG
GGATGACGTCAAGTCCTCATGGCCCTTACGGGTTGGGCTACACACGTGCTACA
ATGGCGGTGACAGTGGGCAGCGAAGGGGCGACCTGGAGCTAATCCCCAAAAG
CCGTCTCAGTTCGGATTGCACTCTGCAACTCGGGTGCATGAAGTTGGAATCGCT
AGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACA
CCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAAGACGGTGCGCTGACCCG
CAA

>Contig_**isolate_9**, Gordonia lacunae strain A4-20 JN627169, Coverage 100%, Identity 99%, 1084 bp: Gordonia terrae strain MRbS27, FJ959396, Coverage 100%, Identity 99%, 1084 bp – (one mismatch)
TGGGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACG
ACGGGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGC
CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGC
CTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTT
TCACCAGGGACGAAGCGTGAGTGACGGTACCTGGAGAAGAAGCACCGGCCAA
CTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGTCCGGAATTA
CTGGGCGTAAAGAGCTCGTAGGCGGTTTGTCGCGTCGTCTGTGAAATTCTGCA
ACTCAATTGCAGGCGTGCAGGCGATACGGGCAGACTTGAGTACTACAGGGGA
GACTGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC
GGTGGCGAAGGCGGGTCTCTGGGTAGTAACTGACGCTGAGGAGCGAAAGCGT
GGGTAGCGAACAGGATTAGATACCCTGGTAGTMCACGCCGTAAACGGTGGGT
ACTAGGTGTGGGTTCCTTTTCACGGGATCCGTGCCGTAGCTAACGCATTAAGTA
CCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGG
GCCCGCACAAGCGGCGGAGCATGTGGATTAATTCGATGCAACGCGAAGAACC
TTACCTGGGTTTGACATACACCAGACGCGGCTAGAGATAGTCGTTCCCTTGTG
GTTGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGG
GTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTATTGCCAGCGGGTTATGC
CGGGGACTTGCAGGAGACTGCCGGGGTCAACTCGGAGGAAGGTGGGGATGAC
GTCAAGTCATCATGCCCCTTATGTCCAGGGCTTCACACATGCTACAATGGCTGG
TACAGAGGGCTGCGATACCGTGAGGTGGAGCGAATCCCTTAAAGCCAGTCTCA
GTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATC
GCAGATCAGCAACGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCC
GTCACGTCATGAAAGTCGGTAAC

>Contig_isolate_10, Sphingopyxis sp. BJGMM-B8, JQ716218, Coverage 100%, identity 100%, 686bp
CTAGGGTTGTAAAGCTCTTTTACCCGGGATGATAATGACAGTACCGGGAGAAT
AAGCTCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGAGCTAGC
GTTGTTCGGAATTACTGGGCGTAAAGCGCGCGTAGGCGGTTTTTTAAGTCAGA
GGTGAAAGCCCGGGGCTCAACCCCGGAATAGCCTTTGAAACTGGAAAACTAG

AATCTTGGAGAGGTCAGTGGAATTCCGAGTGTAGAGGTGAAATTCGTAGATAT
TCGGAAGAACACCAGTGGCGAAGGCGACTGACTGGACAAGTATTGACGCTGA
GGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGATAACTAGCTGTCCGGGTTCATAGAACTTGGGTGGCGCAGCTA
ACGCATTAAGTTATCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGG
AATTGACGGGGGCCTGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGCAGAACCTTACCAGCGTTTGACATCCTGATCGCGGTTACCAGAGATGGTT
TCCTTCAGTTCGGCTGGATCAGTGACAGGTGCTGCATGGCTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCCCTAGTT

>Contig_isolate_11, Agrobacterium tumefaciens, JF681291, Coverage 100%, Identity 100%, 1250 bp
TACCCTTTCCTGCGGAATAGCTCCGGGAAACTGGAATTAATACCGCATACGCC
CTACGGGGGAAAGATTTATCGGGGAAGGATTGGCCCGCGTTGGATTAGCTAGT
TGGTGGGGTAAAGGCCTACCAAGGCGACGATCCATAGCTGGTCTGAGAGGAT
GATCAGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGAGT
GATGAAGGCCTTAGGGTTGTAAAGCTCTTTCACCGATGAAGATAATGACGGTA
GTCGGAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAA
GGGGGCTAGCGTTGTTCGGAATTACTGGGCGTAAAGCGCACGTAGGCGGATAT
TTAAGTCAGGGGTGAAATCCCGCAGCTCAACTGCGGAACTGCCTTTGATACTG
GGTATCTTGAGTATGGAAGAGGTAAGTGGAATTCCGAGTGTAGAGGTGAAATT
CGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTTACTGGTCCATTACT
GACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCGTAAACGATGAATGTTAGCCGTCGGGCAGTATACTGTTCGGTGGC
GCAGCTAACGCATTAAACATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
AAGCAACGCGCAGAACCTTACCAGCTCTTGACATTCGGGGTATGGGCATTGGA
GACGATGTCCTTCAGTTAGGCTGGCCCCAGAACAGGTGCTGCATGGCTGTCGT
CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGC
CCTTAGTTGCCAGCATTTAGTTGGGCACTCTAAGGGGACTGCCGGTGATAAGC
CGAGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCCTTACGGGCTGGGCT
ACACACGTGCTACAATGGTGGTGACAGTGGGCAGCGAGACAGCGATGTCGAG
CTAATCTCCAAAAGCCATCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATG
AAGTTGGAATCGCTAGTAATCGCAGATCAGCATGCTGCGGTGAATACGTTCCC
GGGCCTTGTACACACCGCCCGTCACACCATGGGAG

-Fungal sequences

Isolate 12 – was not possible to amplify the required DNA region from this isolate

>Contig_**isolate_13** Penicillium commune AF455527, coverage 100%, match 100%, 581 bp (Identical to isolate 17)
AAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGT
GAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCG
GCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCC
GCCGAAGACACCCTCGAACTCTGTCTGAAGATTGAAGTCTGAGTGAAAATATA
AATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAA
CGCAGCGAAATGCGATACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGT
CTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGC
GTCATTGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCCCGATCTCC

GGGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTA
TGGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCA
AATTTTTATCCAGGTTGACCTCGGATCAGGTAGGGATACCCGCTGAACTT

>Contig_**isolate_14**, Trichoderma asperellum JN108915 coverage 100%, match 99%, (one mismatch) 595 bp
AAGTCGTAACCAGGTCTCCGTTGGTGAACCAGCGGAGGGATCATTACCGAGTT
TACAACTCCCAAACCCAATGTGAACGTTACCAAACTGTTGCCTCGGCGGGGTC
ACGCCCCGGGTGCGTCGCAGCCCCGGAACCAGGCGCCCGCCGGAGGAACCAA
CCAAACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAGCTCTGAGCAAA
AATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCT
GTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGATCGGCGTTGGGGAT
CGGGACCCCTCACACGGGTGCCGGCCCCGAAATACAGTGGCGGTCTCGCCGCA
GCCTCTCCTGCGCAGTAGTTTGCACAACTCGCACCGGGAGCGCGGCGCGTCCA
CGTCCGTAAAACACCCAACTTTCTGAAATGTTGACCTCGGATCAGGTAGGAAT
ACCCGCTGAACT

>Contig_**isolate_15**, Penicillium species (not possible to say which species as there are 4 species with the same sequence in the database – all of which match 99%) see JN903645 Penicillium granulatum, 579bp
AAAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGT
GAGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCG
GCGGGCCCGCCTTAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCC
GCCGAAGACACCCTCGAACTCTGTCTGAAGATTGTAGTCTGAGTGAAAATATA
AATTATTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAA
CGCAGCGAAATGCGATACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGT
CTTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGC
GTCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCCCCGTCCTCCGATCCCG
GGGGACGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTAT
GGGGCTTTGTCACCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAA
ATTTTTATCCAGGTTGACCTCGGATCAGGTATGGATACCCGCTGAACT

>Contig_**isolate_16**, Penicillium simplicissimum HQ607866 coverage 100%, match 99%, (four mismatches) 563 bp (identical to isolate 18)
AAGTCGTAACAAGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTG
AGGGCCCTCTGGGTCCAACCTCCCACCCGTGTTTATTCTACCTTGTTGCTTCGG
CGGGCCCGCCTCACGGCCGCCGGGGGGCACTCGCCCCCGGGCCCGCGCCCGCC
GAAGACACCAATGAACTCTGTCTGAAGATTGCAGTCTGAGCAGATTAGCTAAA
TCAGTTAAAACTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACG
CAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTC
TTTGAACGCACATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCG
TCATTGCTGCCCTCAAGCACGGCTTGTGTGTTGGGCTTCGCCCCCCGGCTCCCG
GGGGGCGGGCCCGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTAT
GGGGCTTCGTCACCCGCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGACCCCAA
TCAATCTTTCCAGGTTGACCTCGGATCAGGTA

>Contig_**isolate_17,** Penicillium commune GU183165, coverage 100%, match 100%, 553 bp, (Identical to isolate 17)
AGGTTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTG
GGTCCAACCTCCCACCCGTGTTTATTTTACCTTGTTGCTTCGGCGGGCCCGCCT
TAACTGGCCGCCGGGGGGCTTACGCCCCCGGGCCCGCGCCCGCCGAAGACACC

CTCGAACTCTGTCTGAAGATTGAAGTCTGAGTGAAAATATAAATTATTTAAAA
CTTTCAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATG
CGATACGTAATGTGAATTGCAAATTCAGTGAATCATCGAGTCTTTGAACGCAC
ATTGCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCC
TCAAGCCCGGCTTGTGTGTTGGGCCCCGTCCCCCGATCTCCGGGGGACGGGCC
CGAAAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTTGTCA
CCCGCTCTGTAGGCCCGGCCGGCGCTTGCCGATCAACCCAAATTTTTATCCAGG
TTGACCTCGGATCAGGTAGG

>Contig_**isolate_18**, Penicillium simplicissimum HQ607866 coverage 100%, match 99%, (four mismatches) 551 bp (identical to isolate 16)
TTTCCGTAGGTGAACCTGCGGAAGGATCATTACCGAGTGAGGGCCCTCTGGGT
CCAACCTCCCACCCGTGTTTATTCTACCTTGTTGCTTCGGCGGGCCCGCCTCAC
GGCCGCCGGGGGGCACTCGCCCCCGGGCCCGCGCCCGCCGAAGACACCAATG
AACTCTGTCTGAAGATTGCAGTCTGAGCAGATTAGCTAAATCAGTTAAAACTTT
CAACAACGGATCTCTTGGTTCCGGCATCGATGAAGAACGCAGCGAAATGCGAT
AAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAGTCTTTGAACGCACATT
GCGCCCCCTGGTATTCCGGGGGGCATGCCTGTCCGAGCGTCATTGCTGCCCTCA
AGCACGGCTTGTGTGTTGGGCTTCGCCCCCCGGCTCCCGGGGGGCGGGCCCGA
AAGGCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTATGGGGCTTCGTCACCC
GCTCTGTAGGCCCGGCCGGCGCCCGCCGGCGACCCCAATCAATCTTTCCAGGT
TGACCTCGGATCAGGTAGG

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