CRANFIELD UNIVERSITY

ODUKOYA, JOHNSON OLUWASEUN

INFLUENCE OF BIOREMEDIATION ON THE CHEMICAL AND NUTRITIONAL COMPOSITION OF PRODUCE FROM CRUDE OIL-POLLUTED SITES

School of Energy, Environment and Agrifood

PhD Thesis
Academic Years: 2012 - 2015

SUPERVISOR: DR RUBEN SAKRABANI

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ABSTRACT

The influence of crude oil-contaminated and remediated sites on agrifood production is not clearly understood. To address this knowledge gap, the research was divided into two stages involving: (1) assessment of the efficiency of two bioremediation strategies to support hydrocarbons degradation as well as agrifood production with the initial analysis of the experimental materials, and (2) evaluation of the effect of different crude oil remediation intervention values (CRIV) on selected vegetables (*Brassica juncea*, *Brassica oleracea*, *Lactuca sativa* and two different cultivars of *Solanum lycopersicum*). Results from the first stage showed that the crude oil used had a pristane/phytane ratio of 0.98 (within the 0.8 – 3.0 range of most crude oils), higher concentrations of C_{10} – C_{14}, C_{15} – C_{20} and C_{21} – C_{27} alkanes than the C_{28} – C_{36} alkanes including higher concentrations of two of the US EPA priority pollutant polycyclic aromatic hydrocarbons (PAHs) - phenanthrene and anthracene. Four treatments were prepared in which weekly tillage enhanced the degradation of C_{15} – C_{20} and C_{21} - C_{27} alkanes in the Remediation by Enhanced Natural Attenuation (RENA) treatment. The two bioremediation strategies (RENA and bioaugmentation) enhanced PAHs degradation compared with the remediation-study control treatment while only RENA application among the two approaches supported the growth of *B. juncea.* Although there was no statistical significant difference ($p > 0.05$) between the major dietary mineral contents of samples from the various treatments compared with the control treatment samples, RENA application affected the Cr, Zn and Pb contents. Meanwhile, the Ca/P (> 1.0) and Na/K (< 0.60) ratios of all the harvested samples imply that they provide a good source of these minerals for bone formation and would not contribute to high blood pressure. The crude oil used also deterred the attack of juvenile caterpillars of cabbage white butterfly. Findings from the second stage revealed that the yield of the green leafy vegetables including one of the selected tomato cultivars (Micro-Tom), was in most cases impaired at CRIV ≥ 3,000 mg/kg total petroleum hydrocarbon (TPH). Compared with the control treatment samples' composition, crude oil stress at 10,000 mg/kg TPH enhanced the concentration

of K, Mn and crude protein of *B. oleracea* and *L. sativa* as well as the sucrose, total sugars, total phenolics and total flavonoids contents of the latter vegetable. Sucrose was also only detected in M82 tomato cultivar samples from the crude oil-containing treatments. The Cd content of *B. oleracea*, Pb contents of: *L. sativa* and M82 tomato harvested samples were all below the FAO/WHO Codex Alimentarius Commission 2015 recommended maximum levels. However, tartaric acid was only detected in *B. oleracea* and *L. sativa* samples from the 10,000 mg/kg TPH treatment as well as in M82 tomato cultivar samples from the treatment involving CRIV of 5,000 mg/kg TPH. Generally, the yield of these crops in response to crude oil contamination varied in which *B. juncea* had the least tolerance to crude oil stress among the green leafy vegetables tested. Most of the quality parameters in the two tomato cultivars were not affected by CRIV between 750 - 5,000 mg/kg TPH with *p*-xylene having the greatest toxic potential among the VOCs emitted from the 5,000 mg/kg TPH treatment. The research findings, under the experimental conditions, indicated the effectiveness of RENA for the degradation of low molecular weight PAHs and its agricultural benefits. They also suggest that crude oil-contaminated sites at ≤ 3,000 mg/kg TPH present a similar growing environment to a clean site for agrifood production and the possibility that crude oil stress at 10,000 mg/kg TPH could enhance crop quality. Nonetheless, the contribution of bio-accumulated PAHs in these crops to the food chain demands further investigation.

Keywords:

Agrifood, Crude oil Remediation Intervention Values (CRIV), Remediation by Enhanced Natural Attenuation (RENA), Crop quality, Volatile Organic Compounds (VOCs)

DEDICATION

This research project is humbly dedicated to The Word of God, members of my immediate family, including my ever-caring parents - Mr Samuel Odukoya and Mrs Esther Odukoya (who desired that I become a medical doctor) - as well as everyone that helped in making the dream of the successful completion of this PhD programme a reality.

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LIST OF ABBREVIATIONS

%	Per cent
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
AAS	Atomic Absorption Spectrometer
AMOs	Agricultural mineral oils
ANOVA	Analysis of Variance
API	American Petroleum Institute
ATSDR	Agency for Toxic Substances and Disease Registry
b. p.	Boiling point
BCF	Bioconcentration factors
BS	British Standard
BSI	British Standards Institution
BTEX	Benzene, Toluene, Ethylbenzene and Xylenes
C/N ratio	Carbon/Nitrogen ratio
C/P ratio	Carbon/Phosphorus ratio
Ca/P ratio	Calcium: Phoshorus ratio
CCME	Canadian Council of Ministers of the Environment
CELSSs	Controlled Ecological Life Support Systems (CELSSs)
CFU	Colony forming units
CI	Confidence interval
CNS	Central Nervous System
CRD	Completely Randomized Design
CRIV	Crude oil Remediation Intervention Value(s)
DAD	Diode Array Detector
DEQ	Department of Environmental Quality
DF	Dilution factor
DN	Dumas nitrogen
DNA	Deoxyribonucleic acid
DPR	Department of Petroleum Resources

DW	Dry weight
E & P	Exploration and Production
EGASPIN	Environmental Guidelines and Standards for the Petroleum Industries in Nigeria
ELSD	Evaporative Light Scattering Detector
EPA	Environmental Protection Agency
et al.	*et alteri*
F/G ratio	Fructose: Glucose ratio
FAO	Food and Agriculture Organization of the United Nations
g/100g	gram per hundred gram
GAE	Gallic acid equivalents
GC	Gas Chromatography
GC/FID	Gas Chromatogarphy/Flame Ionization Detection
GC/MS	Gas Chromatographic/Mass Spectrometer
HDPE	High Density Polyethylene
HMOs	Horticultural mineral oils
HPLC	High Performance Liquid Chromatography
i.e.	id est – 'that is'
ICP-MS	Inductively Coupled Plasma – Mass Spectrometer
IOM	Institute of Medicine
IR	Infrared Spectroscopy
ISO	International Organization for Standardization
KN	Kjeldahl nitrogen
K_{ow}	Octanol/water partition coefficient
LOD	Limit of Detection
LOQ	Limit of Quantification
m. p.	Melting point
MAF	Ministry of Agriculture and Forestry, New Zealand
mg/g	milligram per gram
mg/kg	milligram per kilogram
ML	Maximum Level
MS	Mass spectrometer

Na/K ratio	Sodium: Potassium ratio
NIST	National Institute of Standards and Technology
NOAA	National Oceanic and Atmospheric Administration
NOSDRA	National Oil Spill Detection and Response Agency
NSW DPI	New South Wales Department of Primary Industries
OECD	Organisation for Economic Co-operation and Development
OPEC	Organization of the Petroleum Exporting Countries
P/S traits	Physical/Sensory traits
PAHs	Polycyclic Aromatic Hydrocarbons
PAL	Phenylalanine ammonia lyase
PAN UK	Pesticide Action Network UK
PDSOs	Petroleum-Derived Spray Oils
PHC	Petroleum Hydrocarbon
PNAs	Polynuclear Aromatic Hydrocarbons
POPs	Persistent Organic Pollutants (POPs)
PTFE	Polytetrafluoroethylene
Pr/Ph	Pristane/Phytane ratio
PUFA	Polyunsaturated fatty acids
QE	Quercetin equivalents
RCBD	Randomized Complete Block Design
RENA	Remediation by Enhanced Natural Attenuation
RMS	Remediation Management System
ROS	Reactive oxygen species
RSPH	Royal Society for Public Health
SD	Standard deviation
SE	Standard Error
SEPCiN	Shell Exploration and Production Companies in Nigeria
SIS	Selah Intermediate School
SMS	Spent Mushroom Substrate
SOP	Standard Operating Procedure
SPDC	Shell Petroleum Development Company of Nigeria Limited
SPE	Solvent Phase Extraction

SRM	Standard Reference Material
TCD	Thermal Conductivity Detector
TD	Thermal desorption
TD/GC/TOF-MS	Thermal Desorption / Gas Chromatography / Time of Flight – Mass Spectrometry
Ti	Transportation index
TLC-FID	Thin-layer Chromatography-Flame Ionization Detection
TON	Total Oxides of Nitrogen
TPC	Total phenolics content
TPH	Total Petroleum Hydrocarbons
TPHCWG	Total Petroleum Hydrocarbon Criteria Working Group
TSS	Total Soluble Solids
UK	United Kingdom
UNEP	United Nations Environment Programme
USA	United States of America
USDA NRCS	United States Department of Agriculture Natural Resources Conservation Service
US EPA	United States Environmental Protection Agency
VOCs	Volatile Organic Compounds
WAT	Week(s) After Transplanting
WHO	World Health Organization

CHAPTER 1
INTRODUCTION

1.1 Project background

Crude oil, the unrefined state of petroleum, is one of the most significant sources of energy (Ftiti et al., 2014; Etornam and Denis, 2015) with high economic importance. The natural resource has played a very important, if not pivotal, role in satisfying the global energy demand (Nicolotti and Egli, 1998). As a result of the numerous benefits and diverse applications of crude oil, the search for it has expanded with positive results in some parts of the world.

Regardless of the socio-economic gains from the discovery of oil in these regions, spills sometimes occur during production operations, or due to technical errors, from transportation and storage faults, as well as from deliberate human acts (Khamehchiyan et al., 2007; Ajagbe et al., 2012) such as that involving the most pronounced form of oil spillage in history that occurred during the Persian Gulf war between August 1990 and February 1991 (Purvis, 1999, cited in Kostreba, 1999, p. 1). In the United States territorial waters, the largest oil spill incidence was also recorded in the 2010 *Deepwater Horizon* Gulf of Mexico oil spill (Levy and Gopalakrishnan, 2010) in which > 200 million gallons of Louisiana light crude oil were released (Ylitalo et al., 2012). Table 1.1 gives a summarized historical account of oil spill cases with the focus on those with serious environmental impact (≥ 200,000 barrels) as provided by NOAA (1992) after which the impacts of crude oil discovery in Nigeria, of Sub-Saharan Africa, is presented.

Table 1-1: Oil spill case histories ≥ 200,000 barrels between 1975 and 1991

SPILL NAME	LOCATION	SOURCE	OIL PRODUCT	BARRELS	DATE
Amoco Cadiz	Brittany, France	Tank Vessel	Arabian light crude, Iranian light crude, Bunker C.	1,619,048	16/03/1978
Arabian Gulf/Kuwait	Persian Gulf, Kuwait	Tank vessels, facilities, pipelines, platforms.	Kuwait crude oil	9,000,000	19/01/1991
Assimi	Oman	Tank Vessel	Light Iranian Crude Oil	379,000	07/01/1983
Athenian Venture	Canada, southeast of Cape Race, Newfoundland	Tank Vessel	Unleaded Gasoline	252,429	22/04/1988
Borag	Keelung, Taiwan	Tank Vessel	No. 4 Fuel Oil	213,690	05/02/1977
Burmah Agate	Galveston Bay, Texas	Tank Vessel	Nigerian Crude, blended crude (high naphtha content)	254,761	01/11/1979
Corinthos	Delaware River, Marcus Hook, Pennsylvania	Tank Vessel	Algerian crude oil	266,000	31/01/1975
Ekofisk Bravo oil field	Norway, North Sea	Platform	Ekofisk crude oil	202,381	22/04/1977
Exxon Valdez	Bligh Reef, Prince William Sound, Alaska	Tank Vessel	Prudhoe Bay Crude	240,500	24/03/1989
Funiwa No. 5	Niger Delta, Forcados, Nigeria	Platform	Nigerian Crude	200,000	19/01/1980
Independenta	Istanbul, Turkey	Tank Vessel	Es Sider Crude Oil	687,785	15/11/1979

Irene's Challenge	Pacific Ocean, 50 miles north of the Hawaiian Islands	Tank Vessel	Light crude oil	237,600	17/01/1977
IXTOC I	Bahia de Campeche, Mexico	Platform	IXTOC I crude oil	3,522,400	03/06/1979 to 23/03/1980
Jakob Maersk	Leixoes, Portugal	Tank Vessel	Iranian crude	637,500	29/01/1975
Khark 5	400 miles north of Las Palmas, Canary Islands	Tank Vessel	Iranian Heavy crude oil	452,400	19/12/1989
Nowruz Oil Field	Persian Gulf, Iran	Platforms	Crude oil	1,904,762	10/02/1983
St. Peter	Cabo Manglares, Colombia	Tank Vessel	Orito Crude Oil	279,000	05/02/1976
Texaco Storage Tank	Bahia Las Minas, Panama	Facility	Venezuelan crude, Mexican Isthmanian crude	240,000	27/04/1986
Urquiola	La Coruna, Spain	Tank Vessel	Light Arabian crude oil, Bunker fuel	733,000	12/05/1976
Venoil	Cape St. Francis, South Africa	Tank Vessel	Iranian heavy crude oil, Bunker fuel oil	219,000	16/12/1977

Adapted from: NOAA (1992)

Impacts of crude oil discovery in Nigeria

Crude oil was first discovered in Nigeria at Oloibiri, Bayelsa State in 1956 (Adati, 2012; OPEC, 2015b) and has since remained the major means of foreign exchange earnings for the country. Other neighbouring states which make up the Nigerian Niger Delta include: Abia, Akwa Ibom, Bayelsa, Cross River, Delta, Edo, Ondo, Imo and Rivers (Adati, 2012). Figure 1.1 shows the map of Nigeria (with the surrounding countries) and the Niger Delta region highlighted in green.

Figure 1-1: Map of Nigeria with the Niger Delta highlighted in green

Adapted from: Integrity (2015)

In 1971, Nigeria became a member of the Organization of the Petroleum Exporting Countries (OPEC) which includes other oil producing countries such as: Iraq, Iran, Kuwait, Saudi Arabia, Venezuela, and Libya, amongst others (OPEC, 2015a). Meanwhile, the main foreign producers in the country are: Chevron, ExxonMobil, Total, Eniagip, Addax Petroleum, ConocoPhillips, Petrobas and Statoilhydro (Adati, 2012).

Despite the various commercial opportunities provided by crude oil discovery within the Nigerian Niger Delta, environmental pollution in the form of oil spills has become a common experience and has also led to reduced interest in the

agricultural sector which was formerly the backbone of the Nigerian economy (Ahmadu and Egbodion, 2013). The continued petroleum exploration activities in the area have increased the damage to the ecosystem with substantial detrimental effects on the surrounding farmlands (Figure 1.2) and water environment (Okpokwasili and Odokuma, 1990; Njoku et al., 2009).

Figure 1-2: Part of spill impact on 10" Diebu Creek – Nun River Pipeline at Oporoma. Picture taken on 26th April, 2015 and provided by SPDC

Source: SPDC (2015c)

In the last 50 years, studies have shown that between 9 to 13 million barrels of crude oil have been spilled in the Niger Delta, making the region one of the most crude oil-damaged parts of the world (Adati, 2012). Table 1.2 is a record of the Nigerian oil spill data as provided by the Nigerian Department of Petroleum Resources (DPR) while Figures 1.3 and 1.4 illustrate the data obtained from the Shell Petroleum Development Company (SPDC) of Nigeria Limited.

Table 1-2: Nigerian Niger Delta oil spill data (Modified after DPR, 2006)

Year	Number of spill incidents	Quantity spilled (m^3)[a]	Quantity recovered (m^3)[a]
1976	128	4,158.62	1,134.37
1977	104	5,227.33	270.76
1978	154	77,791.39	62,234.67
1979	157	110,364.01	10,092.69
1980	241	95,473.44	6,743.72
1981	238	6,792.24	869.69
1982	252	6,809.57	345.22
1983	173	7,687.23	1,010.51
1984	151	6,392.71	261.50
1985	187	1,888.23	273.35
1986	155	2,051.73	87.76
1987	129	5,066.28	971.25
1988	208	1,458.23	310.82
1989	195	1,212.78	342.30
1990	160	2,375.40	332.69
1991	201	16,984.26	442.93
1992	378	8,138.22	234.78
1993	428	1,550.48	466.96
1994	515	4,814.55	371.38
1995	417	10,123.84	494.45
1996	430	7,369.54	188.08
1997	339	12,993.67	No data
1998	399	15,880.47	No data
1999	225	2,687.51	No data
2000	637	13,366.34	No data
2001	412	19,233.64	No data
2002	446	38,414.05	No data
2003	609	5,609.77	No data
2004	543	2,719.31	No data
2005	496	1,706.66	No data
Total	9,107	496,343.07	87,479.88

[a]Original unit = barrels

Source: Okparanma (2013)

Volume unit:
bbls = barrels

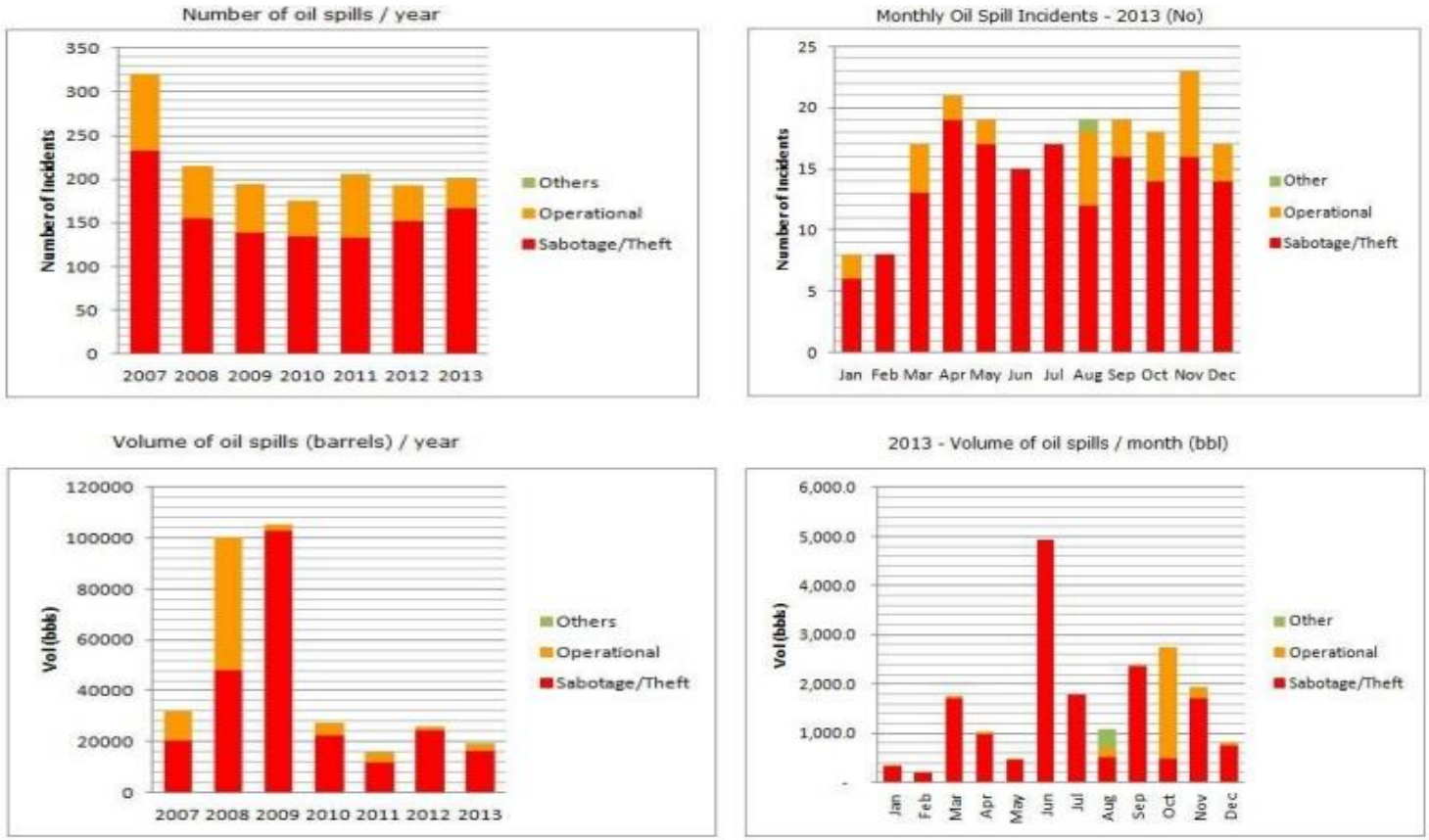


Figure 1-3: Loss of oil at the Niger Delta between 2007 and 2013 as recorded by SPDC

Source: SPDC (2015a)

Volume unit:
bbls = barrels

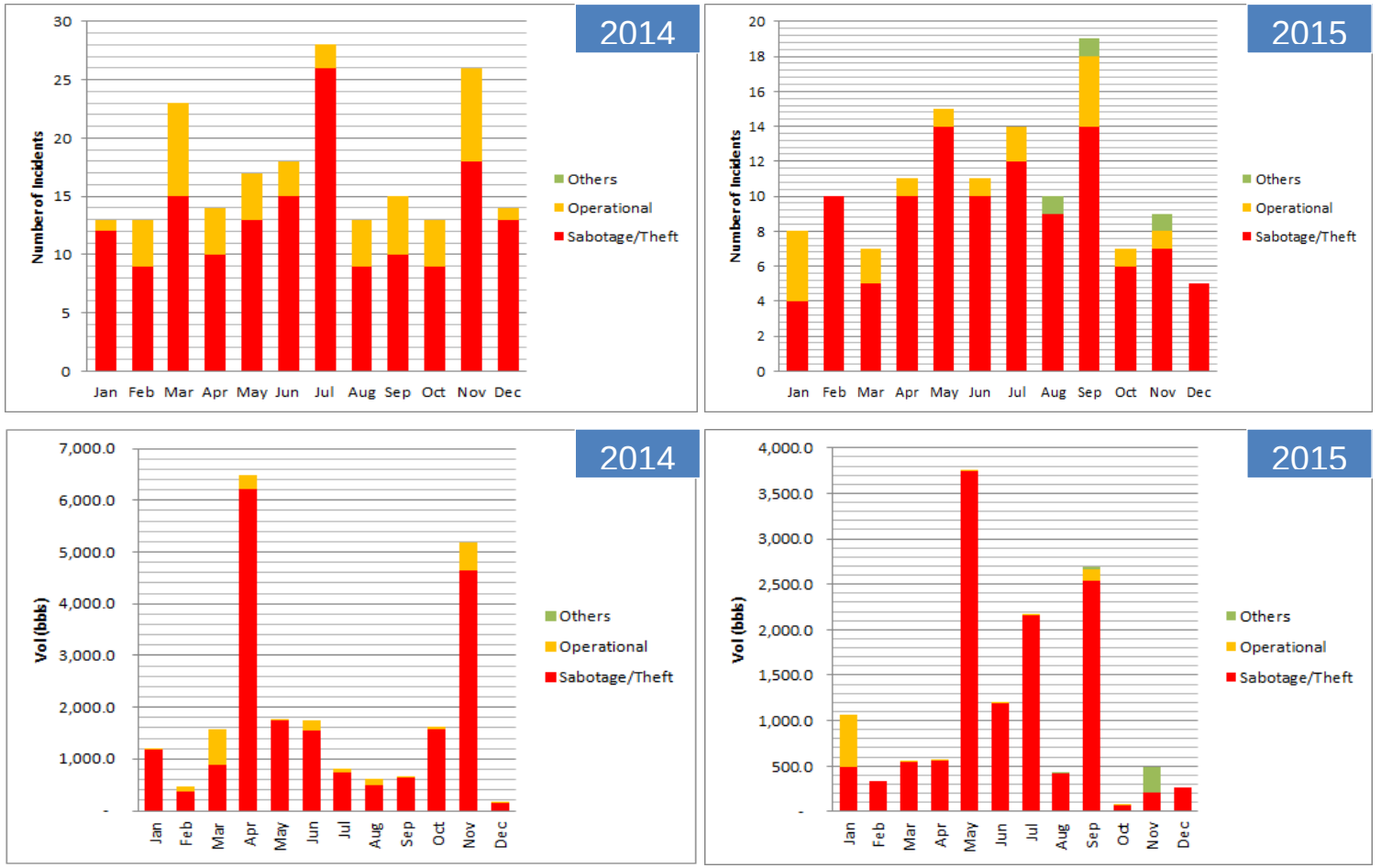


Figure 1-4: Niger Delta oil spill incidents – (top): Monthly oil spill incidents, (bottom): Volume of spills per month

Source: SPDC (2015b; 2016)

In order to evaluate the nature and degree of oil contamination in Ogoniland (a part of the Niger Delta), the Federal Government of Nigeria made a request to United Nations Environment Programme (UNEP) for an independent assessment of the region (UNEP, 2011). This involved several other partners such as community organizations, Rivers state government, laboratories, land owners and universities, in addition to UNEP while SPDC provided the sponsorship (Adati, 2012). In line with this author, the information obtained from the study would assist in the rehabilitation of the region to meet international standards.

1.2 Justification of the research

Food has been identified as one of the likely means of human exposure to polycyclic aromatic hydrocarbons (PAHs) (Hattemer-Frey and Travis, 1991; Audebert et al., 2010; Ball and Truskewycz, 2013) including benzo-a-pyrene (BaP) – the most toxic PAH (Hattemer-Frey and Travis, 1991). These PAHs, which may be from crude oil spills, have negative effects on plants (Oghenejoboh et al., 2008) and Glasson et al. (1999, cited in Oghenejeboh et al., 2008, p. 605) suggest that they may become bioaccumulated with eventual contamination of the food chain.

According to CCME (2008), there are four major routes by which chemical contaminants in the soil environment can be transferred into plants. They could be transported: (1) by a liquid phase transfer from soil solutions through the root, (2) by a form of vapour phase transfer either from the surrounding air to the above-ground plant parts or from soil air spaces to the root, (3) through particle phase deposition on the shoots, and (4) via uptake in oil cells or channels of oil-containing plants. However, a number of processes within the soil such as: adsorption/desorption, biotic and abiotic degradation, volatilization, erosion/runoff and leaching can reduce the amount of the organic contaminants (e.g. PAHs) available for plant uptake (O'Connor, 1996; CCME, 2008) while some may undergo photochemical or chemical transformation (Beck et al., 1996; CCME, 2008). Among other factors, the form of the organic contaminant

in the soil plays a critical role in determining its bioavailability for plant uptake (Beck et al., 1996).

On the other hand, “the general consensus in the literature is that the root uptake pathway of organic contaminants such as hydrocarbons and PAH constituents from the soil by plants is extremely limited” (CCME, 2008, p. 68). De Nicola et al. (2015) also support this as organic chemicals have low water solubility with retention possibilities in soils (Lambert, 1968; Cornelissen et al., 2005; Müller et al., 2007). Figure 1.5 illustrates the various phases through which chemical contaminants generally become transferred into plant leaves via roots, which could lead to food chain contamination.

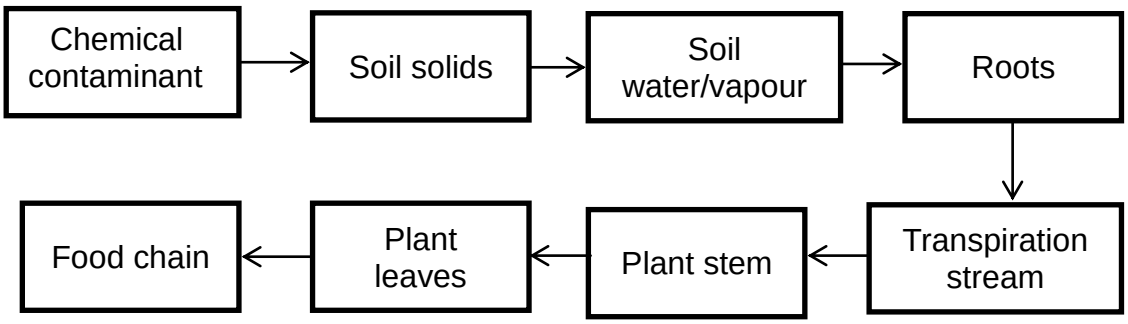


Figure 1-5: Chemical contaminant uptake by roots from the soil

Adapted from: CCME (2008, p. 67)

Surprisingly, despite the numerous accounts of the detrimental effects of crude oil spills on the environment and agrifood production, in line with Salanitro et al. (1997), a global total petroleum hydrocarbons (TPH) cleanup standard for soils contaminated with fresh or weathered crude oils is yet to be set. Calabrese and Kostecki (1993) however recommended target cleanup concentrations such as that of 10 – 10,000 mg/kg TPH (Salanitro et al., 1997) based on PAHs for oil products (details in Chapter 2, Section 2.3.2); petroleum hydrocarbon concentration in the environment above the intervention value of 5,000 mg/kg TPH is also used by several national regulations (Presidency Ministry, cited in Pinedo et al. 2014, p. 1232) to indicate serious contamination (Soil Remediation Circular, 2009; UNEP, 2011; Pinedo et al. 2014).

Although TPH determination is exactly not a measure of petroleum hydrocarbons but that of soluble compounds in certain solvents and detected by various analytical procedures (such as the gravimetric, infrared and gas chromatography) (McMillen et al., 2001), it is commonly adopted by environmental experts as an acceptable regulatory benchmark for assessing petroleum contamination (Xie et al., 1999). The measurement is also defined by the analytical procedure used as different TPH methods would give different TPH concentration of a sample owing to the difference in solvent type, extraction procedure, detection method, and quantification standards (McMillen et al., 2001). In line with Zhu et al. (2001), the gravimetric and infrared spectroscopic methods are the widely accepted TPH techniques for rapid quantification of the oil as a result of their simplicity and low costs. However, these methods are usually associated with low specificity, high detection limits (≥ 10 mg/kg), and sensitivity to matrix effects, which require elective drying and separation steps (Xie et al., 1999).

Practically, the evaluation of TPH has involved the use of: Method 413.1 for oil and grease (a gravimetric method), Method 418.1 (involving infrared spectroscopy) or Modified 418.1 as well as Modified 8015M for Diesel-Range Organics (DRO) and Gasoline-Range Organics (GRO) involving gas chromatography (GC) (Hunt, 1979, cited in McMillen et al., 2001, p. 59). Meanwhile, in addition to these methods, the absence of a unified method for the assessment of petroleum hydrocarbons has led to the development and application of permutations of these methods for estimating TPH by environmental regulatory agencies and individual commercial laboratories (McMillen et al., 2001). Most of these methods are modified forms of the gas chromatographic methods referred to as “modified 8015” (McMillen et al., 2001) as the use of GC methods have been found to greatly advance the determination of TPH (Xie et al., 1999).

On the behaviour of oil in the environment, several authors (Alexander, 1995; Chung and Alexander, 1998; Tang et al.,1998; Northcott and Jones, 2001; Reeves et al., 2001; Huesemann et al., 2004; Tang et al., 2012) have reported the ability of hydrophobic contaminants to become sequestered with

increasing contact time in the soil matrix as a result of their partitioning into organic matter or diffusion into nanopores which reduce their extractability and bioavailability to microorganisms as well as other ecological receptors. This time-dependent decline in bioavailability is often referred to as aging (Aexander, 1995; Chung and Alexander, 1998; Northcott and Jones, 2001; Hongjian, 2009; Tang et al., 2012). Tang et al. (2012) added that most times, these bioavailability limitations or aging effect, which reduces the efficiency of the biodegradation process, makes the attainment of remediation endpoints unachievable. According to Alexander (1995), this reduction in bioavailability and occurrence of chemical sequestration must be put into consideration in the assessment of toxicity, risk determination, and the establishment of cleanup regulations. Also, Alexander (2000) pointed out the possibility of current analytical methods to overestimate the environmental challenge pose by organic pollutants as they measure total but not bioavailable concentrations.

In this author's finding, the bioavailability of organic compounds that have undergone aging (or weathering) in soil is less than freshly added compounds. The weathering process involving: evaporation, dissolution, emulsification, sedimentation, microbial oxidation, and photooxidation lead to the continuous change in the composition of oil (physical and toxic properties) that enters the environment (Mendelssohn et al., 2012). Hence, from the environment point of view, freshly spilled oil is the most important type of oil as a result of its increased level of: volatility, water-soluble components and dispersion from the source, as well as reduced viscosity (Mendelssohn et al., 2012). In other words, fresh oils have higher toxic potential than weathered oils (Mendelssohn et al., 2012). Some of the factors determining the aging behaviour of organic pollutants include the: soil properties, hydrophobic organic compounds, and environmental conditions (Zhao et al., 2009; Tang et al., 2012).

Furthermore, "the development of the 1% TPH guidance level in soil was originally based on the issue of toxicity to plants. If the amount of petroleum hydrocarbon in soil is kept at or below this level, and there are acceptable salt and pH levels, there should not be significant reductions in plant yield, thus meeting landowner requirements" (McMillen et al., 2001, p. 39). Nevertheless,

light or fresh crude oil at this regulatory level (such as 10,000 mg/kg of crude oil in soil), demands stricter management measures (McMillen et al., 2001).

The analysis carried out on 60 crude oil samples obtained from different parts of the world showed that the 16 pollutant priority PAHs were in extremely low concentrations while the analysis of 18 metals in twenty-six crude oils also showed no potential significant human health risk at crude oil in soil concentration exceeding 10,000 mg/kg (McMillen et al., 2001). Consequently, it was concluded that the analysis of PAHs (with the exception of naphthalene) and metals at contaminated sites with TPH below 10,000 mg/kg was not essential (McMillen et al., 2001). This, however, needs further investigation especially as regards to agrifood production for human consumption.

According to SPDC (2010, cited in UNEP, 2011, p. 148), the initial cut-off TPH value of 5,000 mg/kg used at the Nigerian Niger Delta to confirm remediation was changed to 3,000 mg/kg as provided in the Remediation Management System (RMS) document. This new remediation practice was also developed to incorporate the use of an *ex-situ* Remediation by Enhanced Natural Attenuation (RENA) approach (UNEP, 2011) which is a form of bioremediation. Nonetheless, the efficacy of this new remediation method, involving the use of an *ex-situ* RENA strategy, in providing additional improvement to the existing hydrocarbons remediation outcomes in the region has not been practically substantiated (UNEP, 2011).

Lastly, in spite of the slower rate of biodegradation of aliphatic hydrocarbons compared to the aromatics, most research focus have been on the latter distribution and presence in soil (De Pasquale et al., 2012) while previous studies (laboratory, mesocosm and field-works) have sometimes considered remediating crude oil polluted sites for a period of time without having any specific remediation end point. In cases where phytoremediation has been adopted, the plants have mainly been employed for remediation purposes with Noori et al. (2015) indicating the need to evaluate the effect of hydrocarbons exposure on such plants. Hence, it is necessary to: (1) assess the effectiveness of RENA used by SPDC (UNEP, 2011) in supporting petroleum hydrocarbons degradation as well as agrifood production, (2) investigate the effect of different

CRIV on the yield and quality of crops, and (3) provide a better understanding of the effect of the stress caused by crude oil contamination on plants.

1.3 Aim and objectives of the research

The study is targeted towards assessing the influence of petroleum hydrocarbons at oil spill/remediated sites on the chemical and nutritional composition of plants. The response of plants under the selected bioremediation strategies will give an understanding whether bioremediated sites provide similar growing environments as clean sites while examination of the chemical composition on plants has been identified as one of the ways of studying the effect of environmental pollution (Malallah et al., 1996) including those from hydrocarbon-contaminated soil (Malallah et al., 1996; Achuba, 2006).

The research aim was to investigate whether crops from crude oil spill/bioremediated sites have reduced nutritional value but pose no carcinogenic risk.

1.3.1 Objectives

The objectives of this research were to:

1. critique literature on crude oil, bioremediation strategies, remediation intervention values, consequences of PAHs in soils and plants, nutritional composition of plants, and the methodological process that would be useful in achieving the aim of the research;
2. characterise the crude oil and determination of the physicochemical properties of the growing media/composts used for the study;
3. evaluate the efficiency of two bioremediation strategies - RENA and bioaugmentation - for petroleum hydrocarbons degradation and agrifood production;
4. assess the impact of different CRIV on the yield/growth parameters of the selected plants and contribution to VOCs emission; as well as
5. evaluate the effect of different CRIV on the chemical and nutritional composition of the harvested plants.

1.3.2 Research flow

The approach followed in conducting the research is shown in Figure 1.6 while Figure 1.7 gives an illustration of the stages involved in the experimental set-up.

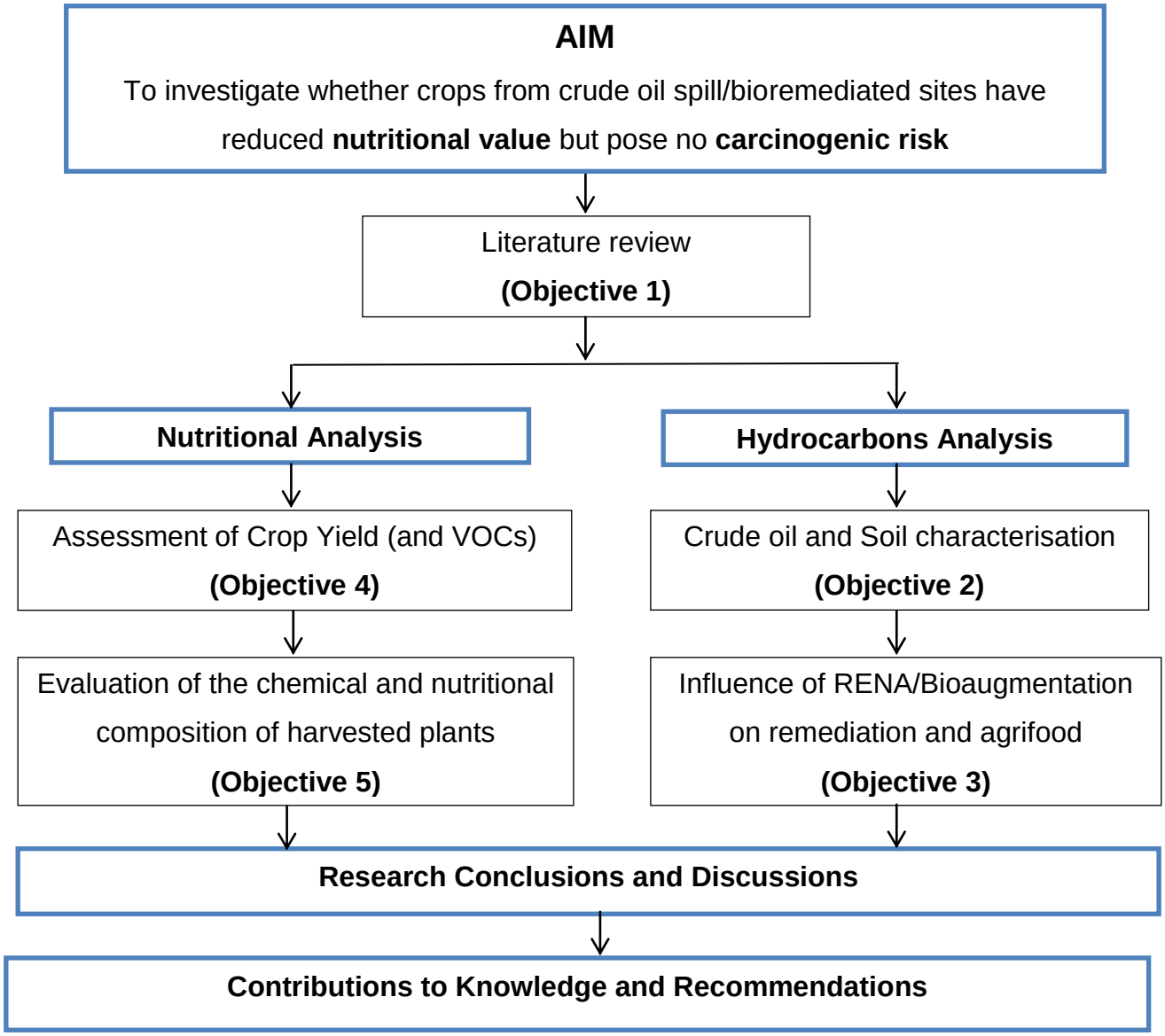


Figure 1-6: The simplified research flow chart

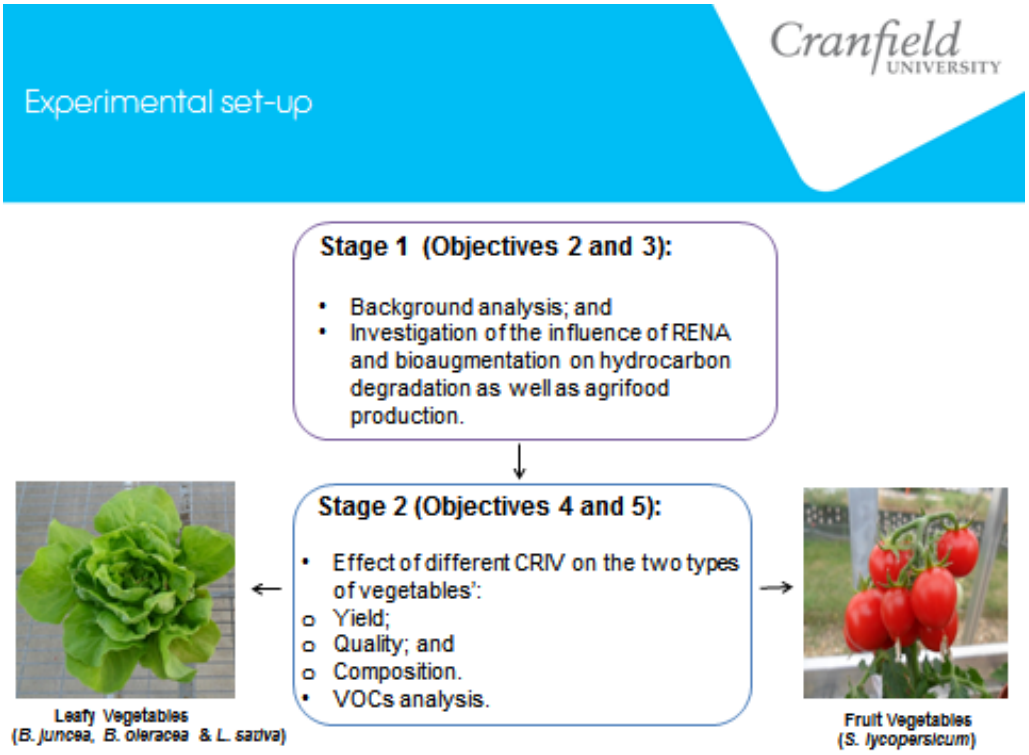


Figure 1-7: Stages in the experimental set-up

CHAPTER 2
LITERATURE REVIEW

2.1 Crude oil

The world's energy demands have led to the high extraction rate of crude oil (Nicolotti and Egli, 1998), the liquid hydrocarbon found in nature (UBS, 2004, cited in UNEP, 2011, p. 36), also referred to as crude petroleum (Plice, 1948). Petroleum which derives its name from the combination of two basic Latin words - 'petra' meaning rock and 'elaion' - oil (UNEP, 2011), is a complex mixture of aliphatic and aromatic hydrocarbons (Mehboob et al., 2009) with different molecular masses (UNEP, 2011; Tang et al., 2011). Although the distribution of the various petroleum hydrocarbons in crude oil varies with the field and well in view (Swingle and Snapp, 1931), the petroleum hydrocarbons occur in gas, liquid or solid form based on the length of the carbon chain (UNEP, 2011). Also, the natural resource which according to Ojo and Adebusuyi (1996, cited in Ekundayo et al., 2012, p. 42) varies in density and can be described as heavy, average or light, is found in three different colours: black, green or light yellow (Onifade et al., 2007b; Ekundayo et al. 2012).

Generally, petroleum hydrocarbons can be divided into four different groups which include the: (1) saturates, (2) aromatics, (3) asphaltenes (phenols, fatty acids, ketones, esters, and porphyrins), and (4) resins (pyridines, quinolines, carbazoles, sulfoxides, and amides) (Cowell and Walker, 1977, cited in Leahy and Colwell, 1990, p. 305) with the saturates (the alkanes and cycloalkanes) being the most dominant in proportion (UNEP, 2011). The aromatic group includes the polycyclic aromatic hydrocarbons (PAHs), some of which have been considered to have carcinogenic properties (UNEP, 2011). In addition to the hydrocarbon constituents, crude oil also contain nitrogen, oxygen and sulphur in small quantities (UNEP, 2011) as well as trace metals occurring partly as organo-metallic compounds comprising the geoporphyrins of V, Ni, Cu and Zn (Gondal et al., 2006). Other metal ions that have been identified are: lead, iron, magnesium, chromium, sodium, molybdenum, cadmium, titanium, manganese, gallium, cobalt, antimony, uranium, aluminium, tin, barium, silver

and arsenic (Khuhawar et al., 2012). According to Gary and Handwerk (2001, cited in Mello et al., 2012, p. 16), the concentration of these elements (metals and non-metals) could be up to 1,000 µg/g. Figure 2.1 shows the chemical classification of petroleum hydrocarbons.

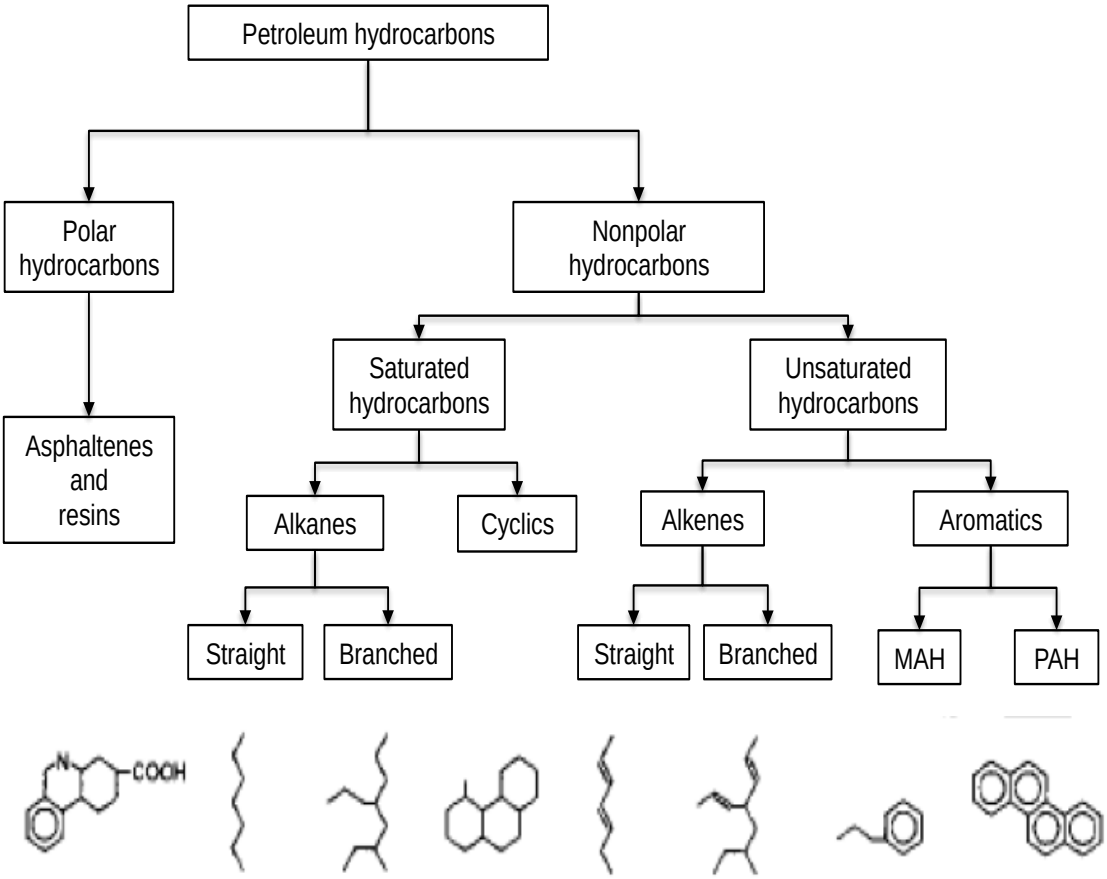


Figure 2-1: Chemical classification of petroleum hydrocarbons
(MAH = Monoaromatic hydrocarbons; PAH = Polycyclic aromatic hydrocarbons)
Adapted from: TPHCWG (1999, cited in Coulon and Wu, 2014, p. 2)

2.2 Environmental pollution when oil spills

Pollution involves the release of elements, compounds or energy at undesirable levels into the environment in a way that negatively affects its functioning or results to an unacceptable risk to man or other objects connected to the environment in any form (Scullion, 2006). This occurs in various ways, and also covers those arising from petroleum hydrocarbons (PHC) which are

one of the most common environmental contaminants found in different parts of the world (Mehboob, 2009; Wang et al., 2012) such as the Nigerian Niger Delta (Adati, 2012), Canada and the United States (Wang et al., 2012). According to UNEP (2011), in the least damaging circumstance like the small spill of a volatile hydrocarbon on dry soil, the properties (chemical or physical) of the soil may not be impaired, but the soil may sometimes be rendered a source of pollution such as when a heavy crude oil spill on clay soil. Further details on the effect of petroleum hydrocarbons on the growing environment and plants are discussed in Section 2.5.2.1.

Polycyclic aromatic hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs), also referred to as polyaromatic hydrocarbons (UNEP, 2011; Ball and Truskewycz, 2013) or polynuclear aromatic hydrocarbons (Boehm, 2005; UNEP 2011), are hydrocarbons with two, three or more fused aromatic rings (Cerniglia, 1992; Okparanma et al., 2011). As a result of their hydrophobic nature (Cerniglia, 1992) and low solubility in water, they are not easily degraded in the environment (Cerniglia, 1992; Okparanma et al., 2011) while the high-molecular-weight PAHs have been found to be more recalcitrant because of the presence of additional rings in their structure which may require more degradation time and in some instances, different/specific microorganisms for such degradation (Silva et al., 2009; Sukor et al., 2012). In line with Maliszewska-Kordybach (1999), they are part of the group of persistent organic pollutants (POPs).

PAHs can be carcinogenic or mutagenic (Okparanma et al., 2011) while sixteen members of this group: naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, pyrene, benz[a]anthracene, fluoranthene, chrysene, benzo[a]pyrene, benzo[b]fluoranthene, benzo[k]fluoranthene, indole[1,2,3-cd]pyrene, dibenz[a,h]anthracene and benzo[g,h,i]perylene, have been classified by the United States Environmental Protection Agency (US EPA) to be among priority pollutants (Latimer and Zheng, 2003, cited in

Okparanma, 2011, p. 5149). Table 2.1 illustrates the two categories of the 16 unsubsitituted PAHs with those having carcinogenic potential indicated.

Table 2-1: List of US EPA 16 Priority PAHs

Lower Molecular Weight PAHs	Higher Molecular Weight PAHs
Naphthalene	Fluoranthene
Acenaphthene	Pyrene
Acenaphthylene	Benzo[g,h,i]perylene
Fluorene	Benz[a]anthracene*
Anthracene	Chrysene*
Phenanthrene	Benzo[b]fluoranthene*
	Benzo[k]fluoranthene*
	Indeno[1,2,3-cd]pyrene*
	Dibenz[a,h]anthracene*
	Benzo[a]pyrene*

***** The asterisk symbol indicates those with carcinogenic possibility

Adapted from: CCME (2010, p. 12)

The division of the PAHs based on their molecular weight provides a good knowledge of the behaviour of the PAHs as their hydrophobic nature, affinity for bioaccumulation, resistance towards biodegradation and recalcitrant ability in the environment, decrease with decreasing molecular weight (CCME, 2010). For instance, low-molecular-weight PAHs such as naphthalene, present a higher level of toxicity to aquatic organisms than the high-molecular-weight PAHs since the former are more water soluble (CCME, 2010).

PAHs may also be classified based on the form of the core ring structure. Those with a core ring structure composed entirely of benzenoid rings (such as: naphthalene, phenanthrene, anthracene, pyrene, benz[a]anthracene, chrysene, benzo[a]pyrene, benzo[g,h,i]perylene, dibenz[a,h]anthracene) are referred to as alternant PAHs while the non-alternants are those with four, five and six-

member, non-aromatic ring arrangements. This latter class include acenaphthene and benzo[k]fluoranthene (CCME, 2010) as shown in Figure 2.2.

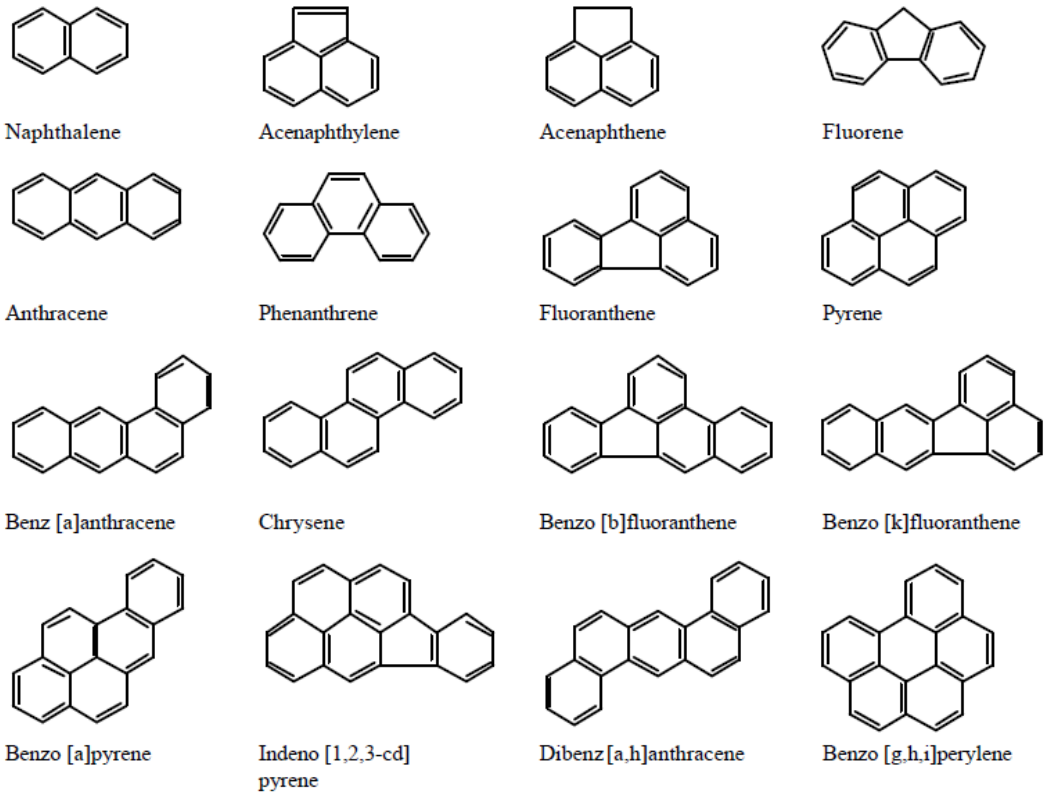


Figure 2-2: Chemical structures of US EPA 16 priority PAHs

Source: McMillen (2001, p. 143)

Introduction of PAHs into the environment

Polycyclic aromatic hydrocarbons (PAHs) are introduced into the environment through natural and man-made sources (Xu et al., 2005; Ball and Truskewycz, 2013;). The natural sources include volcanic eruptions and forest fires (ATSDR, 1995; Ball and Truskewycz, 2013) while man-made sources come from petroleum refining and spills, burning of fossil fuels and wood, coke and charcoal production as well as metal smelting (Latimer and Zheng, 2003, cited in Billiard et al., 2008, p. 5).

These organic contaminants (PAHs) may also be found in higher amount in food samples such as vegetables, fruits and fish from polluted regions when compared to those from less polluted areas (CCME, 2010). Among these PAHs,

benzo[a]pyrene which is widely distributed, is regarded as the most dangerous as a result of its carcinogenic effect on animals (Ayres and Hellier, 1998).

2.3 Remediation strategies

The negative impacts of crude oil spills on the environment and the slow process of the natural reduction in the amount of crude oil at polluted sites have given rise to the need for remediation (Gradi, 1985, cited in Ebuehi et al., 2005, p. 103). According to Soesilo and Wilson (1997), commercially, these remediation technologies may be classified based on:

(1) The way the contaminants are treated, such as the destruction technologies involving incineration.
(2) The treatment methods: This is regarded as the standard classification and in order of increasing cost of implementation, the classification include: (a) biological (i.e. bioremediation), (b) physico-chemical, and (c) thermal treatment technologies.
(3) The placement of the technology: This typically describes where the remediation treatment is taken place and involves: (a) *in situ* (treatment at the actual location/affected environment without any form of excavation), and (b) *ex situ* treatments. In the latter, the treatment is done either on-site or off-site at a place different from the original contaminated site.

Other technologies which have also been adopted for remediation purposes include: containment (to prevent the spread of contaminants) coupled with monitoring and measurement technologies (Soesilo and Wilson, 1997).

2.3.1 Biodegradation of petroleum hydrocarbons

As reported by Venosa et al. (1996), light crude oils undergo biodegradation faster than heavy oils while Atlas and Philip (2005), based on their classification, added that size and solubility also govern the biodegradation of hydrocarbons. The saturates show faster degradation rate while components

with longer chain may present higher resistance to the microbial degradation (Jobson et al., 1972; Leahy and Colwell, 1990).

Generally, although petroleum spilled on or applied to soil presents reduced toxicity as a result of their adsorption to particulate matter, this adsorption tendency may also contribute to their persistence (Leahy and Colwell, 1990). These authors (Leahy and Colwell, 1990), indicated that oxygen, nutrient supply, moisture, and pH are the principal factors controlling the microbial degradation of hydrocarbons in the soil environment while the rates of biodegradation usually increase with increase in temperature. They added that: (1) bacteria and fungi are the chief agents of degradation with the former assuming the major role in marine ecosystems and the latter in the freshwater and terrestrial environments, and (2) communities with a history of exposure to hydrocarbons (referred to as adapted communities) present higher biodegradation rates when compared to new communities in this regard. A summary of the optimum environmental conditions affecting degradation of contaminants as provided by Vidali (2001) is shown in Table 2.2.

Table 2-2: Optimum environmental conditions affecting degradation

Parameters	Required conditions for microbial activity	Optimum value for oil degradation
Soil moisture	25 - 28% of water holding capacity	30 - 90%
Soil pH	5.5 – 8.8	6.5 – 8.0
Oxygen content	Aerobic, minimum air-filled pore space of 10%	10 – 40%
Nutrient content	N and P for microbial growth	C:N:P = 100:10:1
Temperature (^{o}C)	15 – 45	20 – 30
Contaminants	Not too toxic	Hydrocarbon 5 – 10% of dry weight of soil
Type of soil	Low clay or silt content	

Adapted from: Vidali (2001, p. 1167)

2.3.2 Intervention and target values

In line with Calabrese and Kostecki (1993), when considering risk-based soil cleanup levels, chemical-specific soil analyses and risk assessment are carried out with respect to: the potential carcinogenic PAHs, the noncarcinogenic PAH, benzene, toluene, ethylbenzene, and xylene(s), as they are chemicals of concern. The latter compounds [i.e. benzene, toluene, ethylbenzene, and xylene(s)] referred to as BTEX (Pinedo et al., 2014; Fahmy et al., 2015) however attract a special focus when considering spills from gasoline or other low boiling hydrocarbon mixture.

Whereas “remediation standards are clean-up levels that are established to indicate that the concentration of a contaminant at a clean-up level is considered not to pose a threat to human health and the environment” (Soesilo and Wilson, 1997, p. 255), as mentioned in Chapter 1 and as stated in Salanitro et al. (1997), there is yet to be a regulatory guideline both at the federal and state level for a unified TPH cleanup standard for crude oil contaminated soils.

Although a cleanup level of 100 mg/kg of TPH has been used in some states in the United States for hydrocarbons contaminated soils (Bell, 1990, cited in Calabrese and Kostecki, 1993, p. 303), according to Calabrese and Kostecki (1993), the value is not ideal as it is not risk-based and also considering the nature of hydrocarbon mixtures with differing chemical composition as well as degree of toxicity. Hence, risk-based soil cleanup levels which put into consideration the chemical composition of the hydrocarbon mixtures should be used whenever possible and also take priority over the use of generic TPH cleanup levels. Nonetheless, the use of generic TPH target cleanup levels (i.e. mixture-specific TPH target cleanup levels) provided in Table 2.3 could only be employed/considered in cleanup goals (with the use of appropriate uncertainty factor to provide additional margin of safety) when the nature of the hydrocarbon mixture is known. They are also applicable in site remediation plans with little or no information of the PAH content (Calabrese and Kostecki, 1993).

Table 2-3: Recommended TPH Target Cleanup levels based on PAH[a]

Hydrocarbon Mixture	Residential (mg/kg)	Industrial (mg/kg)
70% Gasoline/25% diesel fuel (weathered)	3,689	10,680 (4,108)[b]
Kerosene (weathered)	1,434	4,151
Diesel fuel #2/fuel oil #2 (weathered)	760	2,200 (1,692)[b]
Fuel oil #6 (weathered)	455	1,317
Unleaded gasoline	11,176 (7,890)[b]	32,353 (7,890)[b]
North Slope crude oil	3,762	10,577
Fuel oil #2	2,550	7,383 (4,102)[b]
Kerosene	1,610	4,661
Asphalt	1,382	4,000
Bunker fuel oil #6	732	2,120
Fuel oil #4	559	1,618

[a]Considering conservative 25-30 year soil ingestion and dermal contact exposure scenarios for benzo(a)pyrene toxic equivalents.
[b]Involved the use of additional uncertainty factors.

Adapted from: Calabrese and Kostecki (1993; p. 315)

In Nigeria, impacts of the operations of the oil industry on the surrounding environment are governed by the regulations set-out in the Environmental Guidelines and Standard for the Petroleum Industries in Nigeria (EGASPIN) document issued in 1992 (EGASPIN, 1992, cited in UNEP, 2011, p. 86). Although this document was re-issued in 2002, there is no further guidance provided while the regulations mentioned in the 1992 document still remain operational for the oil industry in the country (UNEP, 2011). In this, two approaches were presented for pollution incidents which include: (a) the use of the prepared American Society for Testing of Materials (E1739-95) Standard Guide for Risk-Based Corrective Action Applied at Petroleum Sites which was reapproved in 2010 or the (b) the application of “intervention and target values” (UNEP, 2011). Conforming to these guidelines, the intervention values are those where the concentration of petroleum hydrocarbons in the environment

presents serious risk to the general ecosystem in which higher concentration beyond the intervention value denotes serious contamination while at the target values, the threat posed by the contamination is believed to have been removed and soil quality required for sustainability regained for human, animal as well as plant life (UNEP, 2011). Following from the Dutch RIVM (National Institute of Public Health and the Environment) as provided in McMillen et al. (2001), the intervention values are applicable to all sites with size more than 25 m^3 while the target values are the desired soil quality levels.

According to UNEP (2011), 5,000 mg/kg TPH is used by SPDC as its remediation criterion for soil. Although there is no reason given for the selection of this value, the National Oil Spill Detection and Response Agency (NOSDRA) assumes that it was from the intervention value of 5,000 mg/kg given in the EGASPIN document. There are however some issues of inconsistency in this document such as the use of 50 mg/kg TPH as a 'target value' for restoration after crude oil spill in one section of the legislation as well as the use of 5,000 mg/kg TPH as an 'intervention value' for remediation closure in the remediation of contaminated land in another section (UNEP, 2011). Notwithstanding, following from the discussion between SPDC and UNEP, the remediation close-out value of 5,000 mg/kg TPH used by SPDC may not have been drawn from EGASPIN, but based on a risk assessment (UNEP, 2011). The new RMS document adopted by Shell Exploration and Production Companies in Nigeria (SEPCiN) in January, 2010 (SPDC, 2010, cited in UNEP, 2011, p. 148) however indicated that this TPH cut-off value of 5,000 mg/kg has been reduced to 3,000 mg/kg and is also used as the new remediation intervention value to show the company's commitment to remediation.

On the other hand, there are different ranges of TPH cleanup standards and regulations (between 100 to 20,000 mg/kg or 0.01 to 2.0%) in the United States while in some part of the country like Texas, Louisiana, Colorado and Michigan, a one percent (10,000 mg/kg) cleanup level is used (Wayne and James, 1999). In keeping with Wayne and James (1999), these various regulations can be linked with one of the three origins: "(1) use of historically developed 1% TPH for protection of plants and water resources, (2) use of TPH numbers from other

regulations such as underground storage tank rules, and (3) current development of more comprehensive risk based evaluations".

Usually, most of these regulations and guidelines use overall TPH which according to Oklahoma DEQ (2012) is employed as it may not be practically possible to assess the hundreds of compounds that could be available. In the view of Pinedo et al. (2014), the TPH range from C_{10} to C_{40} is frequently used as an environmental parameter to evaluate petroleum contamination in the media containing large amount of substances.

2.3.3 Analysis of petroleum hydrocarbons

According to Zhu et al. (2001), the reduction in the levels of spilled oils and target oil constituents is one of the major ways in which the success of bioremediation treatments can be evaluated. Techniques commonly used to assess the petroleum hydrocarbons levels include: gravimetric methods, infrared spectroscopy (IR), thin-layer chromatography-flame ionization detection (TLC-FID), gas chromatography/flame ionization detection (GC/FID), and gas chromatography-mass spectrometry (GC/MS) (Zhu et al., 2001). In line with TPHCWG (1998), the GC-based methods are currently the preferred laboratory technique for TPH determination as they: (1) detect a broad range of hydrocarbons, (2) are both sensitive and selective, as well as (3) can be used for the identification and quantification of TPH. The GC/MS in particular which involves the use of the mass spectrometer is the choice of methods for specific target compound determination, such as PAHs (Wang and Fingas, 1997; Zhu et al., 2001).

Although the IR-based methods which are used in the past are simple, fast and economical, there is a reduction in their current use as a result of the: global ban on Freon production required in the extraction of sample and measurement stages, nonspecificity of these methods, and inability to give information on TPH identification and potential risk (TPHCWG, 1998). The gravimetric-based methods, with similar advantages and disadvantages of the IR-based methods, are more appropriate for very oily sludges and wastewaters which may be difficult to analyze by other sensitive methods. The immunoassay

TPH methods are also becoming well known for field testing applications as they provide a simple and fast way of carrying out *in-situ* TPH quantification (TPHCWG, 1998).

Generally and as pointed out in Chapter 1 (Section 1.2), TPH measurement is defined by the analytical method used (McMillen et al., 2011) while Xie et al., (1999) also noted that there is no single procedure that can be used as a representative, nonspecific technique for TPH determination. Table 2.4 gives a short summary of these TPH analytical methods.

Table 2-4: Summary of TPH analytical methods

TPH Methods	Method Type/ Environmental Media	Typical Carbon Ranges Detected
GC-Based	Primarily laboratory but also field applications – can be adapted for all media.	Normally between C_6 and C_{25} or C_{36} (can be modified for higher carbon numbers).
IR-Based	Laboratory and field screening – most appropriate for water and soil.	Most hydrocarbons with some exceptions (e.g. the volatiles).
Gravimetric	Laboratory – most appropriate for wastewaters, sludges and sediment.	Anything that is extractable (with exception of volatiles which are lost).
Immunoassay	Field-screening – most appropriate for soil and water.	Aromatic hydrocarbons (e.g. BTEX, PAHs).

Adapted from: TPHCWG (1998, p. 20).

2.3.4 The need for bioremediation

The remediation of a contaminated site requires the knowledge of the potential environmental threat/risk which in turn is partly a function of the expected end-use of the site after remediation (Hester and Harrison, 1997).

Although there are different biological, chemical or physical processes that may be employed in remediation (Ebuehi et al., 2005), the combination of these techniques most times give the most efficient remediation process (Armishaw et al., 1992, cited in Scullion, 2006, p. 55). In some cases however, the use of these remediation measures lead to the transfer of pollutants from the sites to the atmosphere (Adekunle, 2011) such as volatile organic compounds (VOCs) present in crude oil (UNEP, 2011).

In the physico-chemical treatment, the soil structure may become damaged at the end of the remediation process (Couto et al., 2010) in spite of the high cost of implementation involved (Ouyang et al., 2005; Couto et al., 2010). This then calls for a better environmentally friendly approach that will be devoid of contaminant transfer. Also, as a result of the adverse effect in the use of common techniques - physical, chemical and thermal processes – in the treatment of oil contaminated sites, as well as cost implications (Frick et al., 1999, cited in Njoku et al., 2009, p. 1), biological techniques have been considered for remediating petroleum hydrocarbons (Njoku et al., 2009). The discovery of the potent ability of fungi and bacteria to breakdown petroleum hydrocarbons (Ogbonna et al., 2007) as well as microbes, in general, capable of degrading organic contaminants (Atlas and Philip, 2005) forms the basis for the use of bioremediation in treating crude oil contaminated sites. The biological treatment (i.e. bioremediation) may also only involve ⅓ to ½ of the total cost when compared to the other treatment technologies (King et al., 1998).

2.4 Bioremediation

According to King et al. (1998, p. 59), “Bioremediation is the application of the normal metabolic processes of microorganisms to alter the chemical structure of organic materials in solid, liquid, or vapour states in order to render the compounds less toxic to humans and the surrounding environment”. In other words, it is a treatment technology involving the use of bacteria and fungi to degrade organic contaminants into non-toxic or less harmful compounds with reduced cost (Soesilo and Wilson, 1997).

Bioremediation, particularly when it can be achieved in situ, has proven to be a cheap and effective method for degrading chemical pollutants considering the need to protect public health, the environment and reduce the cost of decontamination (Atlas and Philip, 2005). The soil in question is also not destroyed and still remains suitable for plant growth (Wilson and Jones, 1993). These (i.e. the reduced cost and undamaged soil) are the major advantages it has over thermal and some physicochemical techniques (Wilson and Jones, 1993). However, the method of bioremediation employed in addressing a spill may not be the best for another as every oil spill is characterized by its distinctive factors such as the type of environment and oil involved (Skipper and Turco, 1995).

In addition, this treatment technology has the following advantages where employed: (a) attractive economics, (b) undisturbed environment (with in situ application), (c) universal (can often treat water, soil, sludges, air), (d) contaminants are destroyed or detoxified, and (e) liability is eliminated. It must also be noted, however, that the technology may not achieve the necessary clean-up standards at all sites, or meet the remediation schedule, be the least expensive or be applied easily for some complex xenobiotic chemicals which resist microbial degradation (like the highly halogenated compounds) as well as metals (King et al., 1998).

When there is complete biodegradation, arising from microbial activity (Atlas, 1981; Ebuehi et al., 2005), the target organic compounds may first be broken down into harmless products such as into organic acids and aldehydes (Atlas and Bartha, 1973, cited in Aldrett et al., 1997, p. 995) but eventually into carbon dioxide and water (Wilson and Jones, 1993; Aldrett et al., 1997; Soesilo and Wilson, 1997; Atlas and Philip, 2005; Okoh and Trejo-Hernandez, 2006) which can be accommodated without harm to the environment and living organism (Atlas and Philip, 2005). This overall process of complete destruction or reduction of the contaminants to its basic mineral constituent is also known as mineralization (Soesilo and Wilson, 1997; King et al., 1998).

In partial degradation, also referred to as biotransformation (Soesilo and Wilson, 1997; King et al., 1998), the organic compound/contaminant is broken

down into simpler daughter compounds (Soesilo and Wilson, 1997; King et al., 1998). The bioremediation of metals also involves a transformation rather than destruction as metals cannot be destroyed, but only change chemically (and sometimes biochemically) in their valence states (King et al., 1998). Hence, bioremediation may not be effective in treating metals in soils, sludges, or in solution (King et al., 1998). Some particular compounds may however resist or exhibit slow degradation in the bioremediation process and are referred to as recalcitrant and persistent compounds respectively (Soesilo and Wilson, 1997).

2.4.1 Biodegradation processes of organic compounds

Typically in the biodegradation process, the microbial degradation of organic contaminants tend to favour the lower molecular weight compounds, hence, in a contaminated site, the lighter molecular weight compounds would be degraded first (King et al., 1998). There are however three metabolic pathways (aerobic, anaerobic and fermentative) by which microbes can reduce or alter hydrocarbon constituents (King et al., 1998).

(a) Aerobic respiration (O_2 as the final electron acceptor)

Among the aforementioned degradation pathways, the aerobic is the most energy efficient and is the mechanism of choice when O_2 is available. The substrate component in the waste matrix is converted to CO_2, H_2O and other chemical species used in the formation of new biomass and in biochemical assimilation and excretion within the respiring organisms (King et al., 1998). Generally, the reaction can be depicted as:

$$\text{Substrate} + O_2 \longrightarrow \text{New Biomass} + CO_2 + H_2O$$

Many organisms that utilize aerobic respiration carry out incomplete oxidation of some substrates resulting in the formation of alcohols, aldehydes, and ketones. Many organic acids can also be produced, such as pyruvic, fumaric, citric, glycolic, oxalic, and α-ketoglutaric acids (King et al., 1998):

$$\text{Substrate} + O_2 \longrightarrow \text{New Biomass} + CO_2 + H_2O + \text{Other Organics}$$

The reaction rates for aerobic metabolism processes are faster and the process is generally easier to control in the field when compared to anaerobic technology (King et al., 1998).

(b) Anaerobic respiration (O_2 not the final electron acceptor)

Under anaerobic respiration, strict and facultative anaerobes metabolize and grow in the absence of oxygen. Strict (or obligate) anaerobes which do not tolerate the absence of oxygen in any way relies on the oxidation of substrates where the final electron acceptor is neither free oxygen nor nitrate while facultative anaerobes are also able to utilize other means of oxidizing carbon for energy and growth in the absence of free oxygen via the use of various anaerobic pathways (i.e. can use the oxygen bound to nitrogen in nitrates for the final electron acceptor) (King et al., 1998). Although these latter (i.e. facultative) anaerobes do not require or use O_2, they sometimes grow better in its presence (King et al., 1998).

In carrying out anaerobic respiration, facultative anaerobes shift metabolic pathways to NO_3 as the final electron acceptor in the absence of molecular oxygen (King et al., 1998). Strict anaerobes become active only in the absence of O_2 by the use of sulfate, carbonate, and specific organics as energy sources (King et al., 1998) which according to these authors include the sulfate-reducing and methanogenic bacteria:

$$\text{Substrate} + NO_3 \longrightarrow \text{Biomass} + N_2 + NO_2 + H_2O + CO_2$$
$$\text{Substrate} + NO_2 \longrightarrow CO_2 + H_2O + N_2$$
$$H_2 + SO_4 \longrightarrow H_2S + H_2O$$
$$\text{Organics} + H_2 \longrightarrow \text{Biomass} + CH_4$$
$$2H_2 + CO_2 \longrightarrow CH_4 + H_2O$$

According to King et al. (1998), the conversion of NO_3 to N_2O and nitrogen gas by some microbes (*Pseudomonas denitrificans*), can be used for the *in situ* bioremediation of aquifers contaminated with nitrates.

On the other hand, some groups of microbes can also utilize sulfate [which can be replaced by sulfite (SO_3) or thiosulfate (S_2O_3)] as the final electron acceptor in the oxidation of organic substrates in which the reduced end product of sulfate reduction is sulfide (King et al., 1998). When there is incomplete oxidation, organic acids are formed:

$$CH_3CHCOOH + SO_4 \longrightarrow CH_3COOH + CO_2 + H_2S$$

The oxidation of molecular hydrogen occurs as:

$$H_2 + SO_4 \longrightarrow H_2S + H_2O$$

Generally, sulfate reducers need low redox potential (-100 meV and below) environment free of oxygen (King et al., 1998).

(c) Fermentation

King et al. (1998) indicated the ability of yeast, fungi, and some specific bacteria to metabolize organic compounds via oxidation-reduction reactions to form less complex organics. According to these authors, the organic compounds are both the electron donors and acceptors in these metabolic reactions while organic substrates are only metabolized if they can be both oxidized and reduced.

They added that several classes of the more complex organics (particularly the carbohydrates) can be utilized during fermentation as they give rise to (i.e. yield) both oxidizable and reducible intermediates; where the various sugars, polysaccharides, organic acids, biogenic excretions, and the lifeless cells of dead microorganisms, are among the fermentable substrates. However, the various fermentation products depend on: the kind of substrate, fermenting organism, amount of available oxygen, as well as the environmental factors (King et al., 1998).

2.4.2 Three bioremediation application approaches

In order to remove the petroleum hydrocarbons from the contaminated site and to possibly restore this site to its initial natural state, three different bioremediation approaches have been employed (King et al., 1998). The first is the use of natural attenuation sometimes referred to as intrinsic or passive bioremediation (King et al., 1998) in which the breakdown of the toxic petroleum hydrocarbons is allowed to take place naturally without any intervention (Atlas and Philip, 2005). The contaminant becomes degraded under natural biodegradation conditions but may not be fast enough (Atlas and Philip, 2005). However, before this method is adopted, the initial evaluation of the site must show: (1) the presence of the desired microbial community, (2) availability of the right nutrients, (3) provision of appropriate/suitable environmental conditions, and, (4) the ability of the contaminants to degrade under natural conditions with time (King et al., 1998).

The two other bioremediation application approaches stated by this author (King et al., 1998) - biostimulation and bioaugmentation – accommodate interventions to provide suitable conditions (Atlas and Philip, 2005; Scullion, 2006; Couto et al., 2010; Adekunle, 2011) for enhanced pollutant degradation (Scullion, 2006). In biostimulation, the natural state of the contaminated-environment is aided to facilitate biodegradation; entails the addition of required nutrients or growth enhancing co-substrates to support the growth of resident microorganisms (Venosa and Zhu, 2003; Scullion, 2006; Ogbonna et al., 2007; Adekunle, 2011). Bioaugmentation, on the other hand, involves the introduction of additional contaminant-degrading microorganisms to the contaminated-site to further enhance the potency of the inherent microbial population (Venosa and Zhu, 2003; Atlas and Philip, 2005; Scullion, 2006; Ogbonna et al., 2007). This latter method has proven to be an effective means of breaking down recalcitrant compounds (Atlas and Philip, 2005) like PAHs (Eggen and Majcherczyk, 1998). Bioaugmentation is also suitable when the population of the hydrocarbon-degrading microorganisms, is not sufficient to bring about effective bioremediation (Forsyth et al., 1995, cited in Venosa and Zhu, 2003, p. 167). Nonetheless, it is important to note that this approach may not provide very

significant petroleum hydrocarbons degradation (Lee et al., 1997; Coulon et al., 2012) and may sometimes present some form of bioremediation ineffectiveness (Cho et al., 1997; Coulon et al., 2012) as indigenous microorganisms are well adapted to their own environment (Bento et al., 2005).

2.4.3 Soil bioremediation requirements

In the view of Flathman et al. (1993), the decision factors guiding the application of bioremediation for a site clean-up include the: (1) type of contaminant and its concentration, (2) environment involved, (3) soil type, (4) state and closeness of the groundwater to the affected soils, (5) required time for the clean-up, and (6) space availability. Okoh and Trejo-Hernandez (2006) however added that microorganisms are the principal component in bioremediation as they are involved in the production of the required enzymes for the degradation of the chemical pollutant.

According to King et al. (1998), in order to achieve a successful bioremediation process in the breaking down of organic contaminants such as the toxic petroleum hydrocarbons into harmless products by microbes, the following factors must be put into consideration:

(1) Presence of water;
(2) Availability of macronutrients: nitrogen and phosphorus (as they are the key limiting nutrients) in the form of ammonia and orthophosphate respectively; and
(3) The existence of some levels of essential minor elements (such as: sulphur, potassium, magnesium, calcium, manganese, iron, cobalt, copper, molybdenum, nickel and zinc) needed in small amounts.

It must however be noted that the macronutrients (N and P) attract the most interest in the field application of bioremediation (King et al., 1998) while this entire biodegradation process also depends on other parameters such as temperature, pH, and moisture content (Soesilo and Wilson, 1997).

2.4.4 Remediation by Enhanced Natural Attenuation (RENA)

Remediation by Enhanced Natural Attenuation (RENA), a land farming treatment technology (Ebuehi et al., 2005; Onifade et al., 2007b; Wegwu et al., 2010) and a form of natural bioremediation (Skipper, 1999, cited in Bento et al., 2005, p. 1050), incorporates the provision of aerobic microbial processes required for the remediation of petroleum hydrocarbons (King et al., 1998). This RENA approach according to UNEP (2011) is given as the principal method of remediating crude oil-polluted sites in the SPDC document via the enhancement of natural biodegrading processes to reduce the level of petroleum hydrocarbons in the environment. The method has been used by SPDC in remediating oil spills in Ogoniland and involves: the provision of aeration via tillage of the contaminated land, use of fertiliser to provide a nutrient support for microbial degradation of the petroleum hydrocarbons, as well as piling of the ploughed soil into neat windrows to increase the level of aeration (UNEP, 2011).

Although land farming has the potential to improve the growth of aerobic soil bacteria leading to faster contaminant degradation (Huang et al., 2004) coupled with the scientific basis for the use of RENA under ideal conditions, the Ogoniland situation presents a contrary result as the method has failed in meeting up with the remediation goals in this region (UNEP, 2011). Consequently, it was recommended that the use of bioremediation or augmentation of natural processes should only be applied after appropriate assessment/characterisation of the polluted sites and provision of control measures for the transfer of oil off-site arising from run-off, infiltration and other processes (UNEP, 2011). Adequate monitoring and supervision of the whole clean-up process is also suggested (UNEP, 2011).

On the other hand, Cho et al. (1997) mentioned the use of bioremediation options such as in-situ land farming, soil piles, and slurry bioreactors for the remediation of lightly contaminated soils with < 5% oil. Rhykerd et al. (1999) added that tillage may reduce remediation time as it increases the bioavailability of the oil for microbial degradation and in general enhances the bioremediation of crude oil spilled on soil. The experiment of Ebuehi et al. (2005) involving

mildly crude oil contaminated farmland settlement in Rivers State (a part of the Nigerian Niger Delta region) and the application of RENA, also showed that RENA reduced the petroleum hydrocarbons in the first 9 weeks of the 10 weeks study period. Consequently, Ebuehi et al. (2005) put forward the use of RENA for the remediation of farmland settlement contaminated with crude oil and also indicated the ability of the interplay of biotic and abiotic factors to achieve significant reduction of the petroleum hydrocarbons during RENA application.

2.4.5 Bioaugmentation with spent mushroom compost for PAHs degradation

White rot fungi (including *Pleurotus ostreatus*) have been linked with the ability to degrade lignin (Kerem et al., 1992; Bezalel et al., 1996) and lignin-related compounds, such as PAHs (Cerniglia, 1992; Bezalel et al., 1996). According to Zebulun et al. (2011), there are four advantages in the use of this class of fungi over bacteria or other methods for bioremediation purposes as white rot fungi can: (1) grow under different environmental conditions which increase their suitability in the environment requiring bioremediation, (2) begin their biodegradation activity without preconditioning to any specific contaminant, unlike in bacteria, (3) degrade a wide range of insoluble and organic contaminants in the environment as a result of their extracellular chemical reaction, and (4) be cultured using cheap nutrient sources like sawdust, agricultural wastes, wood chips etc. Meanwhile, spent mushroom substrate (SMS) has been identified to be applicable for the *in situ* bioremediation of contaminated soils (Buswell, 1994; Eggen, 1999), including those of white rot fungi (Eggen, 1999) which were adopted by Okparanma et al. (2011) in degrading PAHs in Nigerian oil-based drill cuttings. However, in this latter study by Okparanma et al. (2011), the assessment of the degradation ability of SMS on alkanes was not considered.

2.5 Abiotic environmental stresses and food composition

2.5.1 Abiotic environmental stresses

Stress may be referred to as an environmental factor capable of inducing a negative effect on living organisms (Levit, 1972, cited in Hodges and Tiovonen, 2008, p. 155) while Higley et al. (1993, cited in Peterson and Higley, 2000, p. 5) considering the response of plant to these environmental (biotic and abiotic) factors, define stress as “a departure from optimal physiological conditions”. The two forms of environmental factors - biotic and abiotic factors - are referred to as stress factors when there is a shift from the optimum level in their intensity or quantity for a specific plant (Schulze et al, 2005). Under natural conditions, plants are exposed to biotic factors such as those involving infection by pathogens like bacteria, fungi, viruses and nematodes as well as attack by pests (e.g. herbivorous insects) (Ron and Avni, 2004; Atkinson and Urwin, 2012; Suzuki et al., 2014). Abiotic factors arising from factors such as: heat, drought, soil salinity, tropospheric ozone, excess UV radiation (Wang and Frei, 2011), cold etc, also affect the optimal growth and yield of plants (Cramer et al., 2011). However, the response of plants under any of these stresses has been found to depend on the affected tissue or organ (Cramer et al., 2011). Table 2.5 by Cramer et al. (2011) illustrates an estimate of the number of published literature on the impact of abiotic stresses on crop production and further suggests the need to investigate the effect of petroleum-hydrocarbon spillage, an abiotic factor (Nie et al., 2010), on crop production.

Table 2-5: Estimates of the impacts of abiotic stresses on crop production and published research

Stress Type	Percentage of global land area affected	Percentage of global rural land area affected	Number of publications
Abiotic stress		96.5	**35,363**
Water			**4,819**
Deficit or Drought	64	16	4,137
Flooding or Anoxia	13	10	682
Temperature			**9,715**
Cold	57	26	3,798
Chilling			187
Freezing			350
High or heat			5,380
Light			7,659
Low			3,081
High			4,578
Chemical/Soil		50	**12,391**
Salt or salinity	6	6	3,498
Mineral deficiency or low fertility	9	39	222
Mineral toxicity			437
Acid soil	15		3,646
Air pollutants			
Ozone			1,369
Sulfur dioxide			378
NO_x (mono-nitrogen) oxide			2,001
Elevated CO_2			840
Miscellaneous (e.g. wind, mechanical, etc.)			779

Source: Cramer et al. (2011, p. 2)

Abiotic stresses have been identified as the key factors responsible for most of the reduction in agrifood production (Figure 2.3) as well as to play a major role in determining the nutritional value/quality of fruits and vegetables during their growth, harvesting, handling, storage and transport to end users (Kumar et al., 2014). Consequently, investigation on the effects of: production, processing and postharvest stresses on the quality parameters of whole fruits and vegetables, have gained attention (Hodges and Toivonen, 2008). Among all of the possible abiotic stress factors such as drought, salinity, heavy metal, ultraviolet radiations etc (Khan et al., 2014), Tattersall et al. (2007) believes that drought, salinity and cold are the three major environmental factors that can have negative effect on plants.

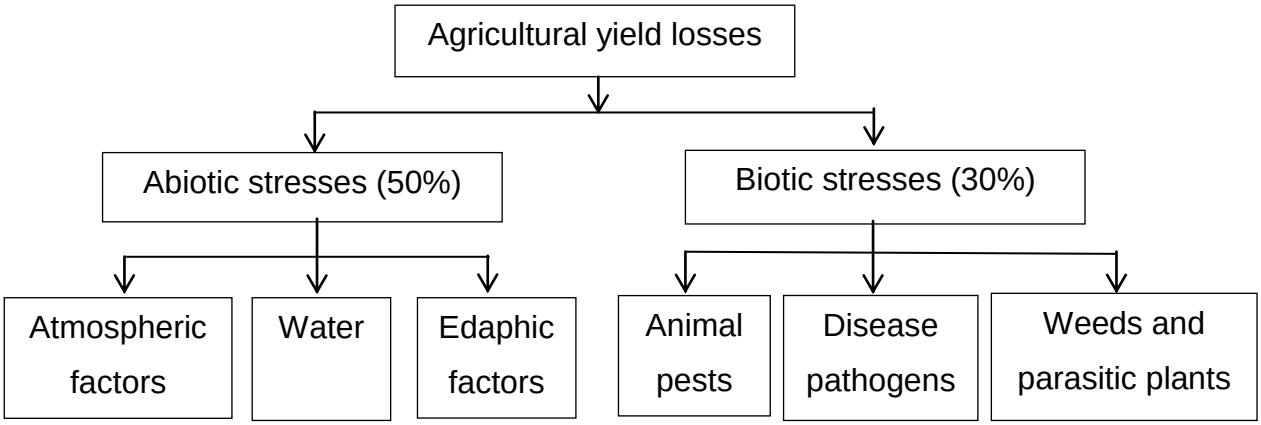


Figure 2-3: Agricultural yield losses due to abiotic and biotic stress

Adapted from: Gaur and Sharma (2014, p. 109)

The impact made by these abiotic stresses have been found to depend on the: (1) the part (tissue or organ) of the plants involved (Cramer et al., 2011; Kumar et al., 2014), (2) crop species, (3) duration, and (4) intensity of the stress (Wang and Frei, 2011) which could result in morphological, physiological, biochemical and molecular alterations within the affected plants (Wang et al., 2001, cited in Kumar et al., 2014, p. 126). Retardation of plant growth may also occur as the plants make effort to conserve and re-partition resources that can become limited under extreme stress conditions (Skirycz and Inzé, 2010).

Effect of abiotic environmental stresses on crop quality

As mentioned, environmental factors, such as: light (energy), moisture (water) and nutrients (Zhou et al., 2009; Kumar et al., 2014) have been known to affect the growth and development of plants as well as their yield and quality (Zhu et al., 2009). Specifically, Demmig-Adams and Adams (2002, cited in Zhu et al., 2009, p. 5495), stated that the nutritional value of agricultural produce such as vegetables, fruits and cereal grains may be increased by enhancing the antioxidant phytochemicals contents of these crops via alterations in their environmental growing conditions. Zhu et al. (2009) added that there is a likelihood that this increase in nutritional values (arising from the short-term exposure of these plants to stress) may not have any negative effect on crop yield as the response of antioxidant metabolism proceed more rapidly than that involving biomass production. In contrast, Wang and Frei, (2011) pointed out that environmental stresses usually have a negative impact on crop yield which have received more attention than modifications in crop quality as the latter (i.e. crop quality) may be difficult to detect as well as present both positive and negative effects.

In addition, Wang and Frei (2011) indicated that all the quality traits of agricultural produce can be influenced by abiotic environmental stress factors which can be linked with the different physiological reactions/responses in the affected plants as a result of: changes in the gene expression and enzyme activity, oxidative stress arising from the accumulation of reactive oxygen species (ROS), accelerated senescence which shortens the crop maturity period and alters the nutrients distribution processes within the stressed plants, reduced water content (markedly under drought, heat and salinity stress) which lead to increase in nutrients content, changes in mineral uptake and translocation, as well as reduced biomass/yield which is the most obvious and identified effect of abiotic environmental stress on agricultural produce. Although some crops respond differently, in most cases, environmental stresses have been found to bring about: (1) decrease in starch concentration, lipids [particularly the polyunsaturated fatty acids (PUFA)], feed value and physical/sensory (P/S) traits, (2) increase in protein and antioxidant contents,

and (3) no clear trend in sugar and mineral contents (Wang and Frei, 2011). Figure 2.4 illustrates a conceptual model of physiological responses of crops under abiotic stress and resultant effect on crop quality.

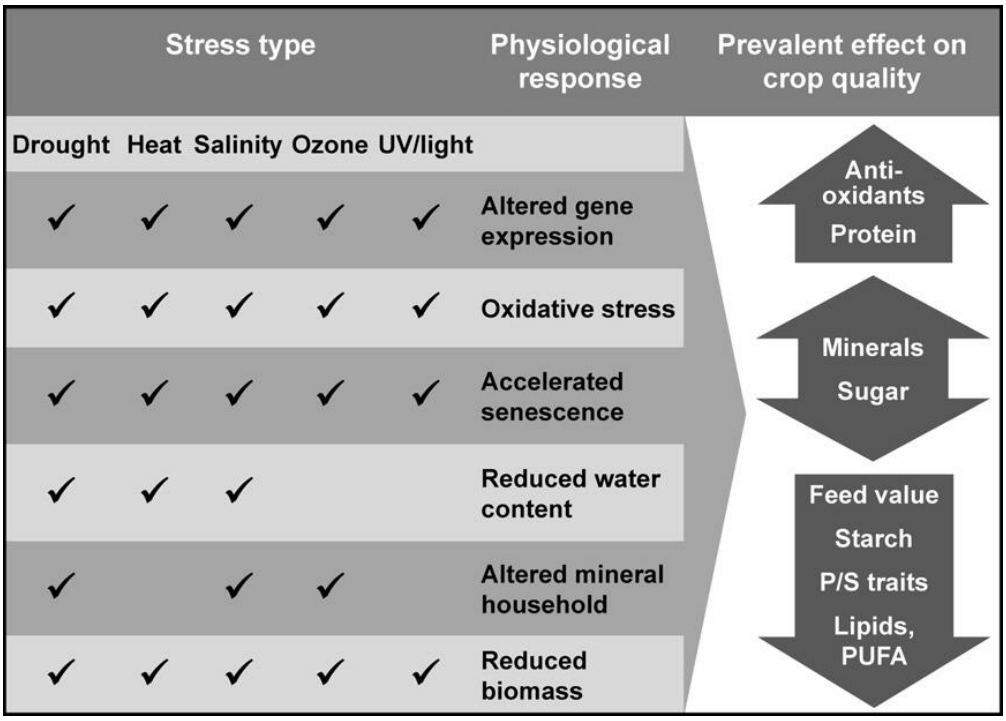


Figure 2-4: Conceptual model of physiological responses of crops stimulated by five types of abiotic environmental stress, and their prevalent effects on quality parameters. Mineral household: mineral uptake and translocation; P/S traits: Physical/sensory traits; PUFA: Polyunsaturated fatty acids.

Source: Wang and Frei (2011, p. 281)

On the other hand, one of the common mechanism by which plants adapt to abiotic stresses encountered in the environment involves the accumulation of compatible solutes which are highly soluble and low molecular weight compounds with no toxic effect at high concentrations (Chen and Murata, 2002). The different form of these compounds accumulated with respect to the type of plant species include: amino acids (like proline), glycine betaine, sugars (such as sucrose, trehalose and sorbitol) or sugar alcohols (like mannitol) (Chen and Murata, 2002; Bartels and Sunkar, 2005; Seki et al., 2007). These compatible solutes are known not to interfere with the normal cellular

metabolism (Sairam and Tyagi, 2004; Seki et al., 2007). Table 2.6 illustrates the osmolytes that accumulate in plants under drought and salinity stresses as provided by Sairam and Tyagi (2004).

Table 2-6: Important osmolytes that accumulate in plants under drought and salinity stresses

Carbohydrate	Nitrogenous compound	Organic acid
Sucrose	Proteins	Oxalate
Sorbitol	Betaine	Malate
Mannitol	Glutamate	
Glycerol	Aspartate	
Arabinitol	Glycine	
Pinitol	Choline	
Other polyols	Putrescine	

Source: Sairam and Tyagi (2004; p. 408)

2.5.2 Crude oil contamination as an abiotic stress factor

2.5.2.1 Effect of petroleum hydrocarbons on the growing environment and plants

The behaviour of petroleum hydrocarbons in the environment determine where they are likely to be found, such as in: air, water, soil, sediment, food or other media that people might come in contact with (UNEP, 2011). In the view of Oghenejeboh et al. (2008), the factors governing the spread/migration of the spilled oil in soil include: the amount of oil spilled, the physical properties of the spilled oil (density and viscosity), and the physical properties of the soil medium itself, particularly its porosity. These factors (i.e. the soil type, quantity and

composition of the spilled hydrocarbon) also determine the level of alteration of the soil physical and chemical properties (UNEP, 2011).

On agricultural lands, Plice (1948; also De Jong, 1980) reported that oil spills can result in reduced plant growth for some time. Petroleum hydrocarbons contamination which is associated with increase in carbon/nitrogen (C/N) ratio and nitrogen deficiency (Merkl et al., 2004), may also lead to a reduction in: plant's stem height, stem density, aboveground biomass, including death of the plant (Hershner and Lake, 1980; Ferrell et al., 1984; Alexander and Webb, 1987, Lin and Mendelssohn, 1996, 1998). Meanwhile, the response of plants to the presence of petroleum hydrocarbons in soil varies (Baker, 1970; Baek et al., 2004; Inckot et al., 2011) and is associated with the: age of plant (Pezeshki et al., 2000), species of the plant involved, type and concentration of the petroleum, time of exposure to the contaminant (Baker, 1970; Inckot et al., 2011) as well as season (Alexander and Webb, 1985; Lin and Mendelssohn, 1996), among others.

For instance, De Jong (1980) referring to the experiment of Carr (1919), indicated the possibility of low concentration of crude oil in the environment to support plant growth. Although this test was conducted in duplicate, Carr (1919) found out that at 0.75% of crude oil addition to the soil, the growth of soybeans was improved. Friend (1948, cited in Plice, 1948, p. 413) also recorded stimulation in the growth of parsley and some specific second-growth grasses when "stove oil" and kerosene were used in spray form as herbicides (rates of 300 to 600 gallons per acre). The deduction made was that the stimulating effects may have been as a result of the bacterial breakdown of the hydrocarbons (Friend, 1948, cited in Plice, 1948, p. 413). Other possible reasons for plant growth-stimulation as suggested by Baker (1970), although subject to further investigation, could be attributed to the release of nutrients from: the oil, oil-killed vegetation, or hormonal influence. Meanwhile, there are claims that this occurrence in plants is as a result of 'petroleum auxin' identified as naphthenic acids, which: (i) improve the yield of different variety of crops, (ii) stimulates photosynthesis, and (iii) increases protein nitrogen (Baker, 1970).

However, most studies regarding the growth of plants in soil containing petroleum hydrocarbons reported a reducing effect (Inckot et al., 2011) while the germination of seeds may also be negatively affected (Adam and Duncan, 2002; Inckot et al., 2011). This reduction in the plant growth could be as a result of: (1) the direct toxic effect of oil on the plants (Baker, 1970; De Jong, 1980); (2) absence of viable seeds leading to lack of germination (Rowell, 1977, cited in De Jong, 1980, p. 187), (3) reduced germination (Udo and Fayemi, 1975, cited in De Jong, 1980, p. 187), (4) unfavourable soil conditions (De Jong, 1980), and (5) inhibition of the bacterial decomposition of the soil organic matter and the associated nutrient remineralization by the toxic components of oil (Pezeshki et al., 2000). Meanwhile, the inhibition of germination may be linked to: oil entering the seed and killing the embryo, or oil coating the seed and hindering the uptake of oxygen and water required for germination (Baker, 1970). Merkl et al. (2004) added that, the reduced seedling emergence could be as a result of toxic effects of the oil or from the adverse soil moisture conditions. Other effects of oil pollution on plants as stated by Baker (1970) include: yellowing and death of oiled leaves, varying sensitivity and recovery rates of perennials etc while a complete elimination may occur at chronic levels. Heavier oils, compared to lighter oils, however present less immediate toxic impact on plants and other organisms (Pezeshki et al., 2000).

On the other hand, Baker (1970) indicated that photosynthesis rate is consistently reduced by oils while the level of reduction in this rate depends on the kind and amount of oil as well as the plant species. From the report of Minshall and Helson (1949, cited in Baker, 1970, p. 38), photosynthesis in parsnip recovered from treatment with petroleum naphtha while that of mustard stopped completely.

Considering the effect of time of spillage on plant response, Baker (1970) reported that the application of an emulsion of light oil on young plants in the light when the stomata are opened will lead to the death of the plant while the same application at night when the stomata are closed would not harm the plants. The same author also indicated that there is an increased level of phytotoxic risk at high environmental temperature (such as during sunny or hot

weather) compared to other times. However, environmental conditions may not have any effect on oils which penetrate easily (Baker, 1970).

2.5.2.2 Mechanistic link with induced abiotic stresses

Physically induced effect

The coating of plant leaves with oil may lead to temperature stress owing to the blockage of the transpiration pathways while the process of photosynthesis in the leaves is negatively affected (Pezeshki et al., 1995; Pezeshki et al., 2000). This blockage of the leaf stomata would reduce the diffusion of oxygen to the root thereby increasing the root oxygen stress (Mendelssohn and McKee, 1988, cited in Pezeshki et al., 2000, p. 131). The penetration of oxygen into the soil can also be restrained by the layer of oil covering the soil surface which results to anaerobic soil conditions for the plant roots (Pezeshki et al., 2000; Inckot et al., 2011) and contributes to oxygen stress on these roots (Ranwell, 1968, cited in Pezeshki et al., 2000, p. 131).

Nonetheless, the level of reduction in the transpiration and photosynthesis process arising from the physical blockage of the stomatal have been found to depend on the extent of oil covering on the plant which is associated with the hydrologic conditions, amount, type and dispersion ability of the spilled oil (Pezeshki et al., 2000).

Chemically induced effect

The chemical impact of oils on vegetation depends on the type of oil (Pezeshki et al., 2000) while the fouling of leaves by oil may have more immediate effect than fouling of the soil surface (Webb, 1994, cited in Pezeshki et al., 2000, p. 132). In the test plant considered by Pezeshki et al. (1995, cited in Pezeshki et al., 2000, p. 132), reduced stomatal conductance with no detectable photosynthetic activity was recorded in the plant shortly after leaf fouling which suggests the potential breakdown of photosynthetic apparatus in leaves directly subjected to oil application. In the view of Pezeshki et al. (2000), this breakdown of leaf structure and/or chlorophyll system may be associated with blocked stomata giving rise to reduced transpiration; consequently

increasing the leaf temperature with the possibility of an adverse effect of the oil on the cellular integrity of the leaf tissue. These authors (i.e. Pezeshki et al., 2000), added that plants may recover from the initial, short-term (often dramatic) adverse effects of oil on leaves while refined products, compared to crude oils, present a different effect on leaves.

Furthermore, as plants like other organisms produce reactive oxygen species (ROS) in response to abiotic and biotic stresses (Noori et al., 2015), environmental contamination arising from petroleum hydrocarbons reduce the availability of essential nutrients (like nitrogen and oxygen) required for plant growth (Ogbo et al., 2009; Adieze et al., 2012) including water (Noori et al., 2014) owing to the surface covering of the hydrophobic contaminant on plant roots, thereby enhancing the production of reactive oxygen species (ROS) and hence, oxidative stress in plants (Sun and Zhou, 2008; Noori et al., 2015).

Basically, petroleum hydrocarbons may affect plants by: (1) upsetting the plant-water relationships, (2) direct effect on plant metabolism such as nutrient uptake (McCown and Deneke, 1972, cited in Lin and Mendelssohn, 1996, p. 206), (3) their toxicity to living cells (Prendeville and Warren, 1977; Lin and Mendelssohn, 1996), and (4) the reduction in oxygen exchange between the atmosphere and the soil which have negative effect on plants (Stebbings, 1970; Lin and Mendelssohn, 1996).

Mechanistic link

Crude oil/petroleum-hydrocarbon contamination, an abiotic factor (Nie et al., 2010), leads to oxidative stress in plants (Noori et al., 2012) and in most cases, the resultant effect of crude oil-induced abiotic stresses on plants also gives rise to oxidative stress as a result of the increase in the production of reactive oxygen species. Figure 2.5 shows the mechanistic link between crude oil contamination/stress and the identified crude oil-induced abiotic stresses.

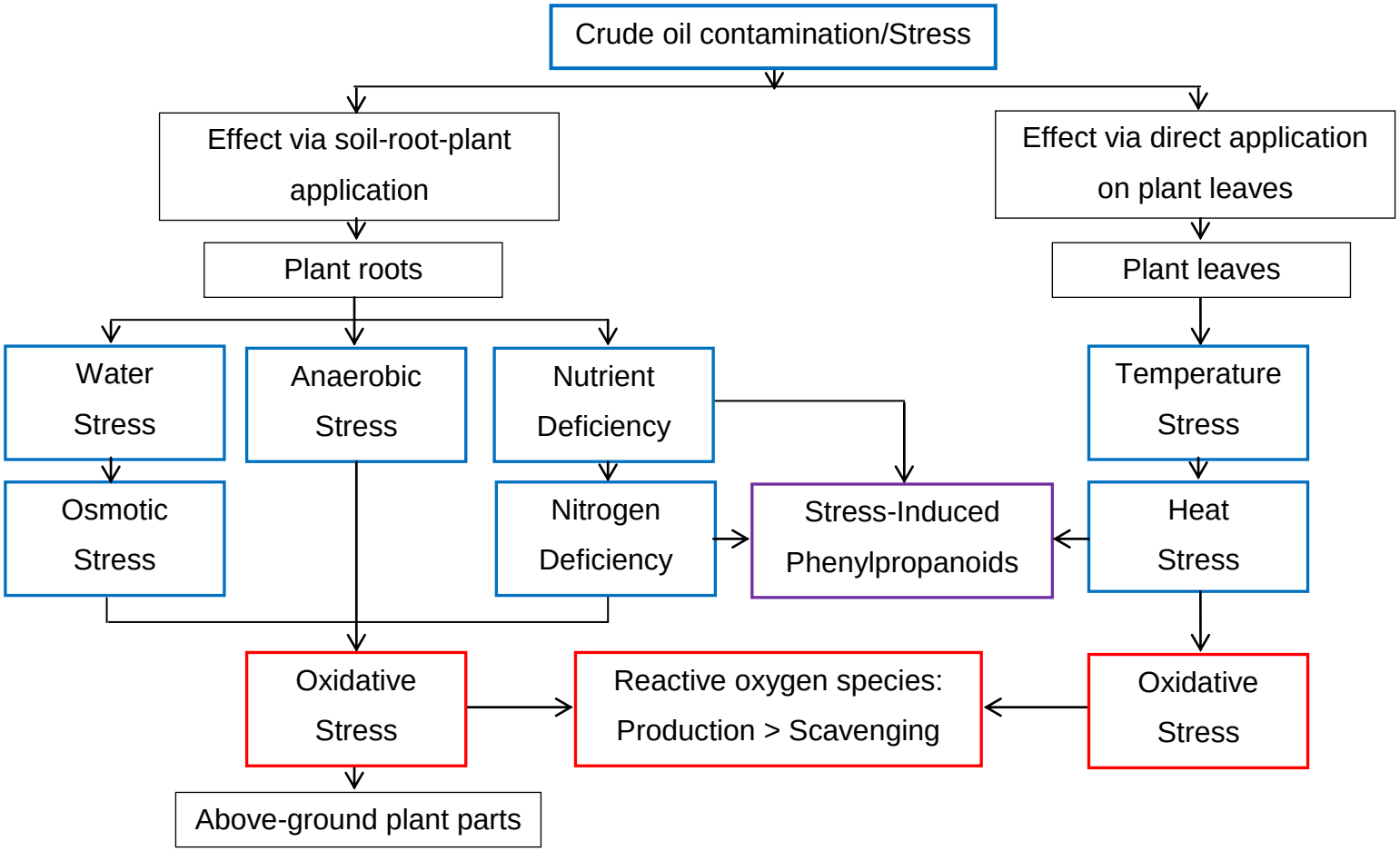


Figure 2.5: Illustration of the mechanistic link between crude oil contamination/stress and the crude oil-induced abiotic stresses as it affects agrifood production.

2.5.2.3 Mode of action and plant response mechanisms to the crude oil-induced abiotic stresses

2.5.2.3.1 Water (drought) and osmotic stresses

"Drought is a meteorological term for a scarcity of water" (Wilkinson and Davies, 2010, p. 510) while water stress on plants occurs when: there is a limitation of water supply to the roots, or when the transpiration rate becomes heightened (Lisar et al., 2012). In other words, water stress arises when the loss of water in transpiration exceeds the absorption rate (Kumar et al., 2014). Photosynthesis and growth are among the key processes affected by drought (Chaves, 1991; 2009) whose effects can be: (1) direct, such as resulting from the alteration in the diffusion of CO_2 in leaves via a decrease in stomatal and mesophyll conductances (Flexas et al., 2004; Chaves et al., 2009), or (2) give rise to secondary effects - oxidative stress (Chaves et al., 2009). Wahid and Rasul (2005, cited in Farooq et al., 2009, p. 188) added that drought is also associated with accelerated leaf senescence and reduction in food production.

Plants response to water stress

Hanson and Hitz (1982, cited in Akinci and Losel, 2012, p. 21) pointed out that plants adaptation to dry environment can be expressed at four different levels which are the: phenological or developmental, morphological, physiological, and metabolic levels while the latter (metabolic or biochemical adaptation) is the least understood. Whereas some responses of plants to water stress occur at the leaf level (Chaves et al., 2002), it is the general response at the whole-plant level, involving carbon assimilation and the distribution of photoassimilates to different plant parts and reproductive ability that eventually determine the survival of these plants and persistence under environmental stress (Pereira and Chaves, 1993, cited in Chaves et al., 2002, p. 907). Nonetheless, carbon assimilation at this whole-plant level always decreases as a result of: (1) reduction in CO_2 diffusion in the leaf, (2) diversion of carbon distribution to non-photosynthetic organs and defence molecules, or (3) biochemical changes in the leaf leading to the down-regulation of photosynthesis (Chaves et al., 2002).

1. Morphological and physiological responses

Ahuja et al. (2010) indicated: stomatal closure, reduction in photosynthetic activity, change in cell wall elasticity, and generation of toxic metabolites leading to plant death, as plants physiological responses to drought.

(a) Crop growth and yield: The experimental results of Timpa et al. (1986) which were reported by Akinci and Losel (2012), revealed that water stress had a reducing effect on the test plants' growth parameters such as: the height, leaf number, leaf area index, fresh and dry weights. As plant growth is achieved via cell division, cell enlargement and differentiation (Farooq et al., 2009), water deficit according to these authors affects the various events (genetic, physiological, ecological and morphological) as well as their complex interactions, which determine plant growth. Figure 2.6 gives a description of possible mechanisms of growth reduction under drought stress as provided by Farooq et al. (2009).

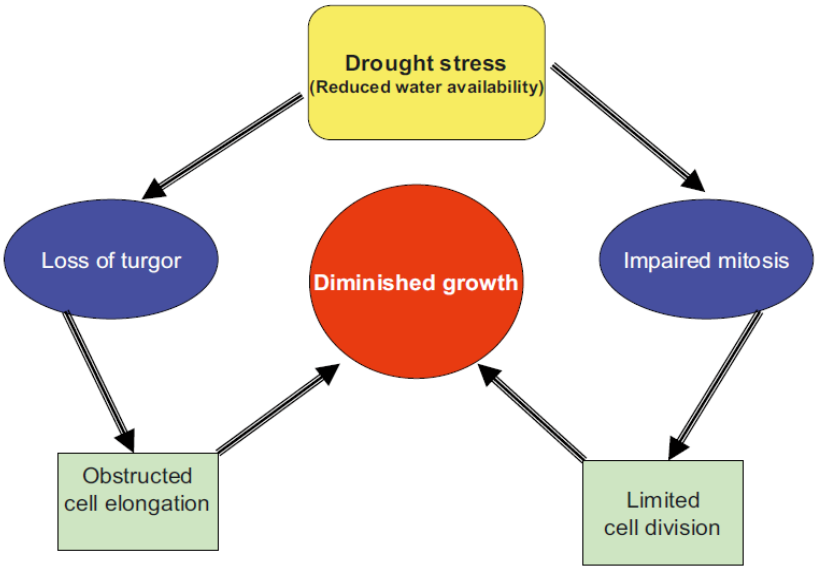


Figure 2.6: Description of possible mechanisms of growth reduction under drought stress.

Source: Farooq et al. (2009)

Specifically, shoot growth has been identified by Sharp (2002) to be very sensitive to conditions of limited water supply. Under this condition, there is a general assumption that shoot growth is more inhibited than root growth (Sharp

and Davies, 1989; Sharp, 2002) as a result of exposure to the dehydrating effects of the atmosphere (Sharp and Davies, 1989).

(b) Water relations: According to Lisar (2012), plant water relations, stomatal closure, gaseous exchange, transpiration and carbon assimilation (photosynthesis) are affected by drought while Farooq et al. (2009) indicated that among the components of plant water relations affected by reduced water availability, stomatal opening and closing is more strongly influenced.

(c) Nutrient relations: Although many nutrient elements are actively taken up by plants, water stress reduces the ability of plant roots to absorb water and nutrients which could be as a result of the decrease in nutrient element demand (Alam, 1999; Akinci and Losel, 2012). The experimental results of Rahman et al. (1971) on the effect of moisture stress on metabolic products and ions accumulation of some plants, which was also reported by Akinci and Losel (2012), revealed that moisture stress led to an increase in total nitrogen as well as favoured the accumulation of ions (K, Na, Ca, Mg and Cl) but with a decrease in P and Fe. Garg (2003, cited in Farooq et al., 2009, p. 188) also indicated that water stress induces an increase in N and a reduction in P; but were of the view that there is no definite effect of water stress on K uptake by plants. Farooq et al. (2009) noted that the availability, uptake, translocation and metabolism of nutrients are reduced by drought stress.

(d) Osmotic adjustment: Plant growing tissues in response to the loss of water by soil, may show osmotic adjustment (Akinci and Losel, 2012) involving lowering of water deficiency and linked to the maintenance of stomatal conductance, photosynthesis, leaf water volume and growth (Turner and Jones, 1980, cited in Akinci and Losel, 2012, p. 20).

Although osmotic adjustment is most often temporary (as plants usually respond quickly to increase in the level of available water), solutes which accumulate with water stress and contribute to osmotic adjustment in non-halophytes, include: inorganic cations, organic acids, carbohydrates and free amino acids (Akinci and Losel, 2012).

(e) Photosynthesis: Continuous rise in water deficiency has been identified by Akinci and Losel (2012) to have a major negative effect on the photosynthesis

of crops and to possibly cause a cessation in the photosynthesis process. According to Cornic et al. (1989), this shortage of water reduces the leaf net CO_2 uptake as a result of stomatal closure as well as a decline in the activity of the photosynthetic machinery.

(f) Assimilate partitioning: Leport et al. (2006 cited in Farooq et al., 2006, p. 190) reported that drought stress often enhances allocation of dry matter to the roots which can support water uptake.

2. Metabolic and molecular responses

(a) Carbohydrate changes: From Chaves (1991) point of view, for moderate level of water stress, the response of plants may be more of regulatory rather than stress-induced damage while a secondary effect of dehydration on photosynthetic carbon metabolism involving change in partitioning towards sucrose may also occur in some plant species. The maintenance or elevated level of sugar in dehydrated leaves may result from the co-ordinated regulation between sucrose synthesis and translocation (Chaves, 1991). Kameli (1990, cited in Akinci and Losel, 2012, p. 21) also added that the accumulation of sugars and some other organic solutes is part of the main effects of water stress.

(b) Plant proteins: One of the biochemical processes affected by water stress in plants is protein synthesis (Hsiao, 1973, cited in Dhindsa and Cleland, 1975, p. 782) while Yancey et al. (1982) identified polyhydric alcohols, free amino acids and their derivatives, as well as combinations of urea and methylamines as the three kinds of osmolyte systems found in all water-stressed organisms except the halobacteria. Petrie and Wood (1938) pointed out that there are two ways in which water content may affect the amounts (on a dry-weight basis) of protein and amino acids, that is, the effect may be as a result of: (1) changes in the concentrations of the nitrogen compounds, or (2) alterations in the rate of one or more reactions in the system owing to the change in water content.

Although water stress is associated with reduction in protein synthesis (Ben-Zioni et al., 1967; Dhindsa and Cleland, 1975; Akinci and Losel, 2012), according to Akinci and Losel (2012) the level of some specific types of proteins and mRNA in water-stressed plants may increase. Dasgupta and Bewley (1984,

p. 1458) in their study considering the effect of water stress on greening leaves, concluded that “reduction of protein synthesis under stress conditions is not at the expense of any particular protein, or set of proteins; rather, all proteins may be affected more or less equally”. The synthesis of a specific stress protein may however be induced under water stress (Dasgupta and Bewley, 1984; Akinci and Losel, 2012).

(c) Plant lipids: Whereas the amount of lipids – one of the key components of the membrane – in plants may be affected by water stress (Akinci and Losel, 2012), there have been contradictory reports on the effect of water stress on plant lipids (Navari-Izzo and Rascio, 1999, cited in Akinci and Losel, 2012; p. 25). Pham Thi et al. (1985) as well as Akinci and Losel (2012) indicated that water deficit hinders fatty acid desaturation which gives rise to a sharp decrease in linoleic and linolenic acid biosynthesis. According to these authors (i.e. Pham Thi et al. 1985; Akinci and Losel, 2012), although this decrease in unsaturated fatty acid biosynthesis occurs in all lipid classes, it is most noticeable in the galactolipid fractions.

(d) Oxidative damage and antioxidants: Drought has been identified by Teotia and Singh (2014) as one of the abiotic stresses that can lead to the production of reactive oxygen species (ROS). These ROS at high concentrations bring about adverse modifications to cell components like the lipids, proteins and nucleic acids (Valko et al., 2006; Birben et al., 2012).

Based on the production of these free oxygen radicals as a usual secondary consequence of environmental stresses which are harmful to cell components, plants have developed several antioxidant systems (both enzymatic and non-enzymatic) for the purpose of scavenging them (Lisar et al., 2012). Some of these antioxidant enzymes include: catalases (CAT), superoxide dismutase (SOD), peroxidases (POD), ascorbate peroxidases (APX), glutathione reductase (GR) and monodehydroascorbate reductase (MDAR); the non-enzymatic plant antioxidants are either ascorbic acid (AA)-like scavengers or pigments (Lisar et al., 2012). Further details on ROS production, oxidative stress and the antioxidant systems are provided in Section 2.5.2.3.5.

Osmotic stress

In line with Zhu et al. (1997), osmotic stress may be used to refer to conditions in which there is a limitation on plants growth and development as a result of shortage of water availability. Although osmotic stress can result from drought, excessive salt in water, chilling and freezing (Zhu et al., 1997), Xiong and Zhu (2002) identified drought and high salinity as the chief causes of the stress to plants under natural conditions.

Plants respond to osmotic stress at the morphological, anatomical and cellular levels (Zhu et al., 1997) which results to: alterations in their development (such as the plant life cycle, limitation of shoot growth and enhancement of root growth), regulation in ion transport as well as metabolic changes which may involve carbon metabolism and the production of compatible solutes (Xiong and Zhu, 2002; Kumar et al., 2014). Some of these responses according to Xiong and Zhu (2002) are triggered by the primary osmotic stress signals while others can be linked to secondary stresses/signals resulting from the primary signals. Examples of the secondary signals are: phytohormones [such as abscisic acid (ABA) and ethylene], reactive oxygen species (ROS) as well as intracellular second messengers (e.g. phospholipids) (Xiong and Zhu, 2002).

Generally, the response of plant to osmotic stresses can be classified into three different forms: (a) involving the maintenance of homeostasis, (b) detoxification of harmful elements, and (c) effort towards growth recovery (Xiong and Zhu, 2002).

2.5.2.3.2 Anaerobic stress

Higher plants, being aerobes, need molecular oxygen from their environment for survival (Vartapetian and Jackson, 1997). However, under certain environmental conditions, there is a possibility of shortage in the supply of O_2 to plant tissues (Hook and Crawford, 1978, cited in Ellis et al. 1999, p. 57). When this happens, that is, when there is restricted aeration of part or all of the plant, “the resulting tissue hypoxia or anoxia inevitably suppresses oxygen-dependant pathways especially the energy-generating system, disturbs functional

relationships between organs such as roots and shoots, and suppresses both carbon assimilation and photosynthate utilization" (Vartapetian and Jackson, 1997, p. 3).

This shortage in the supply of oxygen has more direct effect on underground organs such as the roots and seeds (Armstrong, 1979, cited in Vartapetian and Jackson, 1997); the shoot systems are then indirectly affected as a result of the negative impact of the stress on the root functions upon which the shoots depend (Vartapetian and Jackson, 1997). The mitochondria, has also been identified by Vartapetian et al. (2003) to suffer from oxygen deficiency before other cell organelles are affected.

Natural conditions that could lead to root hypoxia or anoxia (lack of oxygen) include: winter ice encasement, seed imbibition, spring floods and excess rainfall (Blokhina et al., 2003). Most plants tissues can however withstand anoxia only for a short period of time before irreversible damage occurs (Hook and Crawford, 1978, cited in Kennedy et al. 1992, p. 1).

Plants response to anaerobic stress

Depletion of oxygen affects cell physiology; it alters gene expression, energy consumption, cellular metabolism, growth and development (Bailey-Serres and Chang, 2005). Based on findings by the authors, Blokhina et al. (2003) indicated that lack of oxygen majorly results to: a decrease in adenylate energy charge, cytoplasmic acidification, anaerobic fermentation, increase in cytosolic Ca^{2+} concentration, alterations in the redox state and a decline in the membrane barrier function. Low-oxygen (hypoxia) stress is also reported to induce significant changes in the transcriptome as well as a shift from aerobic respiration to anaerobic respiration (Bailey-Serres and Voesenek, 2008, cited in Sunkar, 2010, p. 807).

(a) Pasteur effect: Mustroph et al. (2010) identified Pasteur effect as a common eukaryotic response to oxygen deficiency at the cellular level. According to Winkler et al. (2003, p. 721), this definition "that the rate of fermentation rises when oxygen is excluded" for Pasteur effect by Krebs (1972, p. 2) has long been accepted in place of that given by Pasteur (1861, cited in

Winkler et al., 2003, p. 721) involving “the inhibition of glycolysis by respiration”. Bewley and Black (1978, cited in Kennedy et al. 1992, p. 3) added that most anaerobic-intolerant plants exhibit a pronounced Pasteur effect.

(b) ROS production and oxidative stress: Like many other stress conditions, hypoxia is associated with the excess generation of reactive oxygen species (ROS) (i.e. under oxidative stress) (Blokhina et al., 2003). In line with these authors (i.e. Blokhina et al., 2003), there are however two models which suggest: (1) a decrease in ROS under oxygen deprivation [low NADPH - nicotine adenine dinucleotide phosphate (Birben et al., 2012) - oxidase activity], or (2) an increase in ROS due to inhibition of the mitochondrial electron transport chain.

(c) Gene expression: As the synthesis of several proteins involved in glycolysis and fermentation processes is induced in plants under anaerobic conditions (Umeda and Uchimiya, 1994), Agarwal and Grover (2005) noted that plants response to low O_2-stress condition is via specific alterations in gene expression. Generally, anaerobiosis gives rise to the alteration of gene expression in plants which leads to the accumulation of anaerobic proteins (ANPs) (Sachs et al., 1996) many of which are metabolic pathway enzymes (Agarwal and Grover, 2005).

2.5.2.3.3 Nutrient deficiency

Plants need nutrient elements for their normal growth and development in which the deficiency in any required element will have a significant impact (Chen et al., 2012). Clarkson et al. (2000) also indicated that the level of availability of certain mineral nutrients can alter plants’ transpiration, stomatal conductance and root hydraulic conductivity while the deficiency of any of these three plant nutrients – nitrate, phosphate and sulphate – in the growth medium, would impact the stomatal conductance and root hydraulic conductivity in a similar way.

In line with Kandlbinder et al. (2004), there are two contrasting developmental and metabolic effects that can be induced by nutrient deficiency. At the first instance, which shows the adaptive response, the growth of plants

may decrease in an organised manner in which only the number as well as size of each part of the plant (involving the roots, leaf, shoot, regenerative organs) are reduced while the metabolic activity and 'fitness' are to a large extent unaffected (Kandlbinder et al., 2004). In the second, the unbalanced response gives rise to a disturbed environment and dysfunction of the whole plant - the plant becomes stressed (Kandlbinder et al., 2004).

Plants response to nutrient deficiency

(a) Biosynthesis of stress-induced phenylpropanoids: As stated in Solecka (1997) as well as Dixon and Paiva (1995), environmental (biotic and abiotic) stresses like pathogen attack, wounding, nutrient deficiencies and temperature, among others (see Figure 2.7) are capable of enhancing the levels of phenylpropanoids in plants.

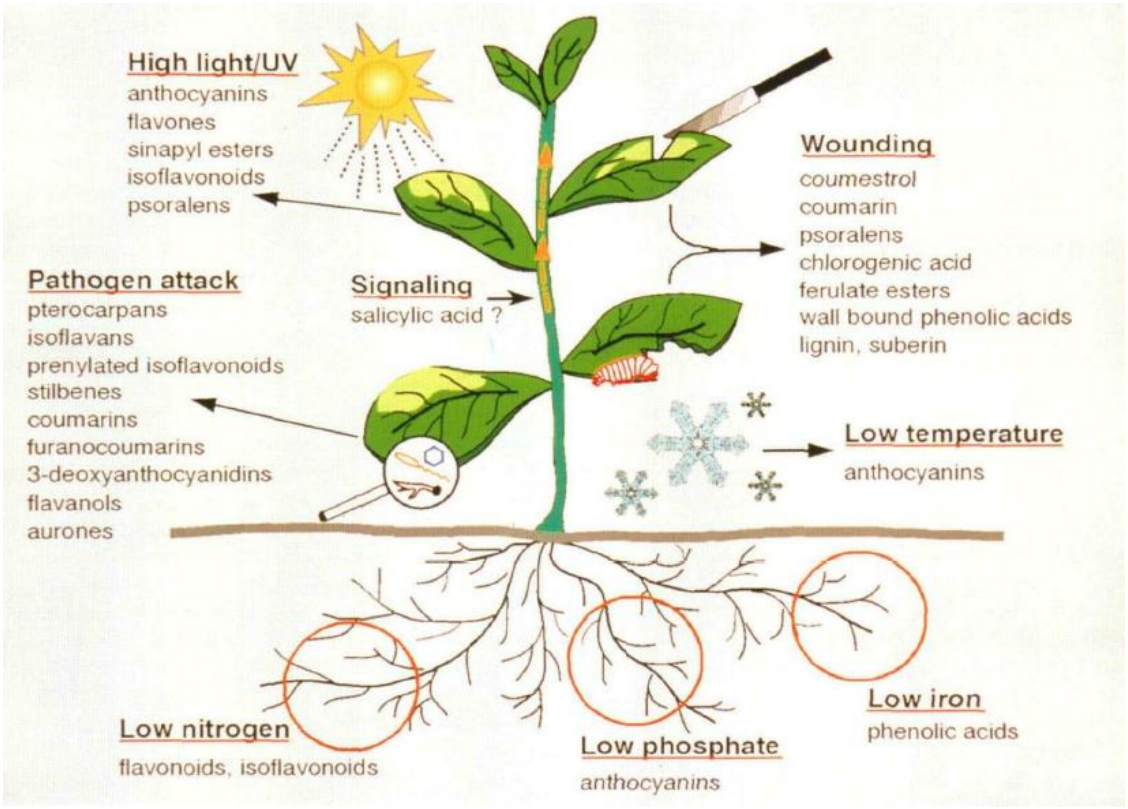


Figure 2.7: Examples of stress-induced phenylpropanoids

Source: Dixon and Paiva (1995)

These phenylpropanoids are plant secondary metabolites (Solecka, 1997) derived from *trans*-cinnamic acid, produced from the deamination of L-phenylalanine via the action of the enzyme – phenylalanine ammonia-lyase (PAL) (Dixon and Paiva, 1995; Solecka, 1997). The deamination of L-phenylalanine catalysed by PAL (Figure 2.8) as well as the biosynthesis of some plant phenylpropanoid compounds (Figure 2.9) as provided by Jones (1984) and Solecka (1997) respectively, are illustrated.

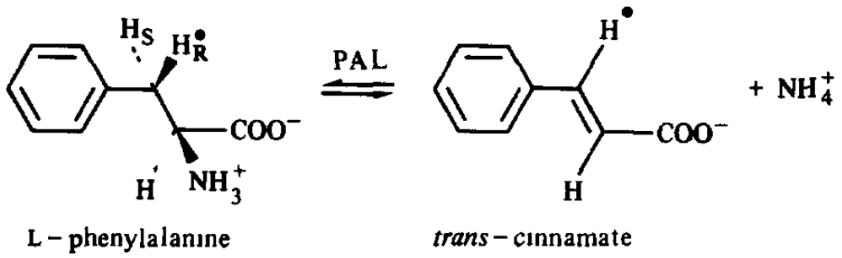


Figure 2.8: The deamination of L-phenylalanine catalysed by PAL.

PAL = Phenylalanine ammonia-lyase.

Source: Jones (1984)

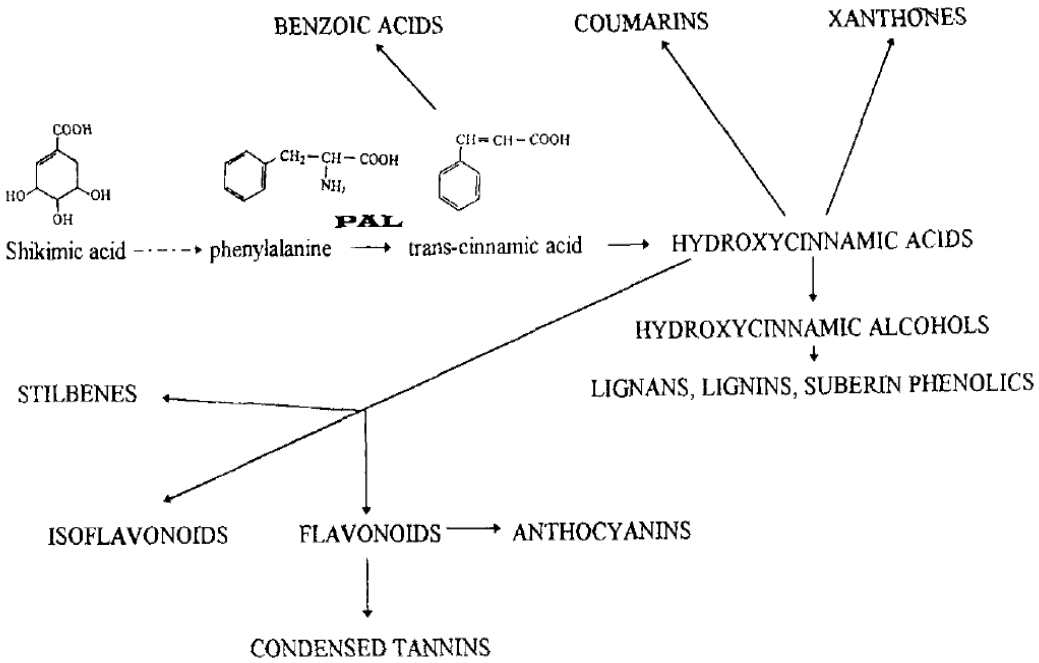


Figure 2.9: Biosynthesis of some plant phenylpropanoid compounds.

PAL = Phenylalanine ammonia-lyase.

Source: Solecka (1997)

In line with Lillo et al. (2008) the shikimate pathway is found in plants and provides phenylalanine for the synthesis of protein and secondary metabolites (like lignin and flavonoids). This shikimate pathway, with distinct patterns of organ as well as tissue activity, however depends on developmental regulation and environmental stimuli (Weaver and Herrmann, 1997). The enzymes of this pathway also respond to nitrogen and amino acid starvation (Weaver and Herrmann, 1997).

On the other hand, Gershenzon (1984) indicated that nitrogen, phosphorus, potassium and sulphur deficiencies, most times lead to higher concentrations of phenolic compounds.

(b) Oxidative stress: Following the acknowledgment of the relationship between macronutrient deficiency and oxidative stress by Tewari et al. (2007), Kandlbinder et al. (2014) in their study found out that N-, P- and S-nutrient deprivation triggered redox changes and induced oxidative stress. Further details on oxidative stress are provided in Section 2.5.2.3.5.

Nitrogen deficiency/stress

Aside from oxygen, carbon, and hydrogen, among other mineral nutrients, plants need greater amounts of nitrogen (N) (Yoneyama et al., 2012) whose deficiency Kovacik et al. (2007) regarded as an abiotic stress factor based on the experimental results of Shin et al. (2005) in which H_2O_2 production occurred in nitrogen-deprived roots. Kovacik et al. (2006) also considers its absence as a form of abiotic stress.

Nitrogen deficiency is associated with increased phenolics concentration (Gershenzon, 1984; Chishaki and Horigushi, 1997; Kovacik et al., 2007) including flavonoids (Bongue-Bartelsman and Phillips, 1995; Stewart et al., 2001; Kovacik et al., 2007) and coumarins (Kovacik et al., 2006; 2007). It is however linked to reduced mass-based protein content (Meyer et al., 2006). In the view of Bongue-Bartelsman and Phillips (1995), the increased deamination of phenylalanine could be responsible for elevated flavonoids content under nitrogen limitation.

In addition, Kovacik et al. (2006) indicated that nitrogen deficiency will affect amino acids and carbon metabolism while Kovacik et al. (2007) as well as Shin et al. (2005) added that nitrogen limitation can encourage the generation of reactive oxygen species (ROS). When the level of these ROS produced exceeds that of removal by the defense mechanisms, oxidative stress occurs in the cell (Sharma et al., 2012).

The indicators of senescence, which is an important outcome of N or P deficiency (Hewitt, 1963, cited in Tewari et al., 2007, p. 314), is reported to be similar to those of oxidative stress which include: net loss of chloroplastic pigments and proteins (Trippi et al., 1989; Casano et al., 1990, 1994; Tewari et al., 2007), lipid peroxidation and membrane alterations - giving rise to a progressive decline in photosynthetic capacity (Casano et al., 1994; Tewari et al., 2007).

2.5.2.3.4 Temperature (heat) stress

Although living organisms recognise temperature beyond the normal optimal as heat stress (Kotak et al., 2007), Wahid et al. (2007) referred the transient increase in temperature, usually 10 - 15^{o}C above ambient, as heat shock or heat stress. These latter authors (i.e. Wahid et al., 2007) added that heat stress occurs when there is an increase in temperature above a threshold level for a period of time which can cause irreversible damage to plant growth as well as development. In other words, heat stress depends on the intensity (in terms of temperature in degrees), time and rate of increase in temperature (Wahid et al., 2007). For instance, very high temperatures may lead to severe cellular injury and cell death within minutes as a result of the damaging effect on cellular organization (Schöffl et al., 1999, cited in Wahid et al., 2007, p. 200). However, at moderately high temperature, injuries or death may still occur but after a long-term exposure (Wahid et al., 2007).

The direct adverse effects of high temperature include: protein denaturation and aggregation, as well as increased fluidity of membrane lipids (Wahid et al., 2007). Meanwhile, enzymes inactivation in chloroplast and mitochondria, inhibition of protein synthesis, protein degradation and loss of membrane

integrity are associated with indirect or slower heat injuries (Howarth, 2005, cited in Wahid et al. 2007, p. 200). The resultant effects of these injuries according to Schöffl et al. (1999, cited in Wahid et al., 2007, p. 200) are: starvation, growth inhibition, reduced ion flux, production of toxic compounds and reactive oxygen species (ROS).

Plants response to temperature (heat) stress

(a) Morpho-anatomical and phenological responses

(i) Morphological symptoms: As temperature stress affects plant growth and metabolism (Hasanuzzaman et al., 2013b), Wahid et al. (2007) as well as Guilioni et al. (1997) reported that high temperature leads to loss in yield. Boyer (1982, cited in Suzuki and Mittler, 2006, p. 46) identified temperature stress (like heat, cold or freezing) as the major cause of the reduction in crop yield while Hasanuzzaman et al. (2013a) added that high temperatures affect the performance and crop quality characteristics.

(ii) Anatomical changes: In the view of Wahid et al. (2007), anatomical changes observed under high ambient temperatures are similar to those experienced under drought conditions and indicated that high temperature impacts anatomical structures at the tissue, cellular and sub-cellular levels in which the associated alterations may give rise to poor plant growth and productivity. At the latter (i.e. sub-cellular) level, major changes occur in chloroplasts, resulting to significant alterations in photosynthesis (Wahid et al. 2007).

(iii) Phenological changes: According to Wahid et al. (2007), heat stress to some extent, affects all plants' vegetative and reproductive stages. However, Slafer and Rawson (1995, cited in Wollenweber et al., 2003, p. 143), indicated that the extent of possible damage on plants exposed to increased temperature depends on the developmental stage of the plant.

(b) Physiological responses

(i) Water relations: Following the experiment of Machado and Paulsen (2001), Wahid et al. (2007) reported that changes in temperature does not affect plants tissue water status when there is sufficient moisture but in the

absence of the latter (i.e. when water is limiting), the plants tissue water status is affected at high temperature. Simões-Araújo et al. (2003) also associated high temperature stress, under field conditions, to reduced water availability which negatively affects plant productivity.

(ii) Accumulation of compatible osmolytes: Several authors (e.g. Bohnert et al., 1995; Hare et al., 1998; Sakamoto and Murata, 2002; Wahid et al. 2007) noted that the accumulation of compatible osmolytes is a basic adaptive mechanism in many plants under abiotic stresses, including extreme temperatures.

(iii) Photosynthesis: Photosynthesis is regarded as the physiological process most susceptible to high temperatures (Wahid et al., 2007) and in line with these authors, high temperature stress has more significant effect on the photosynthetic capacity of C_3 plants than in C_4 plants. Wahid et al. (2007) added that whereas an increase in temperature favours rubisco catalytic activity, a low affinity of the enzyme for CO_2 and its dual nature as an oxygenase hinders the possible increase in net photosynthesis (P_n).

Typically and in line with Stone (2001, cited in Wahid et al., 2007, p. 205), heat stress brings about a reduction in: duration of developmental phases leading to smaller organs, light perception and carbon assimilation processes including transpiration, photosynthesis and respiration. Meanwhile, Suzuki and Mittler (2006) reported that as a result of the physical properties of membranes, membrane-associated processes like photosynthesis and respiration are more sensitive to temperature stress. Berry and Björkman (1980, cited in Salvucci and Crafts-Brandner, 2004, p. 180) also indicated that the inhibition of photosynthesis caused by moderate heat stress (i.e. temperature slightly higher than the optimum level) is reversible while under severe heat stress, damage to the photosynthetic apparatus is permanent.

(iv) Assimilate partitioning: Wahid et al. (2007) pointed out that under low to moderate heat stress, there may be a reduction in source and sink activities giving rise to severe reductions in plant growth, economic yield and harvest index. Under high temperatures, the transport and transfer processes in plants

are affected by assimilate partitioning taking place via apoplastic and symplastic pathways (Taiz and Zeiger, 2006, cited in Wahid et al., 2007, p. 205).

(v) Cell membrane thermostability: In line with Wahid et al. (2007), heat stress increases the kinetic energy and movement of molecules across membranes resulting to loosening of the chemical bonds within molecules of biological membranes. This renders the lipid bilayer of biological membranes more fluid by either protein denaturation or an increase in unsaturated fatty acids (Savchenko et al., 2002, cited in Wahid et al., 2007, p. 205).

In addition, owing to the sensitivity of the integrity and functions of biological membranes to high temperature, heat stress affects the tertiary and quaternary structures of membrane proteins (Wahid et al., 2007). These authors, however, pointed out that the relationship between cell membrane thermostability (CMT) and crop yield under high temperatures depends on the plant species.

(vi) Hormonal changes: In plants, heat stress affects hormonal homeostasis, stability, content, biosynthesis and compartmentalization (Maestri et al., 2002, cited in Wahid et al., 2007, p. 205) while stress hormones [such as abscisic acid (ABA) and ethylene (C_2H_4)] act as signal molecules in the regulation of many physiological properties (Wahid et al., 2007). These authors added that high temperature gives rise to increased levels of ABA which brings about modification of gene expression in response to stress.

(vii) Secondary metabolites: Similar to the reports on plants under nutrient and nitrogen deficiencies, Wahid et al. (2007) indicated that the production of phenolic compounds such as flavonoids and phenylpropanoids is induced by high-temperature stress in which the principal enzyme of the phenylpropanoid pathway is identified to be phenylalanine ammonia-lyase (PAL). These authors also noted that the key acclimatory response of cells to heat stress is associated with the increased activity of PAL. Further details on stress-induced phenylpropanoids are provided in Section 2.5.2.3.3 on nutrient deficiency.

(c) Molecular responses

(i) Oxidative stress and antioxidants: Hasanuzzaman et al. (2012; 2013a) identified the increased production of ROS as one of the key consequences of high temperature stress. Although Wahid et al., (2007) indicated that heat stress

may lead to tissue dehydration as well as induce oxidative stress, in the view of Apel and Hirt, (2004, cited in Zinn et al., 2010, p. 2), both hot and cold temperature stresses induce the production of ROS which at high concentrations will give rise to oxidative damage and, possibly, cell death. Other details on ROS production, oxidative stress and the antioxidant systems are given in Section 2.5.2.3.5.

(ii) Stress proteins: Among the various responses of plants to stresses, Wahid et al. (2007) noted that the expression of stress proteins is an important way plants adapt to environmental stresses, including heat stress.

The major effects of high temperature on plants is summarised in Figure 2.10 as provided by Hasanuzzaman et al. (2013).

Figure 2.10: Major effects of high temperature on plants

Source: Hasanuzzaman et al. (2013)

2.5.2.3.5 Oxidative stress

Oxidative stress is defined as a "state in which oxidation exceeds the antioxidant systems in the body secondary to a loss of balance between them" (Yoshikawa and Naito, 2002, p. 271) and according to Lushchak (2011, p. 176),

"its development is either the reason, or common event of many pathological states, including aging". In line with Lushchak (2011), oxidative stress occurs in situations where the equilibrium between reactive oxygen species (ROS) generation and elimination is upset leading to their enhanced steady-state level. Birben et al. (2012) referred it to a shift in balance between oxidant/antioxidant which favours the oxidants as shown in Figure 2.11. This perturbation of the equilibrium between generation and scavenging of ROS may be caused by various biotic and abiotic stress factors such as: drought, nutrient deficiency, temperature extremes, salinity, herbicides and pathogen attacks, among others (Gill and Tuteja, 2010) which are known to reduce global crop production (Mahajan and Tuteja, 2005).

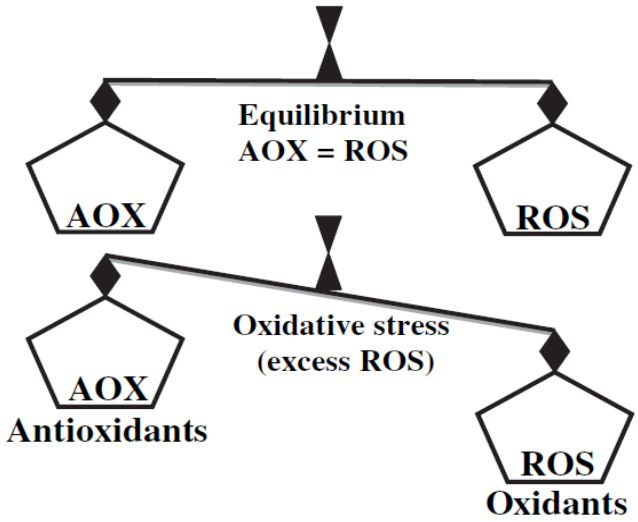


Figure 2.11: Equilibrium between AOX and ROS.
AOX = Antioxidants, ROS = Reactive oxygen species.
Source: Gill and Tuteja (2010)

The ROS consists of a group of free radicals, reactive molecules, as well as ions that are derived from O_2, which in plants, depending on their concentrations, can either be harmful or beneficial (Sharma et al., 2012). At elevated concentrations, ROS bring about damages to biomolecules while at low/moderate concentration, they function as second messengers in intracellular signaling cascades that mediate several responses in plant cells

(Sharma et al., 2012) such as: stomatal closure (Neill et al. 2002; Yan et al., 2007; Sharma et al., 2012;), programmed cell death (Jabs, 1999; Bethke and Jones, 2001; Sharma et al., 2012), gravitropism (Joo et al., 2001; Sharma et al., 2012), and acquisition of tolerance to both biotic and abiotic stresses (Miller et al., 2008, cited in Sharma et al., 2012; p. 5). The role of ROS as second messengers in hormone mediated cellular responses in plants is illustrated in Figure 2.12 while the most common ROS are: singlet oxygen ($^1O_2$), superoxide anion ($O_2^{\cdot -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$) (Sharma et al., 2012).

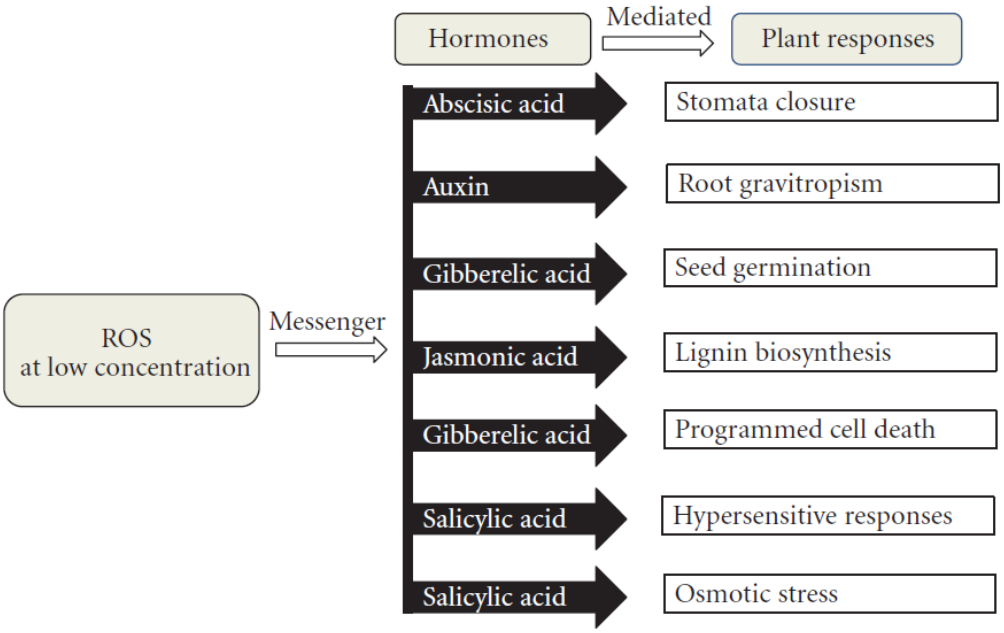


Figure 2.12: Reactive oxygen species (ROS) as second messengers in several plant hormone responses, including stomatal closure, root gravitropism, seed germination, lignin biosynthesis, programmed cell death, hypersensitive responses, and osmotic stress.

Source: Sharma et al. (2012)

As reported by Sharma et al. (2012), in plants, ROS are generated in both unstressed and stressed cells at different locations in chloroplasts, mitochondria, plasma membranes, peroxisomes, apoplast, endoplasmic reticulum, and cell walls. These authors added that they (i.e. the ROS) are

usually produced as a result of the leakage of electrons onto O_2 from the electron transport activities of chloroplasts, mitochondria, and plasma membranes or as byproducts of various metabolic pathways in different cellular compartments.

Generally, under ideal growth conditions, the generation of ROS in organelles is low (Suzuki and Mittler, 2006) but are formed excessively during adverse/stressful conditions (Suzuki and Mittler, 2006; Sun et al., 2007), which can bring about damage to biomolecules like lipids, proteins and deoxyribonucleic acid (DNA) (Sharma et al., 2012) (see Figure 2.13). These reactions according to Sharma et al. (2012) can affect intrinsic membrane properties such as fluidity, ion transport, loss of enzyme activity, protein cross-linking, inhibition of protein synthesis, DNA damage, among others which eventually lead to cell death. Gill and Tuteja (2010) pointed out that in addition to the ability of ROS to damage cells, they can also initiate responses such as new gene expression.

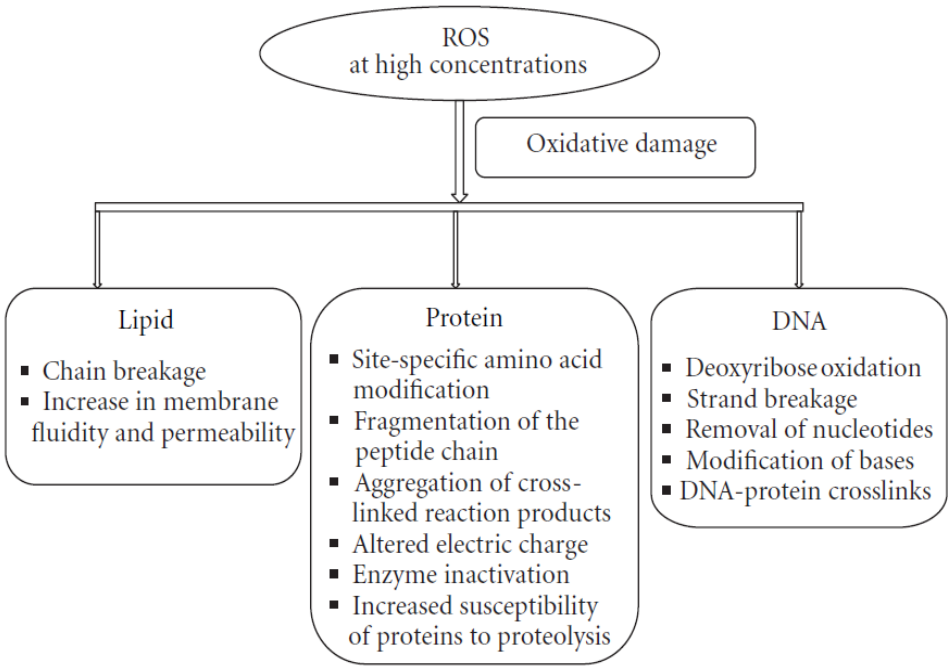


Figure 2.13: Reactive oxygen species (ROS) induced oxidative damage to lipids, proteins, and DNA. DNA = deoxyribonucleic acid.

Source: Sharma et al. (2012)

Plants response to ROS and oxidative stress (antioxidative systems)

According to Gratão et al. (2005, cited in Gill and Tuteja, 2010, p. 910)**,** the balance between the generation of ROS and scavenging at the proper site as well as time, determines whether ROS will act as damaging, protective or signaling factors. Hence, as a result of the multifunctional roles of these ROS, cells must control their levels to prevent any oxidative injury and not to eliminate them completely (Sharma et al., 2012). The scavenging or detoxification of excess ROS is carried out by the antioxidative system comprising the non-enzymatic antioxidants and enzymatic antioxidants (Noctor and Foyer, 1998, cited in Sharma et al., 2012, p. 1). The former class of non-enzymatic antioxidants within the cell comprises: ascorbate (AsA), glutathione (GSH), carotenoids, tocopherols, and phenolics (Sharma et al., 2012) while the latter class (enzymatic antioxidants) include: superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX) and enzymes of ascorbate-glutathione (AsA-GSH) cycle (Noctor and Foyer, 1998, cited in Sharma et al., 2012; p. 2).

Basically, the ability of plants to maintain a high antioxidant capacity to scavenge the toxic ROS (i.e. reduce oxidative stress) implies increased plants' tolerance level to the environmental stresses (Zaefyzadeh et al., 2009; Sharma et al., 2012).

2.5.3 Effect of abiotic/crude oil-induced abiotic stresses on crop quality

2.5.3.1 Nutritional composition of foods and vegetables

2.5.3.1.1 Carbohydrates

Carbohydrates, the most abundant organic compounds available for man as a source of energy (Belitz et al., 2004) and the main solid matter of plants (Fennema et al., 1996), are made up of the elements carbon, hydrogen and oxygen (Birch et al., 1986; Gaman and Sherrington, 1996). Carbohydrates can be classified into three different categories: monosaccharides, disaccharides and polysaccharides (Gaman and Sherrington, 1996) while the three common forms of the sugars (monosaccharides and disaccharides) in foods are: sucrose, glucose and fructose, although there are other sugars (such as lactose

and maltose) which are found in smaller amounts with less sweetness (Birch et al., 1986).

Impact of abiotic/crude oil-induced abiotic stresses

The assessment of the effects of environmental stresses on non-structural carbohydrates pertaining to crop quality are considered from two standpoints: (i) the concentration of starch in starchy staple crops, and (ii) the distributions of sugar in sugar crops, fruits or vegetables while the evaluation of the impact of the different stress factors on sugar contents in crops have attracted varying responses (Wang and Frei, 2011). Although there is no consistent trend in the response of sugars to environmental stresses (Wang and Frei, 2011), many abiotic stresses affect the various sugar processes involving synthesis, composition, metabolism, transport and storage (Cramer et al., 2011).

2.5.3.1.2 Amino acids and Proteins

Proteins, derived from the Greek word – "*proteois*" (meaning: "of the first kind") (Fennema et al., 1996), are different from both carbohydrates and fats as: (1) in addition to carbon, hydrogen and oxygen, they contain nitrogen, sulphur (Birch et al., 1986; Gaman and Sherrington, 1996) and sometimes phosphorus (Gaman and Sherrington, 1996), as well as (2) they have more varied and complicated structures (Gaman and Sherrington, 1996) made up of simple units of amino acids (Birch et al., 1986). Twenty of these amino acids are important in food and exist in three different forms: acid, neutral and alkaline amino acid (Birch et al., 1986). However, not all of these amino acids may be found in some proteins (Fennema et al., 1996).

As a result of the inability of animals to produce proteins from inorganic materials like plants, protein becomes an essential nutrient in animals' diet (Gaman and Sherrington, 1996) while a larger percentage of the proteins consumed by humans are from plants (Fennema et al., 1996). Nonetheless, they are found in low amounts in fruits and vegetables with varying concentration (Fennema et al., 1996).

Impact of abiotic/crude oil-induced abiotic stresses

According to Wang and Frei (2011), the protein contents of crops can be influenced by genetic and the environmental factors. In most cases, adverse growth or environmental stress conditions lead to increase in protein concentration in the harvested plants where research findings showing no effect or lower protein content are not common. This increase in protein content in the stressed plants has however been linked by most researchers with the usual negative effect of stresses on dry matter accumulation (Wang and Frei, 2011).

2.5.3.1.3 Lipids

As much as fats and oils, also referred to as lipids (Gaman and Sherrington, 1996), are similar in many aspects, at “normal” air temperatures, fat is solid while oil is liquid (Birch et al., 1986). In two different ways, fats exhibit similar characteristics with carbohydrates: (1) they contain the same three elements (i.e. carbon, hydrogen and oxygen) (Gaman and Sherrington, 1996) which are linked together, and (2) they help in the provision of energy (Birch et al., 1986) while also acting as a source of essential fatty acids and vitamins (Belitz et al., 2004).

Furthermore, they are found in very low concentrations (less than 1%) in the fresh weight of most fruits and vegetables (Fennema et al., 1996); constituting the chief structural parts of all living cells alongside carbohydrates and proteins. Table 2.7 gives an illustration of the proximate composition of some groups of edible plants.

Table 2-7: Ranges for proximate composition (fresh weight) of crops represented in major economic groups of edible plants

Group	Water, %	Carbohydrates, %	Protein, %	Lipids, %
Fruit	80 - 90	5 - 20	0.5 - 3	0.1 - 0.5
Fleshy vegetables	80 - 90	2 - 20	5 - 7	0.1 0.3
Pulses, seeds	10 - 50	6 - 60	5 - 25	1 - 18
Nuts	3 - 50	10 - 40	3 - 25	2 - 70
Cereals	12 - 14	65 - 75	7 - 12	2 - 6

Adapted from: Fennema et al. (1996, p. 947)

Impact of abiotic/crude oil-induced abiotic stresses

Most of the studies on the effect of environmental stresses on crops have reported a decrease in lipid content, especially for crops under drought, salinity and heat stress (Wang and Frei, 2011). In addition to the effect of these stress factors on the lipid concentration, modifications of the fatty acid composition of the harvested oil have also been identified. In most cases, the environmental stress leads to an increase in the saturation level of the oil fraction which in other words result to a decrease in the amount of (poly)unsaturated fatty acids and an increase in the fraction of saturated fatty acids (Wang and Frei, 2011). These modifications in the fatty acid proportion may be attributed to the: (1) delay in lipid biosynthesis (Mingeau, 1974, cited in Bouchereau et al., 1996, cited in Wang and Frei, 2011, p. 27), (2) alteration of the activities of the enzyme involved (Wang and Frei, 2011; Flagella et al., 2002), and (3) change in the transport of fatty acids from one organelle to the other (Steer and Seiler, 1990, cited in Flagella et al., 2002, p. 229).

2.5.3.1.4 Vitamins

Vitamins are complex organic compounds (Gaman and Sherrington, 1996) required in smaller amounts to maintain normal human health (Birch et al., 1986; Gaman and Sherrington, 1996). Nutrition-wise, they are not synthesized in the body which makes them vital in the human diet (Gaman and Sherrington,

1996) as essential micronutrients (Fennema et al., 1996). According to Birch et al. (1986), five of these vitamins (Vitamin A, B, C, D and E) are the most important in food science and technology with two of these vitamins (Vitamin B and C) and the other three (Vitamin A, D and E) being water-soluble and fat-soluble vitamins respectively. The vitamin content of plants however depends on: variety, growing conditions, postharvest storage conditions, and processing (Fennema et al., 1996).

Vegetables are considered a good source of vitamins (Taura and Habibu, 2009) while vitamin C, an essential nutrient, helps in the prevention of: scurvy (via the synthesis of collagen) and oxidative stress (as an antioxidant) (Liu, 2013). In relation to human nutrition, vitamin C is the most significant vitamin in fruits and vegetables while most of the vitamin C found in human diets are from these groups of food, including potatoes (Lee and Kader, 2000; Liu, 2013).

Aside from the antioxidant abilities of vitamin C, it also aids the absorption of iron from the intestine as well as healing of wounds (Gaman and Sherrington, 1996). Nonetheless, it is easily destroyed by oxygen (especially in the presence of sunlight) and like every other vitamin can be best preserved by freezing (Birch et al., 1986). Vegetables like: lettuce, spinach and carrots also help in the provision of provitamin A (Fennema et al., 1996).

Impact of abiotic/crude oil-induced abiotic stresses

As regards to the impact of stress on antioxidants such as vitamin C, Wang and Frei (2011) pointed out that fewer studies involving the impact of stress on vitamin C content of agricultural produce have been conducted when compared to that of phenolics with controversial results on the former as a result of the complexity of the ascorbate metabolism in plants.

2.5.3.1.5 Dietary Minerals

Like the other categories of nutrients required to support life, dietary minerals are also important (Gupta and Gupta, 2014). They are the other elements aside from carbon, hydrogen, oxygen and nitrogen that are found in foods (Birch et

al., 1986; Fennema et al., 1996) while the total mineral content of foods is a function of the ash value (Vicente et al., 2009).

These dietary minerals are present in relatively low amount in foods (Fennema et al., 1996). They have both direct (as a result of their consumption by human) and indirect effect which deals with their influence on fruit and vegetable quality, including eventual consumer acceptance (Vincente et al., 2009). Among the minerals, potassium is the most abundant in the majority of plants (Fennema et al., 1996), as well as in fruit and vegetables, although nitrogen and calcium have the most impact on horticultural crop quality (Vicente et al. 2009). The latter (Ca) is also the nutrient commonly linked with postharvest disorders (Ferguson et al., 1999; Vicente et al., 2009).

Classification of minerals

Generally, these dietary minerals can be divided into two categories: the major and minor minerals (Nieman et al., 1992) as shown in Table 2.8. The concentrations of the former in plant tissues range from 1,000 to 15,000 µg/g on a dry weight basis while the minor (micro) nutrients are found 100- to 10,000-fold lower than the major minerals (Vincente et al., 2009).

Table 2-8: The major and minor minerals required by the human body

Mineral Nutrient Class	Mineral description	Minerals in the category
Major minerals	These are minerals needed in amounts greater than 100 mg/day	Ca, P, Mg, Na, Chloride, K and S
Minor minerals	These are trace elements needed in amounts no more than a few mg/day	Fe, Zn, I, Fluoride, Cu, Se, Cr, Co, Mn and Mo

Adapted from: Nieman et al. (1992, p. 274)

According to Welch and Graham (2004), ≥ 49 nutrients are required by human for metabolic activities while more than 22 mineral elements, including the major minerals, have also been identified to be vital with iron (Fe), zinc (Zn), copper (Cu), iodine (I) and selenium (Se) needed in small amounts as they could become harmful when present in higher concentrations (Grusak and Cakmak, 2005, cited in Martinez-Ballesta, 2010, p. 296). However, the contribution of other minerals such as: tin (Sn), nickel (Ni), silicon (Si) and vanadium (V) in the human body are yet to be scientifically proven while lead (Pb), mercury (Hg), arsenic (As), aluminium (Al), silver (Ag), cadmium (Cd), barium (Ba) and strontium (Sr) are regarded as contaminants with harmful effect on human health when in excess (Nieman et al., 1992). In relation to health and food safety, most attention are given to: Cd, Hg and Pb as well as the anionic metalloids As and Se (Reilly, 1991, cited in McLaughlin et al., 1999, p. 144).

Generally, the amount of minerals found within the plant is determined by the ability of the absorbed metal to be translocated upward to the different part of the plants such as the leaves and fruits, seeds and other storage organs, while many of these metals are retained in the roots (Szefer and Nriagu, 2007). According to Belitz (2004), the mineral contents of food are governed by: (1) genetic type, (2) climatic condition, (3) agricultural practice, (4) constitution of the soil, and (5) the degree of maturity/ripeness of agricultural products etc. This view is also supported by Szefer and Nriagu (2007) and Martinez-Ballesta (2010) while some plant nutrients (like K and Na), may be involved in the response of plants to salt and water stress (Martinez-Ballesta, 2010).

Heavy metals in foods

According to Farr (2001, cited in Tandi et al., 2004, p. 462), over the years, there has been a continuous increase in the level of heavy metals exposure to man as a result of the rise in industrial activities. The toxicity of these heavy metals results when the rate of their accumulation exceeds that in which they are detoxified by the body (Tandi et al., 2004).

Among the available foods, vegetables are the most prone to environmental pollution as a result of aerial burden while heavy metals may be accumulated in their edible and non-edible parts (Jolly et al., 2013). Practically, the most intake of toxic metals in human beings come from food consumption while the soil to plant pathway is the major means by which human beings become exposed to metal contamination (Jolly et al., 2013). Szefer and Nriagu (2007) noted that toxic metals like Hg, Cd and Pb can be taken up by plants from the soil growing environment.

Vegetables as a source of minerals

Although both vegetables and fruits are referred to as “nutrient-dense foods” (as they are able to provide significant amount of micronutrients, like minerals and vitamins, but relatively few calories), the former has a higher concentration of minerals (Vicente et al., 2009). Meanwhile, increase in their consumption (i.e. fruits and vegetables) has been indicated to enhance mineral regulation and to reduce the development of cardiovascular diseases as well as some forms of cancer (Ismail et al., 2011).

Impact of abiotic/crude oil-induced abiotic stresses

Although the mineral and nutritive quality of crops for human consumption may be altered as a result of abiotic stresses such as high salt level, low water availability and extreme temperatures (Martinez-Mallesta et al., 2010), only few studies have been made on the effect of non-mineral stresses on crops’ mineral composition while there is no clear trend in those documented (Wang and Frei, 2011). Oktem (2008) on analysis of the sweet corn kernels, found that increase in water deficiency resulted to decreased uptake of Fe, Zn and Cu which was linked with water stress and indicated the importance of water in mineral mobilization (Oktem, 2008; Wang and Frei, 2011). As reported by Wang and Frei (2011), reduced biomass, which is a typical occurrence in plants under abiotic environmental stresses, often arise from reduced photosynthetic carbon assimilation and leads to increase in the concentration of other elements, higher crude protein and mineral contents in the stressed plant.

2.5.3.2 Phytochemical composition of foods and vegetables

In addition to the essential nutrients found in plants, they also contain some other bioactive compounds referred to as phytochemicals (Rodriguez et al., 2006). Phytochemicals are the natural non-nutritive bioactive compounds present in plants, including fruits and vegetables (Liu, 2003; Liu, 2004; Gonzalez-Vallinas et al., 2013; Liu, 2013; Acosta-Estrada et al., 2014) with human health benefits (Liu, 2003; Liu, 2004; Gonzalez-Vallinas et al., 2013; Liu, 2013). They are secondary plant metabolites found in relatively small amounts (Rodriguez et al., 2006). More than 5,000 of these individual bioactive compounds have been distinguished (Liu, 2013) with most investigations on the phenolics and carotenoids (Liu, 2004) while the phenolics are the most abundant in the plant kingdom (Saxena et al., 2013).

However, the distribution of these phytochemicals (Figure 2.14) in fruits and vegetables is governed by the biosynthetic pathway (regulated by several enzymes) during their growth and development (Tiwari and Cummins, 2013). Figure 2.15 highlights the factors influencing these phytochemicals' composition.

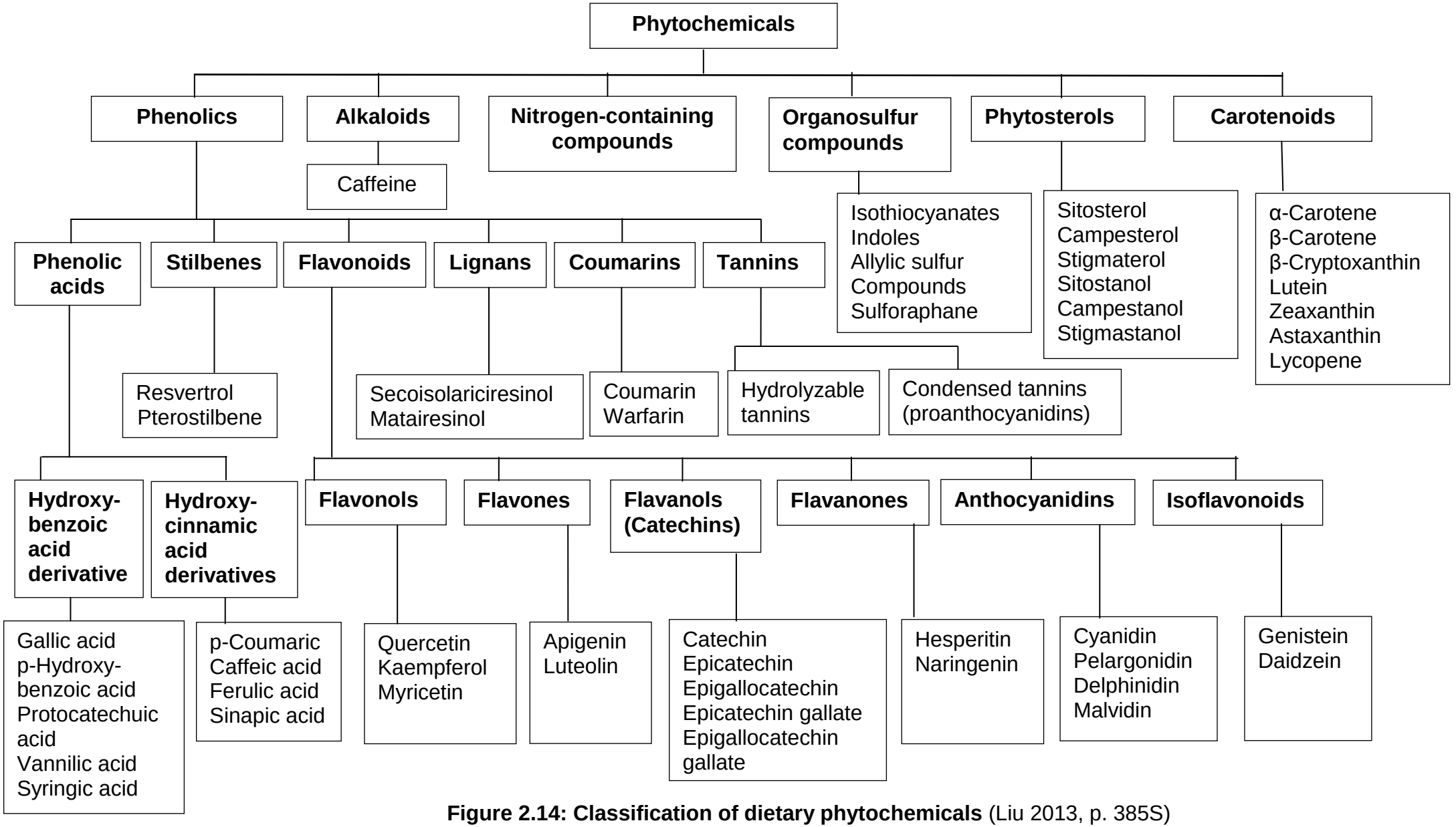


Figure 2.14: Classification of dietary phytochemicals (Liu 2013, p. 385S)

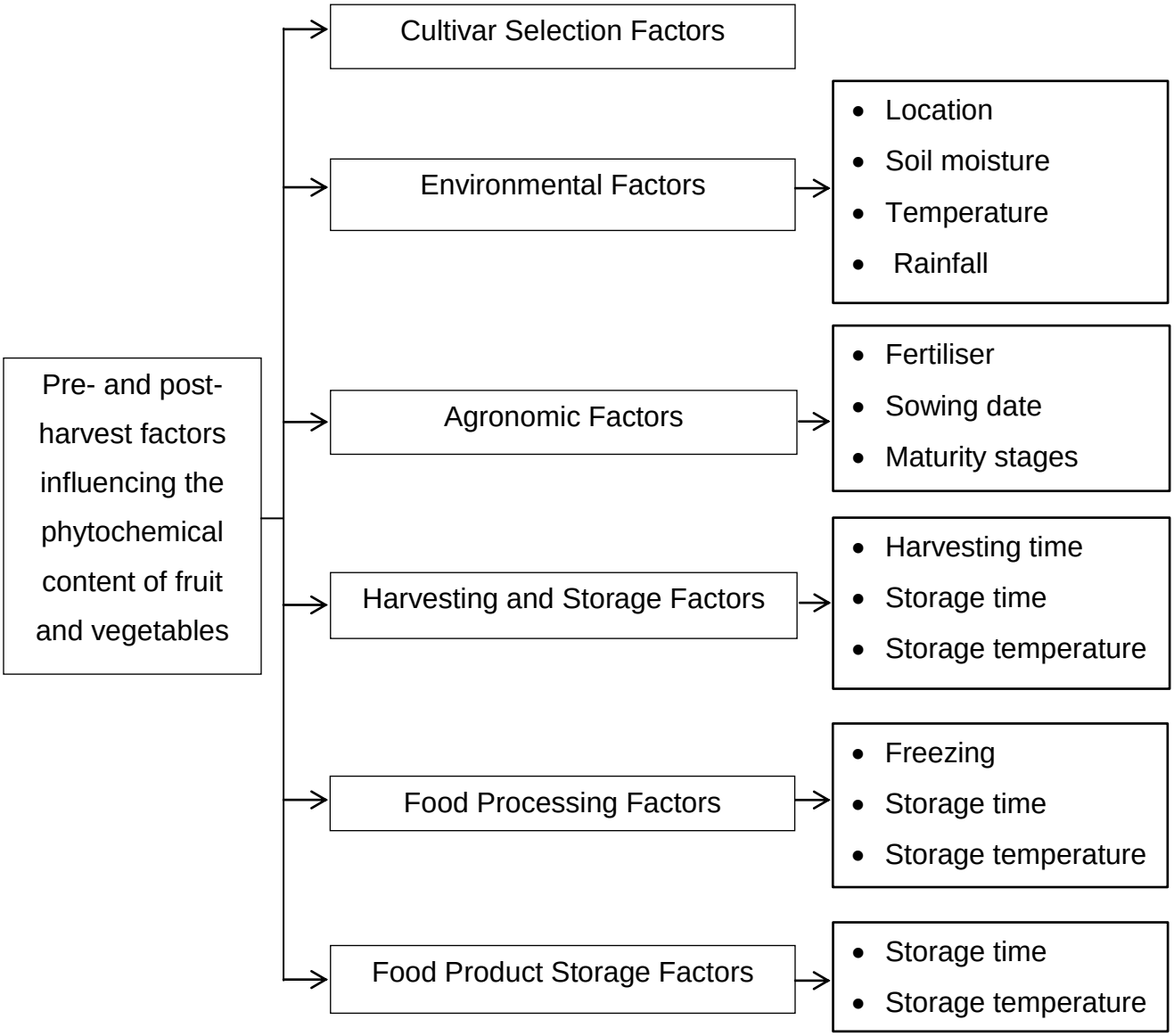


Figure 2.15: Factors influencing the phytochemical contents of fruits and vegetables

Adapted from: Tiwari and Cummins (2013, p. 501)

2.5.3.2.1 Phenolics

As stated in Liu (2013) as well as Dai and Mumper (2010), members of the phenolic group of phytochemicals possess one or more aromatic rings with one or more hydroxyl groups. They are grouped into different classes and subclasses based on their structural arrangement as well as the number and location of the hydroxyl groups including other substituents (Podsędek, 2007).

In plants, this group/class of phytochemicals support: (1) plant reproduction, growth and metabolism processes, (2) protect against biological/environmental threats and (3) contribute to the various plants' colours (Liu, 2004; Liu, 2013).

Although present in small quantities (Vincente et al., 2009), a good measure of dietary phenolics are found in fruits and vegetables while the health benefits in human diet include their ability to reduce the incidences of chronic diseases such as cancer, heart disease and diabetes (Liu, 2013). The three key dietary phenolic groups are the flavonoids, phenolic acids, and polyphenols (commonly referred to as tannins) (King and Young, 1999).

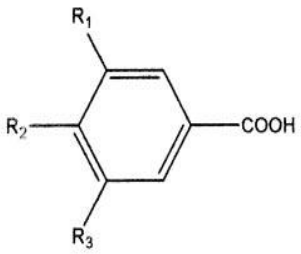
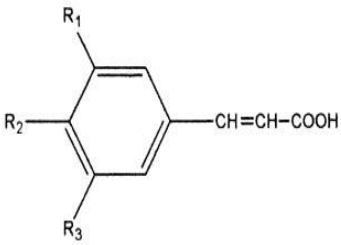
Phenolic acids

Phenolic acids represent 30% of dietary polyphenols (Ramos, 2008; Gonzalez-Vallinas et al., 2013) which contain one carboxylic acid functional group (Gonzalez-Vallinas et al., 2013; Saxena et al., 2013) and are divided into two main subclasses comprising: (1) hydroxybenzoic acid, and (2) hydroxycinnamic acid derivatives (Liu, 2013). In plant foods, the hydroxybenzoic acid derivatives such as *p*-hydroxybenzoic, gallic, syringic, protocatechuic, and vanillic acids are commonly found in bound forms as components of complex structures like lignins and hydrolyzable tannins or found attached to cell walls and proteins (Liu, 2004; Liu, 2013). They can also be present as derivatives of sugar and organic acids in plant foods such as: fruits, vegetables and whole grains (Liu, 2004; Liu, 2013).

However, the hydroxybenzoic acid content of edible plants (with the exception of some red fruits, black radish and onions) is usually low (Shahidi and Naczk, 1995, cited in Mushtaq and Wani, 2013, p. B-342) and are less common when compared to the second category which essentially consist of: *p*-coumaric, caffeic, ferulic and sinapic acids (Mushtaq and Wani, 2013). The latter compounds, i.e. the hydroxycinnamic acid derivatives, are also commonly found in bound form, connected via ester bonds to structural components of the cell wall, like cellulose, lignin, and proteins (Liu, 2004; Liu, 2013). In line with Liu (2013), two members of the hydroxybenzoic acid derivative subgroup (protocatechuic and vanillic acids) and another three from the hydroxycinnamic

acid derivatives (caffeic, *p*-coumaric and ferulic acids) are present in nearly all plant-based foods. The structures of some common phenolic acids are shown in Table 2.9.

Table 2-9: Structure of common phenolic acids

Benzoic acid Derivatives	Substitutions			Benzoic acid
	R_1	R_2	R_3	
p-Hydroxybenzoic	H	OH	H	
Protocatechuic	H	OH	OH	
Vannilic	CH_3O	OH	H	
Syringic	CH_3O	OH	CH_3O	
Gallic	OH	OH	OH	
Cinnamic acid Derivatives	Substitutions			Cinnamic acid
	R_1	R_2	R_3	
p-Coumaric	H	OH	H	
Caffeic	OH	OH	H	
Ferulic	CH_3O	OH	H	
Sinapic	CH_3O	OH	CH_3O	

Adapted from: Liu (2004, p. 3481S)

Flavonoids

Flavonoids account for 60% of dietary polyphenols (Ramos, 2008; Gonzalez-Vallinas et al., 2013) and have attracted most research interest among the groups of polyphenols (Mushtaq and Wani, 2013). They are usually made up of a generic structure with two aromatic rings (A and B rings) connected by 3 carbons that are normally in an oxygenated heterocycle ring, or C ring (Figure 2.16) while their classification into the various sub-groups [i.e. flavonols,

flavones, flavanols (catechins), flavanones, anthocyanidins, and isoflavonoids], depends on the form of the heterocycle C ring (Liu, 2004). The flavonoids that have been identified and found in diets include: flavonols (quercetin, kaempferol, and myricetin), flavones (luteolin and apigenin), flavanols (catechin, epicatechin, epigallocatechin, epicatechin gallate, and epigallocatechin gallate), flavanones (naringenin), anthocyanidins (cyanidin and malvidin), and isoflavonoids (genistein and daidzein) (Liu, 2004; Liu, 2013).

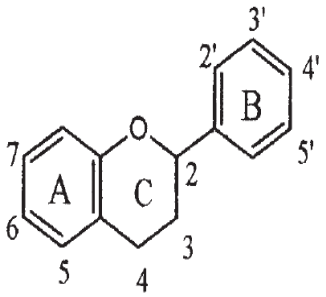


Figure 2.16: The generic structure of flavonoids

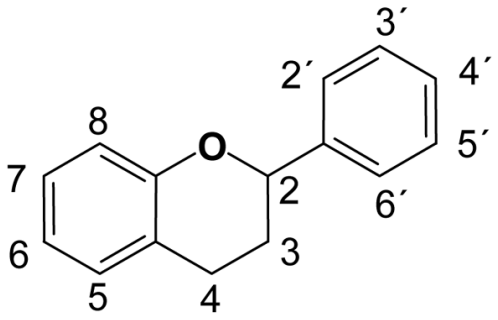
Source: Liu (2004, p. 3480S)

In line with Hollman and Arts (2000), flavonols, flavones and flavanols (catechins) are the three key subclasses of this group of phenolics with the flavonols and flavones having a similar C-ring arrangement comprising of a double bond at the 2 - 3 position. These two flavonoids (i.e. flavonols and flavones) are also commonly found bound to sugars as *O*-glycosides in plants, although, flavones may also be present as *C*-glycosides (Hollman and Arts, 2000). The different sugars (> 80) found bound to flavonoids in plants do so essentially via a β-glycosidic bond to the flavonoid nucleus with a preference for the 3-position (Hollman and Arts, 2000).

On the other hand, among the flavonoids, the anthocyanidins have been linked with the distinctive colours in some fruits, vegetables, and whole grains (Liu, 2013) while quercetin (a flavonol) has been identified as the main flavonoid in fruits and vegetables (Okamoto, 2005; Gonzalez-Vallinas et al. 2013). According to Saxena et al. (2013), flavonoids like luteolin and catechins present better antioxidant effects than the nutrient antioxidants such as vitamin C, vitamin E and β-carotene.

Generally, this class of phytochemicals have the potential to reduce the risk of major chronic diseases (Liu, 2004; Liu, 2013) like: heart disease, cancer, stroke, diabetes, Alzheimer's disease, and cataracts (Liu, 2013). Table 2.10 shows the main groups and some individual compounds of commonly occurring flavonoids as provided by Ramos (2008).

Table 2-10: Main groups and some individual compounds of commonly occurring flavonoids



Group	Compound
Flavonols (R3 = R5 = R7 = OH)	Quercetin (R3′ = R4′ = OH; R5′ = H)
Flavones (R5 = R7 = OH)	Apigenin (R3′ = H; R4′ = OH)
Flavanols (R3 = R5 = R7 = OH)	Catechin (R3′ = R4′ = OH; R5′ = H)
Flavanones (R5 = R7 = OH)	Hesperitin (R3′ = OH; R4′ = OCH_3)
Anthocyanidins (R3 = R5 = R7 = R4' = OH)	Cyanidin (R3′ = OH; R5′ = H)
Isoflavones (R7 = R4' = OH)	Genistein (R5 = OH)

Adapted from: Ramos (2008, p. 508)

2.5.3.2.2 Carotenoids and other plant pigments

Carotenoids are C_{40} isoprenoids with eight isoprene units (Ronen et al., 1999) made up of a long series of conjugated double bonds at the central part of their molecule which account for their shape, chemical reactivity and light absorbing-properties (Liu, 2004; Liu, 2013). The two subgroups of carotenoids, the hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls)

(Podsedek, 2007; Liu, 2013), are the natural pigments linked with the different colours (orange, yellow, red and purple) seen in nature (Burns et al., 2003). This is as a result of their presence in plants (Liu, 2004), such as fruits and vegetables (Burns et al., 2003; Podsedek, 2007; Liu, 2013), microorganisms and animals (Liu, 2004).

Green leafy vegetables have been identified to be rich in carotenoids (Devadas and Saroja, 1980, cited in Raju et al., 2007, p. 1598) while leafy vegetables in general are known to contain the photosynthetic pigments - chlorophylls (Žnidarčič et al., 2011) and carotenoids (Kimura and Rodriguez-Amaya, 2002; Žnidarčič et al., 2011) - which provide a measure of their photosynthetic activity as well as physiological condition (Gamon and Surfus, 1999; Žnidarčič et al., 2011;). According to Podsędek (2007), among the 22 species of vegetables analysed by Müller (1997), kale, red paprika, leaf of parsley, spinach, Lamb's lettuce, carrot, and tomato were found to be very rich in carotenoids (over 10mg/100g edible portion). Liu (2013) also reported orange and yellow vegetables as well as fruits to have high concentration of β-carotene, dark green leafy vegetables (such as spinach, kale, broccoli) to be rich in lutein and zeaxanthin, while significant amount of lycopene can be derived from the consumption of tomatoes, watermelons, pink grapefruits, apricots, and pink guavas.

In plants, carotenoids play key roles in photosynthesis and photoprotection (Liu, 2004; Liu, 2013) with the latter attribute contributing to their antioxidant potential in human health (Liu, 2004). They have been found to be able to prevent lipid oxidation and related oxidative stress when present in sufficient amounts (Liu, 2004). In addition to these, they have provitamin functions (Liu, 2013) such as the pro-vitamin A activity of: β-carotene, α-carotene and β-cryptoxanthin (Rodriguez et al., 2006; Liu, 2013). In terms of human health, Rodriguez et al. (2006) noted that β-carotene, α-carotene, β-cryptoxanthin, lutein and lycopene have been the most studied carotenoids as a result of their presence in human blood.

Although tomatoes and tomato-based products contain other phytochemicals, they are the chief source of lycopene in the diet of many parts

of the world (Rodriguez et al., 2006). According to Kotikova et al. (2009), lycopene and β-carotene are the two principal pigments contributing to the colour of ripe fruits and to a large extent determine the quality perception of fresh tomatoes including their redness. Interestingly, the consumption of lycopene and β-carotene in foods may help in reducing the risk/incidence of prostate (Giovannucci, 1999; Burns et al., 2003; Rodriguez et al., 2006) and lung cancer respectively (Block, 1992; Burns, 2003).

2.5.3.2.3 Other organic acids

In line with Ayaz et al. (2006), the maintenance of the quality of fruit and vegetables is built on organic acids as well as other natural components found in them such as free sugars, amino acids, lipids and minerals which in general also define their nutritive value when consumed by human. In particular, organic acids are employed as an index of authenticity in fruit product (Kelebek, 2010) while both organic acids and phenolic compounds are linked with the organoleptic characteristics of fruits and vegetables (Vaughan and Geissler, 1997, cited in Sousa et al., 2005, p. 9128).

These organic acids, commonly found in fruits and vegetables (Shui and Leong, 2002; Vale et al., 2015), may originate from biochemical processes or from the activities of some microorganisms (such as yeasts and bacteria) (Hernández et al., 2009; Vale et al., 2015). In plant physiology, they act as cofactors, buffering agents, and intermediates of most key metabolic pathways of carbohydrates, lipids and proteins (Koyuncu, 2004; Vale et al., 2015).

As reported by Fennema et al. (1996), the presence of small amounts of organic acids in plants gives them their characteristic acidic or sour taste with citric and malic acids being the two dominant organic acids in plants. Meanwhile, other organic acids present in plants include tartaric acid (in grapes and avocado), oxalic acid (in spinach), isocitric acid (in blackberry), ferulic acid (in cereals), and chlorogenic acid (in apples and pears) (Fennema et al., 1996). The acid level of fruits has however been found to decrease with ripening while some specific acids may respond differently (Fennema et al., 1996).

Generally, organic acids also find application as additives in their use as antioxidant (tartaric, malic, and citric), preservatives (sorbic and benzoic acids) (Cunha et al., 2002; Koyuncu, 2004; Vale et al., 2015) or acidulants (tartaric, malic, citric, and ascorbic acids) (Shui and Leong 2002; Koyuncu, 2004; Vale et al., 2015) in the food industry (Shui and Leong 2002; Vale et al., 2015) as well as in the pharmaceutical industry where they are similarly used as antioxidants, preservatives, acidulants and drug absorption modifiers (Koyuncu, 2004).

Impact of abiotic/crude oil-induced abiotic stresses on antioxidant compounds (phenolics)

According to Wang and Frei (2011), the effects of environmental stresses on the antioxidant composition of harvested agricultural produce have not been well documented. From those reported, in some cases, environmental stress was found to lead to the increase in crops' antioxidant concentration with the phenolics being the most studied antioxidant compound. Among the different studies considered on the effect of environmental stress (like drought and heat) on phenolics by Wang and Frei (2011), a majorty of 25 studies reported an increase in phenolic content while four and ten studies observed a negative and a no clear effects respectively in the crops under investigation. The key enzymes of the phenylpropanoid pathways, such as phenylalanine ammonia lyase, are in most cases responsible for the accumulation of phenolic in crops exposed to environmental stress while a decrease in phenolic content in some crops under environmental stress may occur when there are high amounts of some specific phenolic compounds which are prone to degradation on exposure to stress (Wang and Frei, 2011).

2.5.4 Effect of abiotic environmental stresses on the research selected vegetables

In the view of Gross (1991), a vegetable can be defined at two different levels; at the first (broad level), it refers to any form of plant life or plant product and at the second (narrow level), it implies the fresh edible portion of a herbaceous plant (such as: the roots, stems, leaves and leafstalks, flowers, fruit

or seeds) that are eaten raw or cooked. This view is also supported by Taura and Habibu (2009) who classified vegetables based on the nature of the desired consumed parts into two groups, namely: (a) the leafy vegetables such as: lettuce, amaranth, spinach etc, and (b) the fruit vegetables such as tomatoes, okra, garden egg etc. Figure 2.17 shows the two classes of vegetables based on the consumed part with particular reference to those selected for the research.

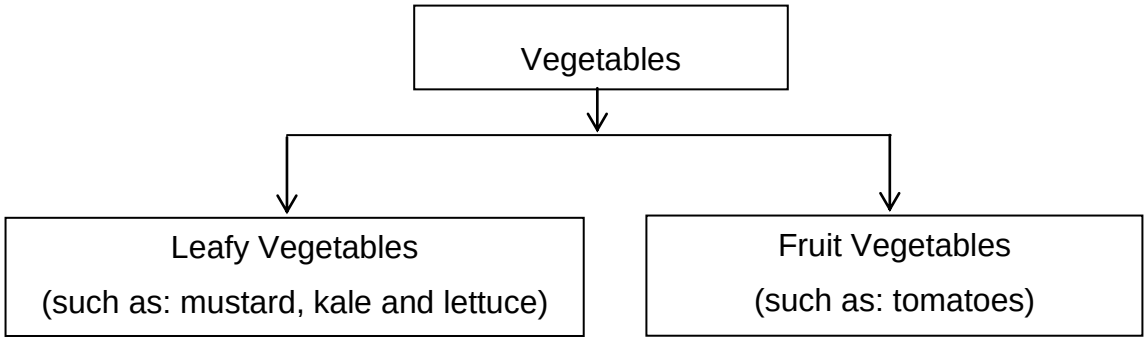


Figure 2.17: Classes of vegetables (based on desired consumed part) with those selected for the research

The two classes of vegetables have high percentage of water when harvested green (NSPRI, 1992, cited in Taura and Habibu, 2009, p. 1) and are considered good sources of vitamins and minerals (Matthew, 1985, cited in Taura and Habibu, 2009) with Ca, K, Fe and Na being the most dominant elements in plants and vegetables (Caunii et al., 2010). They also serve as a good source of phytochemicals which although are not essential nutrients (Temple, 2000; Alarcón-Flores et al., 2013;) are known to have human health benefits (Temple, 2000; Alarcón-Flores et al., 2013; Santos et al., 2014). According to Butnariu (2007, cited in Caunii et al. 2010, p. 45), most vegetables also have high percentage of digestible (starch, sucrose, glucose, fructose) and non-digestible carbohydrates.

From health-related studies, the regular consumption of a diet rich in this class of plants has been found to have human health benefits (Wold et al., 2006; Fernandes et al., 2012) involving the reduction in the incidences of some

form of diseases and cancer which can be attributed to the bioactive compounds (phytochemicals) such as carotenoids, phenolics, alkaloids, nitrogen-containing compounds and organosulfur compounds, they contain (Wold et al., 2006). The antioxidant potentials of this class of food (i.e. fruits and vegetables) depend on: the cultivar or genetic makeup of the plant, the environment in which the crop is grown, the growing season and the type of agricultural practice (Wold et al., 2006).

2.5.4.1 *Brassica juncea* (Mustard)

Mustard, *Brassica juncea* (Mozaffari et al., 2012), is a member of the genus *Brassica* of the family of *Brassicaceae* (Akmal et al., 2011) also referred to as *Cruciferae* (Podesedek, 2007; Pilar et al., 2011). There are about 3,000 species of plants in this family within 350 genera (Pilar et al., 2011) while *B. juncea* is one of the major oil seed crops in the world (Mozaffari et al., 2012) with its leaves also consumed as vegetables (Pilar et al., 2011).

The plant is a rich source of vitamins, minerals, dietary fibres and flavonoids (Xie et al., 2000, cited in Huang et al., 2012, p. 375) while the presence of essential fatty acids with lowest amount of saturated fat in its oil also support its use as an edible oil (Akmal et al., 2011). In addition to the unique contributions of these plants to nutrition, studies have shown that some cultivars of the plant can be used in phytoremediation such as to extract metals like Cd (Kumar et al., 1995; Nouairi et al., 2006; Sharma et al., 2010) and Zn (Ebbs and Kochian, 1998).

The observed values in the experiment of Boomiraj et al. (2010) showed that physiological maturity of mustard varied between 116 to 134 days while nitrogen is the most required nutrient by the plant (Mozaffari et al., 2012). This indicates that petroleum hydrocarbons contamination which is associated with nitrogen deficiency (Merkl et al., 2004) as indicated in Section 2.5.2.1, will have a negative effect on the growth of this plant. Generally, the output of the plant is affected by some abiotic (Akmal et al., 2011) and biotic stresses (Manju et al., 2004; Akmal et al., 2011) which include insect-pests as well as diseases (Manju et al., 2004).

2.5.4.2 *Brassica oleracea* L. *acephala* group (Kale)

Kale (*Brassica oleracea* L. *acephala* group) (Prior and Cao, 2000; Cartea et al., 2008) an annual crop and a green leafy vegetable (Emebu and Anyika, 2011) also belongs to the *Brassicaceae* family (Zhang et al., 2012). Members of the brassica group, including kale, have been linked with nutritional (Sikora and Bodziarczyk, 2012) and health-promoting advantages (Emebu and Anyika, 2011; Sikora and Biodziarczyk, 2012). As a result of these health-promoting properties, the plant is referred to as “hospital too far” in Delta State of Nigeria (Emebu and Anyika, 2011) which is a part of the Nigerian Niger Delta region. It is known to be a good source of fibre, minerals, vitamin C, carotenoids and some other bioactive compounds (Sikora and Bodziarczyk, 2012) such as the glucosinolates (Cartea et al., 2008; Fernandes et al., 2012) commonly found among *Brassica* species with anticancer potentials (Cartea et al., 2008).

The plant is easy to grow and can be cultivated under low temperature conditions (Emebu and Anyika, 2011). It however demands a lot of water during growth (Sikora and Bodziarczyk, 2012) and also vulnerable to pests that attack the cabbage family (Emebu and Anyika, 2011). The size of the plants depends on the variety grown but is mostly 12 - 36 inches wide and 12 - 24 inches high. It reaches maturity in approximately 2 months although this depends on temperature of the growing environment (Damrosch, 2004, cited in Emebu and Anyika, 2011, p. 190).

Impact of abiotic/crude oil-induced abiotic stresses

Brassica juncea, *Brassica napus* and *Brassica campestris*, have been identified as the three plants among the *Brassica* species that often encounter drought and osmotic stresses as a result of their wide cultivation (Alam et al., 2014). According to Dixon (2007, cited in Tong et al., 2014, p. 190), some types of *Brassica juncea* can tolerate unfavourable environmental conditions like drought and poor soil fertility. In most cases, however, the nutritional quality improvement under drought stress lead to reduced yield (Tong et al., 2014).

2.5.4.3 *Lactuca sativa* (Lettuce)

Lettuce, *Lactuca sativa* (Page et al., 1987), is a member of the Compositae (*Asteraceae*) family (Romani et al., 2002; Nomaani, Rahma Said Salim Al et al., 2013) and its cultivation could first be linked to Egypt (Romani et al., 2002; Nomaani, Rahma Said Salim Al et al., 2013). Currently, it is one of the most widely consumed vegetables in the world as a result of its health benefits (Nomaani, Rahma Said Salim Al et al., 2013) and also a major food crop within the European Union as well as the United States of America (Baslam et al., 2013).

Cultivated lettuce can be classified into five different categories based on their vegetative morphology, namely: iceberg or crisphead, romaine or cos, butterhead, stem, and leaf lettuces (Hu et al., 2005) which also require different number of days before harvest (Table 2.11). The plant is easily cultivated in countries with relatively low temperatures (Nomaani, Rahma Said Salim Al et al., 2013) and is also one of the plant species considered for the study regarding human life support in space via controlled ecological life support systems (CELSSs) (Wheeler et al., 1994).

Table 2-11: Average days required to harvest the different lettuce type

Lettuce type	Average days to harvest after seedling
Crisphead	70 – 80
Butter and Bibb	50 – 60
Romaine	60 – 70
Leaf	50 - 60

Source: Seaman (2015) and Douglas (2001)

Aside from the macrominerals (such as K, Na, Ca and Mg) and trace elements (like Fe, Mn, Cu and Zn) found in lettuce as part of its nutritional composition (Kawashima and Soares, 2003; Pinto et al., 2014), the vegetable also contains phenolic antioxidants, vitamins A and C (Romani et al. 2002). Those obtained from agricultural sites may have some other non-essential toxic

elements such as: Ni, As, Cd and Pb, among others below the permissible limits (Pinto et al., 2014). Boon and Soltanpour (1972, cited in Jordão et al., 2013, p. 765) also noted the ability of this vegetable to accumulate heavy metals.

According to Seaman (2015) lettuce on average, reaches maturity in 60 days with even spacing to achieve uniform maturity. Although the different varieties vary in their tolerance to heat and cold, the vegetable grows optimally in cool-season and will germinate in soil temperature as low as $0^{o}C$ while at soil temperatures above $26.7^{o}C$, the seed will not germinate until the temperature cools. High temperatures are detrimental, leading to disorders such as premature bolting, tip-burn, and brown rib (Seaman, 2015). Whereas there is a higher percentage of germination in seeds grown in the greenhouse compared to the field, planting lettuce in the greenhouse favours plant pathogen which may not be easily controlled. The availability of nutrients to the plant is maximized by maintaining a soil pH between 6.0 and 6.2 (Seaman, 2015). Table 2.12 illustrates the degree of nutrient demand by lettuce.

Table 2-12: Crop nutrient requirements

	Nutrient Needs		
	Lower	**Medium**	**Higher**
Crop	Bean	Cucumber	Broccoli
	Beet	Eggplant	Cabbage
	Carrot	Brassica greens	Cauliflower
	Herbs	Pepper	Corn
	Pea	Pumpkin	Lettuce
	Radish	Spinach	Potato
		Winter squash	Tomato

Source: Mohler and Johnson (2009, cited in Seaman, 2015, p. 8)

Impact of abiotic/crude oil-induced abiotic stresses

As antioxidants, which include some phytochemicals, are induced by plant as a defense mechanism when exposed to environmental stress (Oh et al., 2009), these authors (i.e. Oh et al., 2009) found out that controlled/regulated

mild environmental stresses involving heat shock (40^{o}C for 10 min), chilling (4^{o}C for 1 d) or high light intensity (800 µmol m^{-2} for 1 d) increased the total phenolic content and antioxidant capacity of lettuce while they had negligible effect on the plant growth. The experimental outcome also revealed that some specific flavonoid derivatives (phenolic compounds), such as quercetin-3-*O*-glucoside and luteolin-7-*O*-glucoside, may be induced in lettuce when exposed to some environmental stresses, particularly high light intensity. They further pointed out that both the amount and composition of phenolic compounds in lettuce may be affected by environmental stresses while increase in the phenolic content of lettuce under these mild stresses would also lead to enhanced antioxidant potential and consequently promote the plants' health-benefits.

2.5.4.4 *Solanum lycopersicum* L. (Tomato)

Tomato (*Solanum lycopersicum* L.) (Albacete et al., 2008; Ilahy et al., 2011), a major source of antioxidants (Riahi and Hdider, 2013), is one of the world's most important (Naika et al., 2005), popular (Beckles, 2012) and consumed vegetable (Anza et al., 2006). According to Bergougnoux (2014), in terms of importance, the crop ranks seventh after maize, rice, wheat, potatoes, soybeans and cassava. It is a member of the *Solanaceae* family; rich in minerals, vitamins, essential amino acids, dietary fibres (Naika et al., 2005) as well as antioxidant compounds such as: β-carotene, lutein, vitamin C, vitamin E and phenolic compounds etc (Dumas et al., 2003) which could be affected by cultural and abiotic factors (Riahi and Hdider, 2013).

Generally, the quality of tomato plants has been classified into two amongst others: organoleptic (defined by physiochemical parameters) and nutritional quality which relates to human health (Anza et al., 2006) while its other processed forms include purees, juices and ketchup (Naika et al., 2005). The cultivar type determines the shape of the tomato fruit (Naika et al., 2005) as well as the lycopene content (Hart and Scott, 1995; Rao and Agarwal, 2000). The red tomatoes have lycopene but with a lesser amount of provitamin A when compared with yellow tomatoes (Naika et al., 2005).

Adequate space preparation is required in the planting process as Naika et al. (2005) identified three different types of tomato plants: tall or indeterminate (which keep growing after flowering), semi-bush or semi-indeterminate and the determinate (which stops growing after flowering). The plant needs a cool and dry climate for optimum productivity at temperatures between 21 and 24^{o}C, soil depth of 15 to 20 cm, pH of 5.5 – 6.8 and sufficient nutrient supply (Naika et al., 2005). However, the plant tissues may be negatively affected at temperatures below 10^{o}C or higher than 38^{o}C (Naika et al., 2005). Table 2.13 shows the temperature requirement of tomato fruits.

Table 2-13: Temperature requirements for tomato fruits production

Stages	Temperature (^{o}C)		
	Minimum	Optimum range	Maximum
Seed germination	11	16 - 29	34
Seedling growth	18	21 - 24	32
Fruit set	18	20 - 24	30
Red colour development	10	20 - 24	30

Source: Naika et al. (2005)

Generally and according to Young et al. (1993), tomato quality assessment is mainly focused on sugars and organic acids determination as they are the chief components of tomato total and soluble solids.

Impact of abiotic/crude oil-induced abiotic stresses

Although light and temperature have been identified as the two common environmental factors that affect the nutritional value of tomato (Dorais et al., 2008), according to Lapushner et al. (1986, cited in Veit-Köhler et al., 1999, p.

584), under water and salinity stress conditions, the sugar and acid contents of tomato fruits may increase. However, when the stress becomes severe, the plant and fruit growth become impaired (Veit-Köhler et al., 1999). The sugar content which contributes to improved fruit quality may increase under water stress (as a result of a decrease in tomato water content) but also lead to decrease in marketable fruit yield (Veit-Köhler et al., 1999). Following from this, Wang and Frei (2011), reported that the increase in the sugar concentrations of tomatoes under drought and salinity stress in some reported studies could be as a result of concentration effects as the analysis were done on a fresh weight basis.

Under mild conditions of water stress, the fruit quality may be enhanced without any reduction in marketable fruit yield (Veit-Köhler, 1999) while the availability of excess water may have a negative effect on the tomato soluble solid content as well as the concentration of reducing sugar in the fruit (Favati et al., 2009). In line with Shalhevet and Yaron (1973 cited in Mitchell et al., 1991b, p. 215), the improvement in tomato fruit quality arising from water deficit and saline water irrigation are often linked with reduced yields. Kim et al. (2008b) however indicated that tomatoes produced under these stress conditions (water or salt) usually develop fruits with richer colour in addition to their enriched flavour.

2.6 Identified research gaps

From the literature review carried out, the following research gaps were identified:

1. Current knowledge on RENA, a land farming treatment technology (Ebuehi et al., 2005; Onifade et al., 2007b; Wegwu et al., 2010), is mostly associated with remediation with poor understanding on how it supports agrifood production.
2. No practical verification of the effectiveness of the new remediation intervention value of 3,000 mg/kg TPH as provided in the SEPCiN RMS document (UNEP, 2011, p. 149).

3. Although the soil remediation intervention value indicates the concentration at which the functional properties of the soil for human, animal and plant life are seriously impaired or threatened (Soil Remediation Circular, 2009; UNEP, 2011), there is dearth of information on the effect of intervention values stipulated in various national environmental regulations on the yield and composition of crops.
4. There are several published reports on the influence of abiotic stresses on agrifood production (see Table 2.5) and crop quality. However, majority of the studies on the effect of crude oil/petroleum hydrocarbons on plants have mostly considered the impact on plant growth/yield (e.g. Kuhn et al., 1998; Adam and Duncan, 2002; Baruah et al., 2004; Adieze et al., 2012; Inckot et al., 2011) and few crop quality parameters (e.g. Baruah et al., 2004; Chupakhina and Maslennikov, 2004; Noori et al., 2012). Some of these experiments also involved the use of only one test plant (e.g. Carr, 1919; Kuhn et al., 1998; Achuba, 2006; Inckot et al., 2011; Baruah et al., 2014) limiting the extensive application of the research findings.
5. As crude oil is known to contain volatile organic compounds (VOCs) (UNEP, 2011), there is a need to have an understanding of those associated with the commonly used soil remediation intervention values.

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Source of experimental crude oil

The Libyan crude oil with sulphur content (0.14% wt.) and specific gravity (0.842 g/ml) close to that of the Nigerian light crude oil (Grimmer et al., 1983) was employed for the study. It was obtained from a Libyan oil-field in early 2013, and was used as received.

3.1.2 Sources of growing media and seeds for the bioremediation mesocosm study

According to Zhu et al. (2001), mesocosm, or a pilot scale system, is a 'scaled'-link between microcosm and field study, and has been used in assessing the efficiency of different bioremediation treatment methods. The John Innes No. 2 compost (John Innes Manufacturers Association, Berkshire), spent *Pleurotus ostreatus* mushroom compost (Marlborough Mushrooms, UK) and the *Brassica juncea* seeds (Herbiseed, England) as shown in Figure 3.1 were used for the mesocosm experiment in Chapter 4 involving the selected bioremediation strategies.

Figure 3-1: Materials used: (a) – John Innes No. 2 Compost; (b) – Spent *Pleurotus ostreatus* mushroom compost, and (c): *Brassica juncea* seeds

3.1.3 Sources of growing media and seeds for the different CRIV experiments

John Innes No. 2 compost for potting-on house and vegetable plants by Hurstridge (Surrey, UK) was used to grow the leafy vegetables in Chapter 5 while for the experiment involving the two tomato cultivars in Chapter 6, the general purpose peat-sand growing medium of William Sinclair Horticulture Ltd (Lincoln, UK) containing 95% peat (William Sinclair Holdings PLC, 2008) was utilized. The two growing media are shown in Figure 3.2.

The seeds of the leafy vegetables used: Oriental Mustards (*Brassica juncea*), Kale Starbor F1 Hybrid (*Brassica oleracea*) and Lettuce Butterhead Analena (*Lactuca sativa*) were purchased from Thompson & Morgan (Suffolk, United Kingdom). Micro-Tom WSS1414 and M82 tomatoes (*Solanum lycopersicum*) varieties seeds were provided by the Plant Science Group of Cranfield University (United Kingdom).

Figure 3-2: Growing media used: (a) John Innes Compost No. 2, and (b) Sinclair All Purpose Growing Medium

3.1.4 Rationale behind the choices of growing media and test crops

Use of commercial potting compost

The use of commercial potting compost (with up to 1.5% organic carbon) for this research is supported by OECDiLibrary (2006) guidelines for testing the effects of contaminants on plants while Lawrence (1955) recommended the use

of standardized John Innes compost to overcome the possible variations that could arise in the use of different growing media with varying compositions. This has been used by several other authors (Anderson and Mansfield, 1979; Ashenden, 1985; Magrath et al., 1994; Bean et al., 1997; Rahman et al., 2012) and was referred to as standard soil by Ashenden (1985). Kim et al. (2008a) also used commercial potting soil to study the effect of salinity stress on romaine lettuce (*Lactuca sativa* L.).

For the tomatoes, the Sinclair all-purpose growing medium containing 95% peat (Figure 3.2b) was used because according to Naika et al. (2005), tomato is susceptible to drought and its yield decreases significantly after short periods of water deficiency. The increased level of peat (which is acidic) in this growing medium, allows for improved available water holding capacity (John Innes Manufacturers Association, 2010). Although the John Innes compost also contains peat, the loam (sterilised soil) is the main ingredient (John Innes Manufacturers Association, 2010).

Plant selection criteria

Among the numerous available vegetables, *Brassica juncea* (mustard), *Brassica oleracea* (Kale), *Lactuca sativa* (lettuce) and two cultivars of *Solanum lycopersicum* (tomato) were selected for this research based on the following criteria:

(a) They belong to the group of plant families: Brassicacea (Cruciferae), Asteraceae (Compositae) and Solanaceae which have found plant testing applications as indicated in OECDiLibrary (2006);
(b) From literature, they are possible sources of the nutritional and bioactive compounds of interest;
(c) The selected leafy vegetables can be grown in the greenhouse and harvested within a short time as pointed out in Chapter 2, Section 2.5.4.1, 2.5.4.2 and 2.5.4.3 for *B. juncea*, *B. oleracea* and *L. sativa* respectively;
(d) According to Kuhn et al. (1998), *S. lycopersicum* (tomato) is a sensitive plant and was specifically chosen for the determination of the effect of remediation intervention values at ≤ 5,000 mg/kg TPH.

3.2 Methods

Generally, the methods used for the analysis of the soils and plants were based on British and International Standards. Cost-effective protocols which: are updated over time, give best results, and have been published in scientific journals, were also employed where applicable in the analysis of the experimental samples.

3.2.1 Crude oil characterisation

3.2.1.1 GC/MS analysis of the crude oil

For the assessment of the alkane and PAHs distributions in the whole crude oil, the Libyan crude oil was subjected to GC/MS analysis. In this, 0.1 g of the crude oil was dissolved in 10 ml of hexane/acetone (1:1) mixture and passed through a solvent phase extraction (SPE) tube. This was then made up to 50 ml using hexane/acetone (1:1) with 0.7 ml of this extract, 0.1 ml of 5 µg/ml PAHs internal standard solution and 0.2 ml of 10 µg/ml alkane internal standard solution added into GC vial prior to the GC/MS analysis. The analysis was done with five replicates while the operating conditions of the hydrocarbons instrumental analysis are provided in Section 3.2.4.5.

3.2.1.2 Fractional distillation of the crude oil

In order to evaluate the impact of the crude oil during usage on the environment, the distribution of the different fractions was assessed. An All-glass Dufton column was used for the fractional distillation of crude oil under laboratory conditions (Figure 3.3). Crude oil (200 g) was weighed into a clean 500-ml round bottom flask and placed in a heating mantle. Different fractions of crude oil were collected in calibrated receiving tubes as the temperature increased from the room temperature to 270^{o}C to determine the volume and weight of the fractions obtained. The fractions were collected at different boiling temperatures and the collecting tubes changed as soon as there is cessation in distillate droplets due to exhaustion of the fraction at that particular temperature range. Heat losses were minimized by insulating the column with aluminium foil.

Figure 3-3: Fractional distillation of crude oil under laboratory conditions

3.2.2 Growing media/composts physicochemical properties determination

3.2.2.1 Adjustment of the John Innes No. 2 compost pH for the bioremediation mesocosm study

The initial pH of the growing medium (i.e. John Innes No. 2 compost), 5.31 ± 0.01, for the bioremediation experiment was found below the range of 6.5 - 8.0 recommended by Vidali (2001) for effective oil degradation (see Chapter 2, Table 2.3). Hence, liming was carried out using Westland garden lime to neutralise the pH to a range suitable for the bioremediation process.

The liming involved the addition of 20 g of the soil-based compost with varying amount of garden lime as shown in Chapter 4 (Table 4.2) in order to generate a straight line graph (Chapter 4, Figure 4.3) from which the amount of lime to be added to the bulk of soil-based compost can be deduced. This then led to the manual uniform full-depth mixing of 16.9 g of lime with 1 kg of soil-based compost to give a pH of 6.60 ± 0.03 (Chapter 4, Table 4.4).

3.2.2.2 pH determination

The procedure according to Sparks et al. (1996) was initially used for the pH determination. In this, 10 g of the air-dried sample was weighed in a 100-ml beaker. Deionized water (10 ml) was added, mixed well and allowed to stand for 10 min. The suspension was swirled and the electrodes inserted to measure the pH. The HANNA instruments HI 8519N pH meter (Figure 3.4) equipped with glass and reference electrode was used.

Figure 3-4: HANNA Bench-Top pH/mV meter, model H18519, Portugal

This procedure was later replaced with the standard operating procedure (SOP) based on the British Standard BS International Standard for Standardization ISO 10390:2005 (BSI, 2005) for the determination of soil pH. In this, 10 ml of the air-dried soil sample was scooped into a labelled 100-ml glass bottle using a 10-ml brass scoop. Deionized water (50 ml) was added to the soil in the glass bottle (i.e. 1:5 volume fraction) and shaken on a side-to-side shaker (set at 300 min^{-1}) for 60 min. The samples were then left to stand for 1 h and the pH measured after calibrating the pH meter with pH 4.0, pH 7.0 and pH 10.0 buffers according to the manufacturer's instructions.

3.2.2.3 Soil moisture (water) content determination

The British Standard BS 7755: Section 3.1:1994 Determination of dry matter and water content on a mass basis by a gravimetric method which is identical to ISO 11465:1993 was used for the soil moisture (water) content assessment. This procedure entails the weighing of 5 g of the air-dried soil sample into a

labelled porcelain crucible with the mass of the crucible as m_0 and that of the crucible plus soil as m_1. Thereafter, the crucible and soil were dried in an oven set at 105^{o}C for 24 h. The crucible and contents were then removed from the oven, cooled in a desiccator, removed and immediately weighed to record the mass m_2.

The moisture (water) content (W_{H2O}) was then calculated using the following expression and the value recorded.

$$\%\ \text{Soil moisture (water)content} = \frac{\mathrm{m1} - \mathrm{m2}}{\mathrm{m2} - \mathrm{m0}} \times 100$$

where:

m_0 = mass (g) of the empty crucible;

m_1 = mass (g) of the crucible plus air-dried soil;

m_2 = mass (g) of the crucible plus oven-dried soil.

3.2.2.4 Water holding capacity determination

The modified Hilgard method of Mabuhay et al. (2003) and Ahn et al. (2008) was followed with the weighing of 10 g of the growing media/composts of known initial moisture content weighed (W_1) and placed in a beaker. This was soaked in water for 2 d while excess water was drained through Whatman Number 2 filter paper. The saturated growing media/composts were weighed again (W_s). The amount of water retained by the dry growing media/composts was calculated as water holding capacity (g water/g dry material) and calculated as:

$$\text{Water holding capacity} = \frac{[(\mathrm{Ws} - \mathrm{W1}) + \mathrm{MC1}\ \times \mathrm{W1}]}{[(1 - \mathrm{MC1}) \times \mathrm{W1}]}$$

where MC_1 = Initial moisture content of sample

3.2.2.5 Electrical conductivity

The determination of electrical conductivity on a 1:5 soil:water extract based on the British Standard BS 7755: Section 3:4:1995 Determination of specific electrical conductivity identical to ISO 11265:1994 (BSI, 1995a) was conducted.

The principle involves extracting the air-dried growing media/composts with water at an extraction ratio of 1:5 (m/V) to dissolve the electrolytes after which the electrical conductivity of the extract is then measured.

Each of the samples (20 ± 0.01g) was weighed into a 290-ml polycarbonate bottle. Demineralized (deionized) water (100 ml) was added and the bottles shaken on a side-to-side shaker (set at 300 min^{-1}) for 30 min. Centrifugation of the bottles at 3500 rpm for 20 min was carried out using Sorvall® Legend RT centrifuge machine and the supernatant filtered through Whatman Number 2 filter paper. The electrical conductivity of the filtrate obtained was then measured using the JENWAY conductivity meter 4310 model (Figure 3.5).

Figure 3-5: JENWAY Conductivity meter, Model 4310, Essex, UK

3.2.2.6 Total carbon determination

The procedure followed (BSI, 1995b) is based on British Standard BS 7755 Section 3.8: 1995 for the determination of organic and total carbon after dry combustion which is identical to ISO 10694:1995.

The growing media/composts were first dried at 40^{o}C for approximately 48 h after which they were ground and passed through 2.00 mm sieve. These were then passed through Retsch®ZM 200 fine grinder operated at a speed of 16,000 revolutions per minute (rpm). The fine ground samples were dried in the oven at 105^{o}C for 2 h.

Less than 40 mg of each of the samples was weighed into a small Elementar Zinnschiffchen 6 × 6 × 12 mm tin boats (Elementar Analysensysteme GmbH, Germany) capsule and packed tightly. They were then loaded in the automatic carousel sampler of the Elementar Vario EL III analyzer (Figure 3.6) which is suitable for analysing carbon, hydrogen, nitrogen, sulphur and oxygen while acetanilide (C_8H_9NO; MERCK, Germany) was used as the standard.

The carbon present in the samples was first oxidised to carbon dioxide (CO_2) after heating to a temperature of at least 900^oC on a flow of oxygen-containing gas that is free from carbon dioxide which also allows for the complete decomposition of any carbonates that could be present. The amount of carbon dioxide released from this combustion process was then quantified using the thermal conductivity detector (TCD).

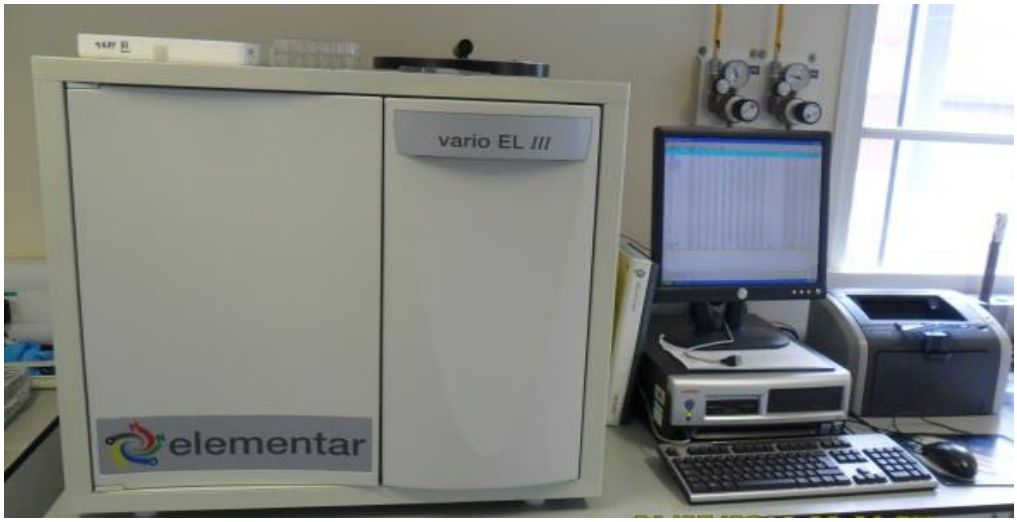


Figure 3-6: Vario EL III, CHNOS Elemental Analyzer
(D-63452, Hanau-Germany)

3.2.2.7 Total organic carbon determination

This determination followed a similar procedure used for the total carbon determination except for the addition of 3 to 4 drops of 4 mol/l hydrochloric acid to the sample first in the Elementar Silberschiffchen silver boat (Germany) to remove the carbonates in the sample. They were then transferred back to the oven to dry at 90^oC for 4 h. Thereafter, they were re-packed in the tin boats before introduction in the Vario EL III, CHNOS Elemental Analyzer (Figure 3.6).

3.2.2.8 Total nitrogen determination

The SOP based on British Standard BS EN 13654-2:2001 (BSI, 2001) Soil improvers and growing media – Determination of nitrogen – part 2: Dumas method was used in evaluating the amount of total nitrogen present in the oven-dried soil samples. In this, the soil samples ≤ 40 mg was weighed into the Zinnschiffchen 6 × 6 × 12 mm tin boats and were treated in a similar way as in the total carbon determination (Section 3.2.2.6) as well as heated to a temperature of ≥ 900^{o}C in the presence of oxygen gas. During the oxidised combustion, mineral and organic compounds in the sample produce the oxidation products NO_x, in addition to molecular nitrogen (N_2). The nitrogen oxide produced was then quantatively reduced to molecular nitrogen (N_2) by copper in the reduction tube which also binds excess oxygen. Thereafter, the measurement of the total nitrogen in the sample was done using the TCD as provided in the Elemental analyzer (Figure 3.6).

3.2.2.9 Total phosphorus determination

The procedure used is based on USEPA method 3051 (US EPA, 1997) and the principle involves treating the sample with a nitric/hydrochloric acid mixture using a microwave digestion system while the determination of the phosphorus content is by a spectrometric measurement in solution.

About 0.25 ± 0.001 g of each of the fine ground samples was weighed in a weighing boat and transferred into a labelled microwave digestion liner. ZeroStat 3 mittypro pistol was used to neutralise static during the addition of the sample. Six millilitres of nitric acid (specific gravity of 1.42; Fisher Scientific, UK), 1 ml of hydrochloric acid (specific gravity of 1:18; Fisher Scientific, UK) and 2 ml of demineralized (deionized) water were added to the sample in the digestion liner. The liners were left overnight in a fume cupboard for digestion to take place. Thereafter, the liners were inserted into the pressure vessel and the screw cap closed by hand tightly. The blank digest was also carried out.

One of the sample vessels was sealed using the p/T sensor and placed in rotor position 1. The remaining vessels were evenly spread within the rotor and

transferred into the Antor Paar (Perkin Elmer) Multiwave 3000 microwave reaction system (Figure 3.7) with the lid to cover and fume cupboard on.

Figure 3-7: Multiwave 3000 Microwave Reaction System, (Antor Paar GmbH, Graz, Austria)

At the end of the digestion, filtration of each of the samples was carried out using Whatman 542 filter paper into 100-ml volumetric flask. The liners and the seals were rinsed with demineralized water with the filtrate made up to 100-ml volumetric mark. The filtrate was transferred into bottles and kept in the refrigerator (6^{o}C) prior to analysis.

On removal from the refrigerator, the samples were left for 30 min before usage. Five millilitres of each of the working standard (0, 1, 2, 3, 4, 5, 6 and 7 µg/ml P) was pipetted into a labelled 100-ml beaker. Twenty millilitres of 0.15% m/v ammonium molybdate reagent and 5 ml of (1.5%) ascorbic acid solution were added, swirled and allowed for colour development (blue colouration) for 2 h. The same procedure was repeated for each of the samples. The absorbance of the standards and the samples were then read at 880 nm using the Thermo Electron Corporation, Nicolet Evolution 100 UV/Visible spectrophotometer (Figure 3.8).

Figure 3-8: Nicolet Evolution 100 UV/Visible Spectrophotometer, (Unicam Limited, UK)

3.2.3 Microbial population determination

3.2.3.1 Samples and serial dilutions preparation

For the determination of the microbial population of the growing media/composts for the bioremediation experiment in Chapter 4, 1 g of each of the John Innes No. 2 compost and spent *Pleurotus ostreatus* mushroom substrate was separately taken and suspended in a universal bottle containing 9 ml of sterile reverse osmosis water supplemented with TWEEN® 80, SigmaUltra. The samples were vortexed for 10 min to extract the microorganisms resulting to one in 10 dilution (10^{-1}). Serial dilutions were made by adding 1 ml of 10^{-1} dilution into another universal bottle containing 9 ml of the initially prepared sterile reverse osmosis water to give one in 100 dilution (10^{-2}). The procedure was repeated until a 10^{-5} dilution was achieved.

3.2.3.2 Media preparation

For fungal growth, Malt Extract Agar (Oxoid Ltd., England) was prepared following the manufacturer's instructions with the addition of a small amount of the antibacterial agent - chloramphenicol (Fisher Scientific, UK) while half-strength Nutrient Agar (Oxoid Ltd., England) with the small addition of cyclohexamide (Sigma, USA) as an antifungal agent, was used for culturing bacteria. The media were autoclaved at 126^{o}C, allowed to cool, and poured into 90 mm triple vent (3V) petri dishes.

Each dilution (100 µl) was spread onto the prepared agar plates for microbial counts using a sterile spreader and incubated at 25^{o}C. After the incubation period, the Gallenkamp Colony Counter (Figure 3.9) was employed for counting the colonies and the first countable dilution was used for the calculation using the following expression:

$$\text{Colony Forming Units (CFU/g compost)} = \text{Average Number of Countable Colonies} \times 10 \times \text{Dilution factor}$$

Figure 3-9: Gallenkamp Colony Counter

3.2.4 Soil hydrocarbons analysis

3.2.4.1 Artificial contamination of growing medium with the crude oil

From literature, the determination of the effect of crude oil contamination on plants has involved artificial contamination with the soil by uniform mixing or application at a uniform rate to the surface of the soil (De Jong, 1980). Hence, the crude oil was thoroughly mixed with the soil to achieve homogeneity (Merkl et al., 2004; Pérez-Hernández et al., 2013) using the modified method of Brinch et al. (2002) for spiking soil samples with organic compounds. In this, the required amount of crude oil is added to 25% of the total soil sample mixed appropriately and then added to the remaining 75% of the soil with thorough mixing to achieve a homogeneous sample. This procedure was used as the initial dissolution of hydrophobic compound in organic solvents before spiking

may present some form of solvent effects (Brinch et al., 2002) on the microbial population of the growing media/composts.

3.2.4.2 Extraction of petroleum hydrocarbons

According to Brassington (2008), the ultrasonic extraction method reduces time, cost and solvent usage with extraction efficiencies of ≥ 95% capability. Consequently, the modified sequential ultrasonic solvent extraction technique of Risdon et al. (2008) was employed for the extraction of petroleum hydrocarbons.

The growing medium/compost (5 g) was initially dried with 5 g of anhydrous sodium sulphate (Fisher Scientific, UK) in a 50-ml centrifuge tube. This was followed by the addition of 4 ml of acetone (Fisher Scientific, UK) and sonication for 2 min at 20^{o}C. Acetone (6 ml) and 10 ml of hexane (Fisher Scientific, UK) were added to the samples and sonicated for 10 min. The solvent and the growing media/composts were mixed, centrifuged for 5 min at 3,000 rpm while the supernatant was passed through Supelco Discovery® DSC-Sci 3-ml SPE tube (Sigma-Aldrich, USA). This was followed by a sequential extraction step involving re-suspension of the samples in 10 ml of acetone/hexane (1:1) mixture, sonication for 15 min at 20^{o}C, centrifugation for 5 min at 3,000 rpm and decantation into the SPE tubes. The mixture of acetone/hexane (1:1) was then used to make the final extract volume to 50 ml and homogenized by manual shaking. 0.1 ml of 5 µg/ml PAHs internal standard, 0.2 ml of 10 µg/ml alkane internal standard and 0.7 ml of the sample extract were then added in 2-ml Agilent GC glass vial and crimp capped with polytetrafluoroethylene (PTFE) seal for the GC/MS analysis. Figure 3.10 shows some of the stages involved in the sequential extraction process prior to the GC/MS analysis.

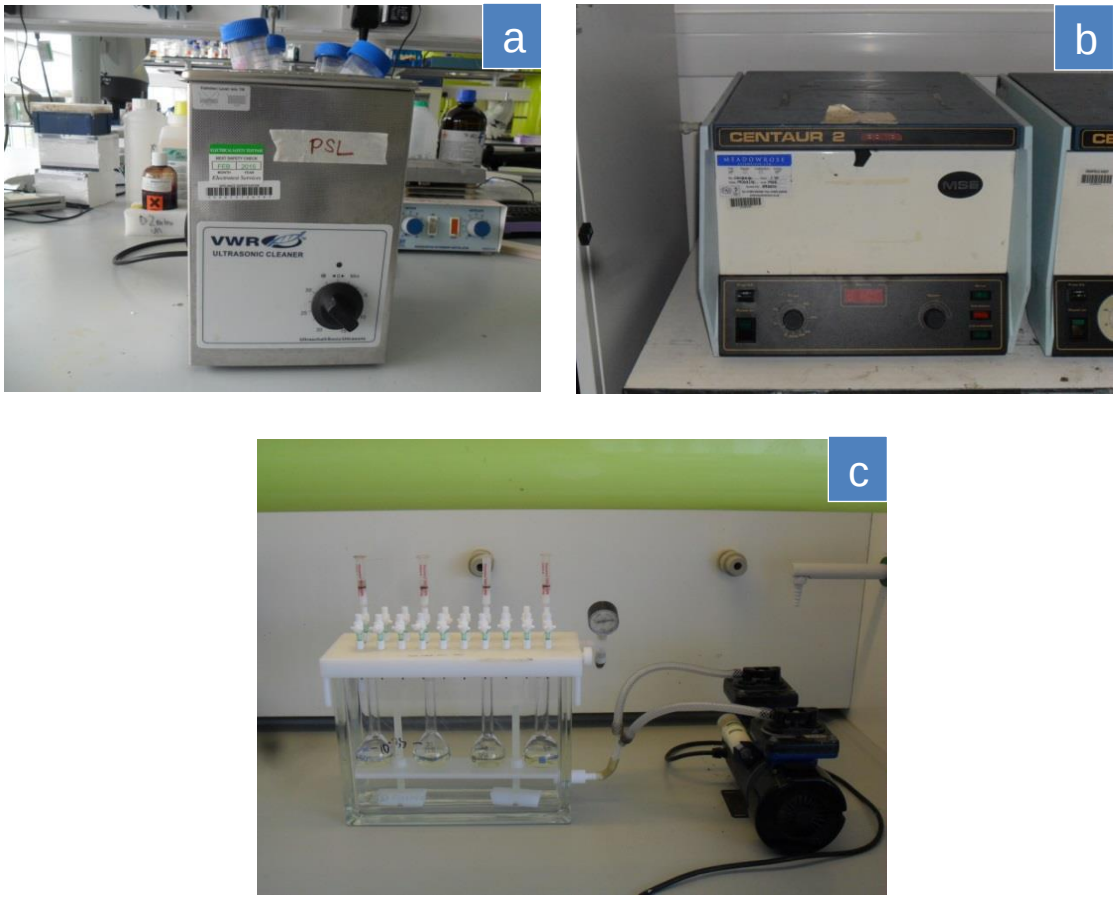


Figure 3-10: Some stages in the soil hydrocarbons' sequential extraction process prior to the GC/MS analysis: (a) Sonication, (b) Centrifugation, and (c) SPE vacuum extraction

3.2.4.4 Limits of detection and quantification

The Limit of Detection (LOD) is defined as the lowest concentration or weight of an analyte (chemical constituent of interest) that can be measured at a specific confidence level. Whereas, the Limit of Quantification (LOQ), referred to as Limit of Quantitation by Mitra (2003), is the lowest concentration level at which a measurement is quantitatively meaningful (Mitra, 2003). The LOD and LOQ were calculated as three and ten times the signal/noise ratio respectively (Liguori et al., 2006; Rubert et al., 2013).

In practice, ten injections of the least concentration of the standards 0.01 µg/ml and 0.5 µg/ml for the PAHs and alkane respectively were made into the GC/MS and quantified. The standard deviation obtained from the quantification of the results was multiplied by 3 and 10 to give the LOD and LOQ respectively.

3.2.4.5 Hydrocarbons instrumental analysis

The quantification of the alkanes (C_{10} - C_{36}) and US EPA priority PAHs (essentially phenanthrene and anthracene) in the extracted soil samples was carried out using the Agilent Technologies 6890N Network GC system coupled to Agilent Technologies 5973 Network mass selective detector operated at 70 eV in positive ion mode. The column used was Phenomenes ZB-5HT (30 m × 0.25 mm internal diameter, film thickness of 0.10 µm). Manual injection in splitless mode with a sample volume of 3 µl was applied. The oven temperature was increased from 60^{o}C to 220^{o}C at 20^{o}C/min then to 310^{o}C at 6^{o}C/min using the full scan mode (range m/z 50 - 500) for quantitative analysis of the target alkanes and PAHs. Quantification of each compound was carried out by integrating the peak at specific m/z using the internal standard method. A calibration solution mix was used in carrying out a priori 5-level calibration. C_8 - C_{40} alkanes calibration standard with alkane internal standard solutions of Decane-d_{22} and Nonadecane-d_{40} (Sigma-Aldrich Company Ltd., UK) were used in making the alkanes calibration solution mix while EPA 525 PAH Mix A standard solution (Sigma-Aldrich Company Ltd., UK) and the PAHs internal standards of Naphthalene-d_8, Anthracene-d_{10}, Chrysene-d_{12} and Perylene-d_{12} solutions from the same manufacturer were used for the PAHs calibration solution mix. The soil extract samples, the blank and calibration standards were thereafter analysed using the Agilent Technologies gas chromatography-mass spectrometer (GC/MS) (Figure 3.11).

Figure 3-11: Agilent Technologies GC/MS

3.2.5 Volatiles analysis

Headspace samples were prepared for the volatiles analysis involving *Brassica juncea* in Nalophan® bags filled with zero grade air and incubated at an average temperature of 26^{o}C for 10 min. A 500 ml volume of the resulting headspace was drawn into a previously conditioned thermal-desorption (Tenax TA 35/60, Carbotrap 20/40) tube. The plants were place in the Nalophan® bags (Figure 3.12b) before the collection of the volatile samples at a flow rate of 100 cc/min for 5 min with the use of GilAir Plus air sampling pump (USA). A travel blank sample was also collected following the same procedure (see Figure 3.12a).

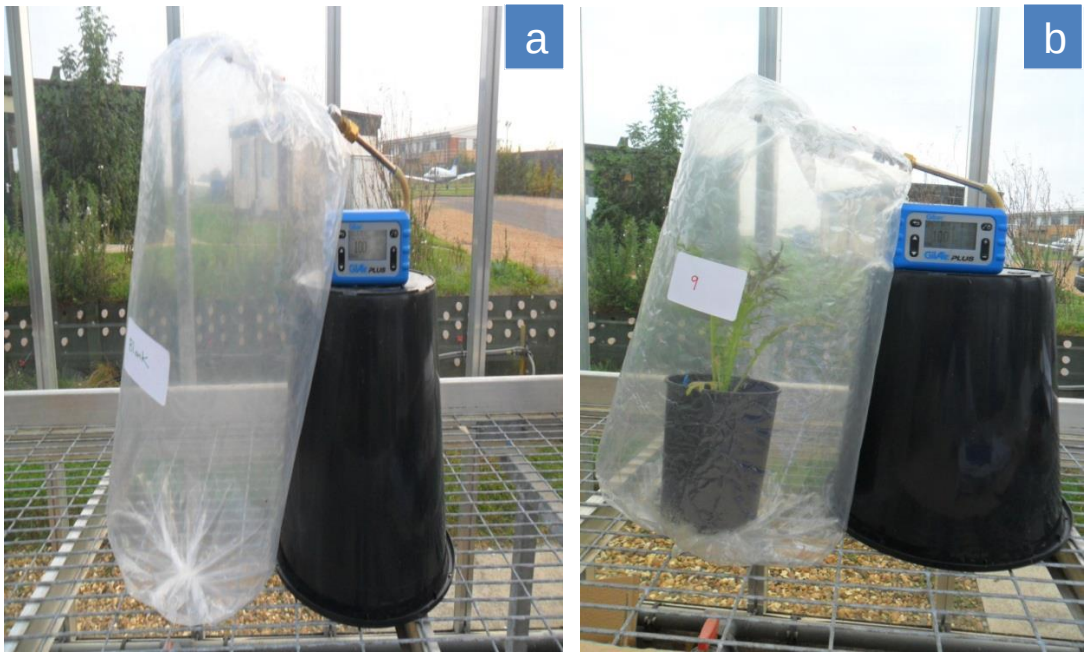


Figure 3-12: Drawing of 500ml volume of headspace from: (a) The travel blank, and (b) The plant sample

The collected volatile samples were thereafter taken to the laboratory with the preparation of two additional thermal desorption tubes (for the blank and the internal standard). Following this, an internal standard solution containing 50 ng d8-toluene (Supelco Cat No. 48593) in methanol was added to each thermal desorption tube according to the manufacturer's instructions (Markes International Ltd, Llantrisant, UK). Headspace samples were analysed by ToF-GC-MS. Analytical instrumentation comprised a Series 2 Unity thermal desorber with Series 2 Ultra autosampler connected to an Agilent model 7890 gas chromatograph and ALMSCO Bench-ToF-GC-MS (Markes International, Llantrisant, UK) (Figure 3.13). The gas chromatograph was fitted with a DB5 column of dimensions 60 m x 0.4 mm x 0.25 mm (Agilent Technologies UK Limited, Stockport, UK) while the carrier gas was CP grade helium (BOC gases, Guildford, UK) passed through a combined trap for removal of hydrocarbons, oxygen and water vapour.

Figure 3-13: The Markes/Agilent TD/GC/TOF-MS

Thermal desorption (TD) tubes were initially purged for 1 min in order to remove air and water vapour then desorbed at 300°C for 8 min onto the secondary cold trap (Materials emission trap, U-T12ME-2S) which was initially maintained at -10°C. Once desorption was complete, the secondary trap was heated at the maximum available rate to 300°C and maintained for 3 min whilst the effluent was transferred to the GC via a transfer line maintained at 150°C. Constant-flow operation was used, at 1.2 ml/min. Initial GC temperature was 35°C held for 1 min, then increased at 2°C/min to 75°C, 5°C/min to 140°C and 10°C/min to 300°C where it was held for 10 min. The eluted products were transferred to the MS via a line maintained at 200°C where they were subjected to electron ionisation, the ion source temperature being maintained at 200°C. The MS was operated from 33 – 480 amu at an effective scan rate of approximately 2.2 Hz.

3.2.6 Greenhouse experimental design

The planting of the crops was done at the Cranfield University Research Glasshouse Facility, Cranfield, United Kingdom. For the experiment involving the influence of the selected bioremediation strategies on hydrocarbons

degradation and plant yield, Pöpplemann TEKU 5 litres pots capable of containing 2 kg soil-based compost were used while standardized John Innes Number 2 compost was used as the potting/growing medium. The experimental design - completely randomized design (CRD) - involved the preparation of four different treatments in which two incorporated the bioremediation strategies. Further details on the composition of each of this treatment are provided in Chapter 4, Section 4.3.1.

For the experiment involving the CRIV, 10 plant seeds were sown into three different pots to make a replicate. Three replicates of the individual treatment (control inclusive) were then distributed in a Randomized Complete Block Design (RCBD) as it is one of the widely used complete block design in agriculture (Raza and Masood, 2009). The plants in each of the pots were thinned to one plant for the *B. juncea*, *B. oleracea* and *L. sativa*. The transplanting of the two cultivars of *S. lycopersicum* also involved one plant per pot while all the pots were watered as recommended in agronomic practices. Collection trays were placed beneath each pot in order to retain leachate and were re-applied immediately to the same pot. The plant growth parameters were measured throughout the planting period followed by laboratory analyses of the harvested agricultural products. Further details of the experimental design involving the effect of the different CRIV on the leafy vegetables and tomatoes are provided in Chapter 5 (Section 5.3.1) and Chapter 6 (Section 6.3.1) respectively.

3.2.6.1 Growth parameters and postharvest measurements

In the course of the greenhouse experiments, different growth parameters were measured. For the green leafy vegetables and tomatoes, the average plant height, average number of leaves ≥ 2.0 cm (weekly), stomatal conductance using the Leaf Porometer Model SC-1 (Decagon Device, Inc., USA), percentage seed germination and number of fruits produced were recorded as applicable.

At the point of harvesting of the tomatoes, total number of fruits per plant, total fruits mass from each pot treatment as well as other immediate

postharvest measurements such as the colour and firmness were measured. The Chroma meter CR - 400 (Konica Minolta Sensing, Inc., Japan) calibrated on a white plate was used in evaluating the chroma (C*) (saturation), hue angle (h^o) (colour) (Kim et al., 2008a) and lightness (luminescence) of the harvested samples while the Instron Bluehill® 2 (System ID Number 5542K3233; assembled in USA) equipment was employed for the firmness determination. In addition, the total soluble solids (TSS) of the harvested tomatoes were measured as typically done in industries using the ATAGO® Palette Digital Refractometer PR - 32 α (Brix 0 ~ 32%) (ATAGO Co. Ltd., Japan).

At the end of each of the experiment, the above-ground plant parts of each plant were excised at their base while the roots were removed from the growing medium and washed (Merkl et al., 2005) with tap water as well as rinsed repeatedly with deionized water (Langer et al., 2010). The above-ground plant parts weight and fresh root weights for all the harvested plants in the experiment involving the effect of different CRIV on the selected leafy vegetables were also measured. Figure 3.14 shows the various measuring instruments adopted in the parameters' assessment.

Figure 3-14: Growth and postharvest parameters measuring instruments: (a) the leaf porometer, (b) the chroma meter, (c) the palette digital refractometer, and (d) the Instron Bluehill® 2 system

3.2.6.2 Harvesting and plant samples preparation

Immediately after harvesting the plants, similar ways of preparing vegetables for consumption were followed (Wennrich et al., 2002) with other forms of preservation treatments required for the experimental analysis. Also, in order to overcome the possible 'concentration effect', as plants under some stress conditions have been found to have lower water content (Wang and Frei, 2011),

and to effectively compare the nutritional/phytochemical content as well as active accumulation of these components (Krauss et al., 2006) in the harvested agricultural produce, especially in the tomatoes, the quantitative chemical assessments were carried out on a dry weight basis. To achieve this, these samples were first thoroughly washed with water to remove soil and surface particles followed by snap-freezing with liquid nitrogen. Thereafter, they were freeze-dried for 7 d, ground into powder and kept at -40^{o}C prior to chemical analyses. The stages involved in the preparation of the harvested plant samples prior to instrumental analysis are shown in Figure 3.15.

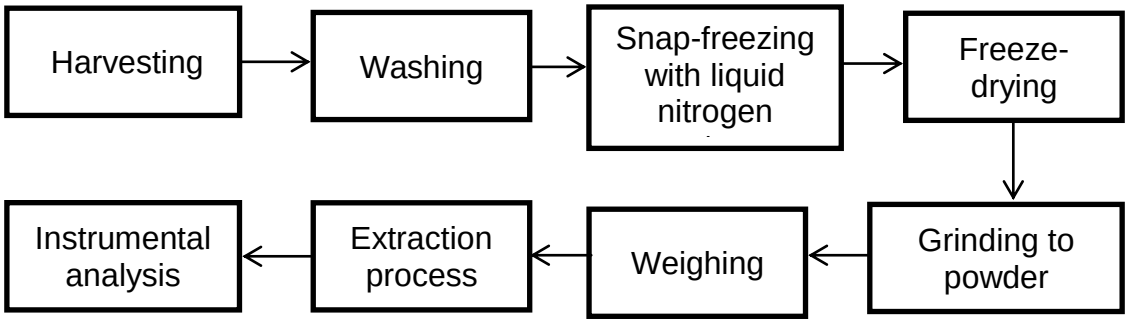


Figure 3-15: Stages involved in the preparation of the harvested plant samples prior to instrumental analysis

3.2.6.3 Rationale behind the assays taken on the harvested agricultural products

As mentioned in Chapter 2, the production of plant foods (like vegetables) containing essential nutrients and phytochemicals (Rodriguez et al., 2006), can be affected by abiotic stresses (Cramer et al., 2011; Kumar et al., 2014). Although the two classes of vegetables (leafy and fruit vegetables) considered in this study have high concentrations of: digestible carbohydrates (such as fructose, glucose and sucrose) (Butnariu, 2007, cited in Caunii et al., 2010, p. 45), vitamins (Taura and Habibu, 2009), some major and minor minerals (Caunii et al., 2010), their composition, such as the vitamin (Fennema et al., 1996), mineral (Belitz, 2004; Szefer and Nriagu, 2007; Martinez-Ballesta, 2010)

including the phytochemicals (Tiwari and Cummins, 2013) produced in relatively small amounts (Rodriguez et al., 2006), can be affected by the environmental growing conditions.

Furthermore, two of the selected leafy vegetables, *B. juncea* (Kumar et al., 1995; Nouairi et al., 2006; Sharma et al., 2010) and *L. sativa* (Boon and Soltanpour, 1972, cited in Jordão et al., 2013, p. 765) have been linked with phytoremediation potential as a result of their ability to accumulate heavy metals. Meanwhile, owing to the high levels of sugars and organic acids in tomato total as well as soluble solids, studies on the quality of tomato fruits have mostly been on these compounds (Young et al., 1993).

Hence, as crude oil stress like every other form of stress is capable of inducing a negative effect on these vegetables, the various assays carried out were used in examining the responses of these plants under different level of crude oil contamination.

3.2.7 Analysis of agricultural produce

3.2.7.1 Nutritional composition analysis

3.2.7.1.1 Extraction and quantification of non-structural carbohydrates (sugars)

Modified methods of O'Donoghue et al. (2004) and Chope et al. (2007) were used for the extraction and quantification of sugars. As chromophores and fluorophores are not found in carbohydrates (Chaplin and Kennedy, 1986, cited in Shanmugavelan, 2013, p. 112), the Evaporative Light Scattering Detector (ELSD) was preferred for detection since all particles are able to exhibit photon scattering (Shanmugavelan et al., 2013). This detector also has a higher sensitivity compared to the typical detection involving the use of refractive index (Terry et al., 2005; Chope et al., 2007).

For the extraction and quantification, 3 ml of High Performance Liquid Chromatography (HPLC) grade methanol: water (62.5: 37.5; v/v) mixture was added to 150 mg of the freeze-dried vegetable samples in a 7-ml vial. The vial was then placed in a low speed shaking water-bath (Figure 3.16) at 55^{o}C for 15 min and agitated every 5 min during the water-bath extraction for 20 s to prevent layering. The samples were thereafter allowed to cool and filtered

through Cronus 25 mm PTFE 0.2 µm syringe filter. The extracts obtained were stored at -40^{o}C until required. Prior to immediate HPLC analysis, a ten-fold dilution was made with the vegetable extract using HPLC grade water (1:9, df = 10).

Figure 3-16: Bath and Shaker (Nickel-Electro Ltd, England)

The obtained extract filtrate (20 µl) was then injected into the Agilent Technologies 1260 Infinity HPLC system and ELSD (Agilent Technologies, Germany) (Figure 3.17) for the analysis which involved the use of GRACE Prevail carbohydrate ES 5µ (250 mm × 4.6 mm, Serial number: N1450709 and Part number: 35101) column.

The mobile phase was HPLC grade water and acetonitrile at a gradient elution from 20:80 (%) to 50:50 (%) and run at a flow rate of 1.0 ml/min as well as column temperature of 30^{o}C. For the ELSD, nitrogen was used as the nebulizing gas at a pressure of 3.5 bar and drift tube temperature of 40^{o}C. The sugars concentrations in the extract were then quantified against the calibration standards of glucose (Fisher Scientific, UK), fructose and sucrose (Sigma-Aldrich, USA) within the range of 0.05 mg/ml to 2.50 mg/ml. The LOD and LOQ as explained in section 3.2.4.4 using the least concentration of the standard were also calculated.

Figure 3-17: Agilent Technologies 1260 Infinity HPLC system and ELSD

3.2.7.1.2 Determination of protein content

Based on available equipment, the Dumas method (which is an alternative method to the Kjeldahl method) was employed in evaluating the total nitrogen content (g/100g) in the harvested plant samples. The Dumas (combustion) method (Simonne et al., 1997) has the advantage of evaluating all the forms of nitrogen in the sample (Mariotti et al., 2008) and according to Simonne et al. (1997), gives a true reflection of total nitrogen. However, the classical Kjeldahl method, which involves wet chemistry (Simonne et al., 1997), is used as a reference method for the determination of nitrogen content in foods (Wiles et al., 1998; Mariotti et al., 2008) as well as crude protein assessment (Simonne et al., 1997).

The Dumas nitrogen determination of these samples was carried out as explained in Section 3.2.2.8 but with the use of freeze-dried plant samples. In order to convert the Dumas nitrogen (DN) value to the Kjeldahl nitrogen (KN) and to avoid the possible overestimation of nitrogen as well as crude protein by the Dumas method (Simonne et al., 1997), the mean KN: DN ratio of 0.89 ± 0.28 (mean ± S.D) obtained for vegetables and vegetable products by Simonne et al. (1997) was used for the adjustment.

On the other hand, as a result of the inaccuracy of the traditional method of using a nitrogen-to-protein conversion factor of 6.25 in multiplying the nitrogen content of food as a measure of the protein content (Salo-väänänen and Koivistoinen, 1996; Mariotti et al., 2008), Salo-väänänen and Koivistoinen (1996) suggested that the analysis of protein content of foods should be done either by direct quantitative analysis of the amino acids present in the food or indirectly by the use of appropriate nitrogen-to-net (true) protein conversion which takes into consideration the group which the food of interest belongs. Hence, the obtained nitrogen content from the previous conversion of the DN to KN was multiplied by the average specific conversion factor of 4.40 for vegetables, mushrooms, and leaf proteins as indicated by Mariotti et al. (2008) (instead of the traditional 6.25) to express the nitrogen content in the harvested vegetable samples as a measure of protein content. Consequently, the following expression was used in calculating the crude protein content in the harvested above-ground plant parts:

$$\text{Crude Protein Content} = \text{Dumas nitrogen} \left(\frac{\text{g}}{100\text{g}}\right) \times (0.89 \pm 0.28) \times 4.40$$

3.2.7.1.3 Minerals (metals) analysis

Soil samples

As a result of the use of aqua regia, the Atomic Absorption Spectrometer (AAS) was employed for the quantification of the metals in the soil samples (particularly Al, Cd and Pb) while P was analysed using the colorimetric method as explained in section 3.2.2.9.

For these metals' (i.e. Al, Cd and Pb) determination, the SOP used was based on the US EPA Method 3051 and British Standard BS 7755: Section 3.13:1998 which is identical to ISO 11047:1998. In this, 0.5 g ± 0.001 g of each of the soil sample was weighed in a labelled microwave digestion liner with the addition of 6 ml of hydrochloric acid (specific gravity of 1.18; ~ 37%) and 2 ml of nitric acid (specific gravity of 1.42; 70%) (Fisher Scientific, UK) using another dispenser. An empty liner without the addition of sample was used as the blank while NIST 1515 Apple leaves standard reference material was also similarly

treated. All the liners were left to digest overnight in the fume cupboard at room temperature.

Thereafter, the liners were inserted into the pressure vessel with the screw cap hand-tight into the Antor Paar (Perkin Elmer) Multiwave 3000 microwave reaction system (Figure 3.7) with the lid to cover and fume cupboard on. After the digestion and cooling down of the samples, Whatman 541 filter paper was used in collecting the filtrate obtained in separate 100-ml volumetric flask. Rinsing of the liner and seals down with deionised water were also done as necessary while also making up to volume with deionised water. The filtrates were then transferred into 150-ml transparent bottles and taken to the AAS laboratory for analysis.

The PerkinElmer Atomic Absorption Spectrometer (AAnalyst 800) (Figure 3.19b) was used for the determination. In this, the samples were aspirated into the flame where light of a particular wavelength is allowed to pass through the flame which gives the concentration of the desired metal in the sample. Appropriate standard solution range for each of the metal analysis was also prepared involving similar acid matrix as the sample. Table 3.1 illustrates the different wavelength used for the analysis of the metals by the AAS.

Table 3-1: Wavelengths used for the AAS metals analysis

Metal	Wavelength used (nm)
K	766.5
Fe	248.3
Al	309.3
Cd	228.8
Pb	283.3

Plant samples

Dry mass (0.5 g ± 0.1 mg) of each of the above-ground plant parts and root samples was weighed into the microwave digestion liner. Empty liner without the addition of sample was used as the blank. Five millilitres of nitric acid was

added to each of the microwave digestion liner in a fume cupboard and left uncovered overnight to digest at room temperature. Five millilitres of hydrogen peroxide solution (100 volumes >30% w/v) (Fisher Scientific, UK) was thereafter added and the samples left to stand at room temperature for 2 h.

Final digestion of the samples was carried out using the Microwave Accelerated Reaction System (Model MARS®, CEM Corporation, USA) (Figure 3.18) while the National Institute of Standards and Technology (Gaithersburg, MD, USA) NIST 1515 Apple Leaves Standard Reference Material (SRM) was also similarly treated to assess the suitability (Kawashima and Soares, 2003) and accuracy of the method used (Fadigas et al., 2010).

Figure 3-18: Microwave Accelerated Reaction System (Model MARS®, CEM Corporation, USA)

After the digestion process and subsequent cooling of the samples, the preparation of the filtrate in a similar manner to that of soil samples was carried out, however, the filtrate obtained were transferred to the Inductively Coupled Plasma - Mass Spectrometer (ICP-MS) laboratory for analysis using Perkin-Elmer Elan 9000 ICP-MS (USA) as shown in Figure 3.19a.

For the ICP-MS analysis, 10 ml of the filtrate was pipetted into 15-ml centrifuge tube and 100 µl of the internal standard, rhodium (5 ppm), was added to each of the sample with appropriate shaking.

On the other hand, as a result of the high concentration of K in the plant and root samples, the AAS was used for the K determination in the harvested plant samples. The same instrument (AAS) was also employed for the analysis of the Fe content in the plant samples to avoid possible interferences that may occur through the use of argon plasma in the ICP-MS.

Figure 3-19: Instruments used for the minerals (metals) analysis: (a) The ICP-MS, and (b) The AAS

The expression of Ghosh and Singh (2005), Abdu et al. (2011) as well as Ondo et al. (2013) for assessing the bioconcentration factor (BCF) was used as an indicator of the ability of the leafy vegetables used in this study to accumulate Al, Cd and Pb as a function of the metal concentration in the different soil treatments. This is also referred to as metal transfer factor (Abdu et al., 2011; Ondo et al., 2013) and calculated as:

$$\text{Bioconcentration factor (BCF)} = \frac{\text{Concentration of metal in plant tissue } (\frac{\text{mg}}{\text{kg}})}{\text{Concentration of metal in soil } (\frac{\text{mg}}{\text{kg}})}$$

Also, the transportation index (T_i) showing the ability of the plant to translocate metals from the roots to the leaves was evaluated following the expression of Ghosh and Singh (2005):

$$\text{Transportation index} = \frac{\text{Concentration of metal in plant leaves } (\frac{\text{mg}}{\text{kg}})}{\text{Concentration of metal in plant roots } (\frac{\text{mg}}{\text{kg}})} \times 100$$

3.2.7.2 Phytochemicals composition analysis

3.2.7.2.1 Determination of total phenolics content

The Howard Davies extraction method was used for extracting the total phenolics present in the harvested above-ground plant parts. In this, 150 ± 0.5 mg of the freeze-dried powdered sample was weighed in a 7-ml vial with the addition of 3 ml of ethanol (HPLC grade; Fisher Scientific, UK): water (80:20, v/v). After adequate mixing, the samples in the vials were placed in a shaking water bath at 70^{o}C for 2 h and removed every 30 min for 20 s vortexing. Then, the samples were allowed to cool and filtered through Cronus 25 mm PTFE 0.2 µm syringe filter.

Sodium carbonate (Fisher Scientific, UK) (250 mg/ml) was prepared using 100% deionised water involving adequate stirring and filtration via Whatman 1 qualitative filter paper. The calibration standard of 5.0 mg/ml gallic acid (Sigma-Aldrich, USA) was also prepared using ethanol: water (10:90, v/v) from which other calibration standard concentrations of: 0.05, 0.10, 0.15, 0.25 and 0.50 mg/ml were prepared.

Thereafter, for the quantification of total phenolic content in the samples, 20 µl of the gallic acid standard or sample filtrate was measured in a cuvette with the addition of 3.2 ml of deionised water, 200 µl of Folin and Ciocalteu's phenol reagent (Fisher Scientific, UK) and 600 µl of the previously prepared sodium carbonate solution. The sample blank (20 µl) was also similarly treated. All of these were mixed in the cuvette and left in the dark for 2 h at room temperature for the colour to develop. The absorbance was then measured at 765 nm with

the use of Helios Gamma UV/Visible spectrophotometer (Unicam Limited, UK) (Figure 3.20). The concentration of the total phenolics in the harvested samples was then calibrated against gallic acid.

Figure 3-20: Helios Gamma UV/Visible spectrophotometer (Unicam Limited, UK)

3.2.7.2.2 Determination of total flavonoids content

For this analysis, the previously explained Howard Davies extraction method in section 3.2.7.2.1 (for the total phenolics determination) was adopted for the extraction of the total flavonoids in the freeze-dried powdered sample using a similar amount of 150 ± 0.5 mg.

Following this, 40 mg/ml of sodium hydroxide (Fisher Scientific, UK) was separately prepared using the solvent mixture of ethanol: water (50:50, v/v) with proper mixing. The calibration standard of quercetin (Sigma-Aldrich, USA) was also similarly prepared within the range of 1.0 mg/ml and 0.005 mg/ml.

Thereafter, for the quantification of total flavonoids in the samples, 100 µl of the quercetin standard or sample filtrate was measured in a cuvette with the addition of 3 ml of the previously prepared sodium hydroxide solution. The samples were mixed in the cuvette and left for 10 min at room temperature for colour development and the absorbance subsequently measured at 420 nm using the spectrophotometer (Figure 3.20). The sample blank (100 µl) substituted for the quercetin standard or sample filtrate was also used.

Calibration against the quercetin standards was then carried out to determine the total flavonoid concentration in the above-ground plant parts.

3.2.7.2.3 Extraction and quantification of non-volatile organic acids

The modified method of Terry et al. (2007) was used in the extraction and quantification of organic acids present in the above-ground plants parts. In this, 300 ± 0.5 mg of the individual freeze-dried powdered sample was weighed into a 7-ml vial with the addition of 3 ml of HPLC grade water. The slurry was allowed to stand at room temperature (25^{o}C) for 5 min followed by vortexing for 30 s. In order to ensure easiness of the filtration process, the samples were centrifuged at 4000 rpm for 10 min at 4^{o}C. Thereafter, filtration using the Cronus 25 mm PTFE 0.2 µm syringe filter was carried out. An appropriate amount of the filtrate obtained was dispensed into HPLC vials and kept at -40^{o}C prior to analysis.

The vegetable extract obtained (20 µl) was then injected into Alltima HP C18 AQ 5µ (GRACE) 250 mm × 4.6 mm (Serial number: 614110890 and Part number: 87820) column with OPTI-GUARD® 1 mm guard column. Analytical grade of 25 mM KH_2PO_4 (Fisher Scientific, UK) in HPLC grade water was used as the mobile phase while its pH was adjusted to 2.5 using phosphoric acid (Acrōs Organics, USA). The separation was achieved using Agilent Technologies 1200 series HPLC system (Germany) (Figure 3.21) at isocratic conditions for 10 min at a flow rate of 1.5 ml/min and column temperature of 35^{o}C. At a wavelength of 210 nm, the eluted organic acids were detected by means of a photodiode array detector (DAD Serial No. DE60556253, Agilent Technologies).

Thereafter, the desired non-volatile organic acids concentrations in the samples' extracts were then quantified against the calibration standards of citric acid (Fisher Scientific, UK), L(+) tartaric acid, L-ascorbic acid (vitamin C) and L(-) malic acid (Sigma-Aldrich, USA) within the range of 0.025 mg/ml and 1.0 mg/ml by comparing their peak areas using the ChemStation rev.B.04.01 software. The 0.025 mg/ml standard was used in deducing the LOD and LOQ

(Section 3.2.4.4) while Figure 3.21 shows the Agilent Technologies 1200 series HPLC system used for the organic acids analysis in the samples.

Figure 3-21: Agilent Technologies 1200 Series HPLC System (Germany)

3.2.7.2.4 Quantification of chlorophyll and carotenoids contents

The modified method of Mackinney (1941), Arnon (1949), and Ghani (2011) was used in quantifying the chlorophyll a, chlorophyll b and total carotenoid contents of the green leafy vegetables' samples, in semi-darkness (Wellburn, 1994). For this determination, 200 mg of the freeze-dried and fine ground samples were extracted with 3 ml of acetone (Fisher Scientific, UK): HPLC grade water (80:20, v/v) solvent mixture with agitation for 2 min at room temperature. Thereafter, 4 ml of the extraction solvent mixture was added followed by vortexing for 2 min before filtration. The equations of Wellburn (1994) for spectrophotometers with the resolution range of 1 – 4 nm were then used for the determination of chlorophyll a (Chl_a), chlorophyll b (Chl_b) and total carotenoids (C_{x+c}) contents of the obtained extract (in µg/ml) using 10 mm LP optical glass sample cell (HACH company, Loveland).

$$Chl_a\ (\mu g/ml) = 12.21A_{663} - 2.81A_{646}$$

$$Chl_b\ (\mu g/ml) = 20.13A_{646} - 5.03A_{663}$$

$$C_{x+c}\ (\mu g/ml) = (1000A_{470} - 3.27C_a - 104C_b)/198$$

Where A_{470}, A_{646} and A_{663} are the absorbance of the extract measured at three different wavelengths of: 470 nm, 646 nm and 663 nm respectively.

3.2.7.2.5 β-Carotene and lycopene determination

The extraction method of Nagata and Yamashita (1992) as used by other authors (Guimarães et al., 2013; Vinha et al., 2014) was employed for the determination of β-carotene and lycopene contents of the tomato samples with their modified equations. The pigments in 1 g of the tomato fruit samples were extracted with 20 ml of acetone-hexane mixture (4:6, v/v) by vigorous shaking for 60 s followed by filtration. The absorbance of the filtrate obtained was then measured at four different wavelengths: 453 nm, 505 nm, 645 nm and 663 nm using glass cuvettes on the Helios Gamma UV/Visible spectrophotometer (Figure 3.20).

The pigments content were calculated using the following equations:

β-carotene (mg/100ml) = $0.216 \times A_{663} - 1.220 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$;

Lycopene (mg/100ml) = $-0.0458 \times A_{663} + 0.204 \times A_{645} - 0.304 \times A_{505} + 0.452 \times A_{453}$

The obtained pigments content were then further expressed in mg per 100 g of sample with A_{453}, A_{505}, A_{645} and A_{663} being the absorbance at the corresponding wavelength respectively.

3.2.5.3 Bio-accumulated PAHs extraction and analysis

Among the considered attempts for the extraction of PAHs from the harvested vegetables samples which included the use of Soxhlet extraction apparatus and coffee filters (Figure 3.22), the modified method of Orecchio (2007) was adopted based on its reported PAHs extraction average recovery of 82%.

One gram of the vegetable samples was extracted with 8 ml of dichloromethane with the use of an ultrasonic bath and filtering through a solvent-rinsed glass wool and anhydrous sodium sulphate. The extraction process was repeated three times after which the vegetable extracts obtained were concentrated with the use of miVac Quattro concentrator (Genevac Limited, UK) at a thermostatic bath temperature of 35^{o}C. The concentrated extracts were then dried under a weak nitrogen flow while the dry residue obtained was dissolved in 0.5 ml solution containing the PAHs internal standards and capped in GC injection vials prior to the GC/MS analysis.

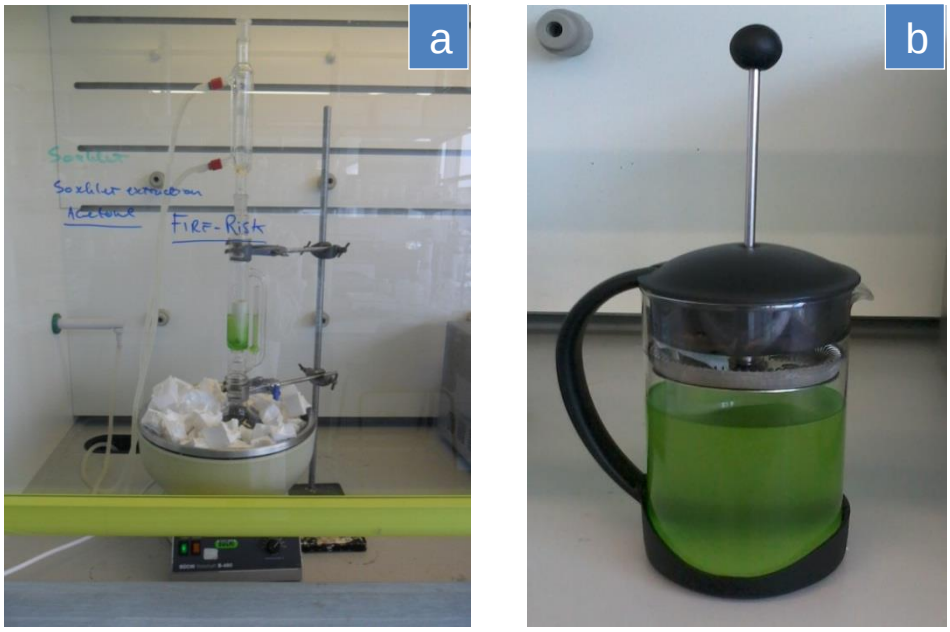


Figure 3-22: The other approaches considered for the extraction of bio-accumulated PAHs from the harvested plant samples: (a) Soxhlet extraction apparatus, and (b) Coffee filters used

CHAPTER 4
EFFECT OF SELECTED BIOREMEDIATION STRATEGIES ON HYDROCARBONS DEGRADATION AND AGRIFOOD PRODUCTION: A MESOCOSM STUDY

4.1 Chapter Summary

The impact of crude oil remediated sites on both agrifood production and the nutritional composition of plants is poorly understood. In this chapter, the efficiency of two selected bioremediation strategies and their agricultural benefits was investigated. The mesocosm study involved: the initial chemical examination of the crude oil and compost/growing media, the preparation of four treatments (planting and remediation-study controls inclusive), and the planting of *Brassica juncea*.

Experimental results indicated that the crude oil used had a pristane/phytane ratio within the range of most crude oils, low concentration of C_{28} – C_{36} alkanes but higher concentrations of two of the US EPA priority PAHs. The pH and moisture content of the soil used for the experiment were within the acceptable level for oil degradation. After 7 d of incubation, Tukey *post-hoc* analysis showed a significant reduction ($p < 0.05$) in phenanthrene concentrations following bioremediation (RENA and bioaugmentation) treatments compared with the remediation-study control whereas anthracene was below the limit of quantification following RENA treatment. Only the *B. juncea* plants from the bioaugmentation treatment showed a significant difference in plant height compared with those from the control treatments after 24 d of planting before a pest attack. One-way ANOVA also revealed that there was no significant difference ($p > 0.05$) in the concentration of major dietary minerals of all the harvested treatment samples. The research findings suggest that RENA performs a dual function during the remediation of petroleum hydrocarbons contamination by favouring the growth of plants without reducing the mineral composition of the agricultural products, with the exception of chromium and zinc.

4.2 Introduction

For years, crude oil spillage has been a recurring experience in different part of the world, especially in oil-producing regions. Records have shown adverse effects of the introduced toxic petroleum hydrocarbons in the environment on living organisms and human health. Consequently, the quest for efficient and economical strategies to break down petroleum hydrocarbons at contaminated sites has led to the development of remediation methods, including bioremediation which involves the use of microorganisms. Among the different bioremediation treatment approaches, natural attenuation, otherwise known as intrinsic or passive bioremediation, is considered when the other options - biostimulation and bioaugmentation - have been found to be too costly or unsuitable (King et al., 1998). Bioaugmentation, in which hydrocarbon-degrading microorganisms are introduced at the contaminated site to enhance the ability of the inherent microbial population to break down contaminants (Venosa and Zhu, 2003; Ogbonna et al., 2007), is often employed for the bioremediation of recalcitrant compounds (Atlas and Philip, 2005) which include PAHs (Eggen and Majcherczyk, 1998). Sometimes, this artificial introduction of microbial consortium may not support bioremediation effectiveness (Cho et al., 1997; Coulon et al., 2012).

On the other hand, Remediation by Enhanced Natural Attenuation (RENA), a land farming treatment (Ebuehi et al., 2005; Onifade et al., 2007b; Wegwu et al., 2010) and bioremediation technology (Ebuehi et al., 2005), has been suggested as a viable option in remediating crude oil-polluted agricultural lands (Wegwu et al., 2010) as it supports the provision of aerobic microbial processes required for the remediation of petroleum hydrocarbons (King et al., 1998).

The goal of this study was to compare these two aforementioned bioremediation strategies, i.e. the use of RENA to break down petroleum hydrocarbons and to also promote agrifood production at crude oil remediation sites with bioaugmentation via the use of improved degradation abilities of *Pleurotus ostreatus* (Bezalel et al., 1996; Okparanma et al., 2011; Zebulun et al., 2011; Sukor et al., 2012) spent mushroom compost (Okparanma et al., 2011). This mesocosm study assessed the effect of these remediation methods

on hydrocarbon degradation and the yield as well as the mineral composition of *Brassica juncea*, a member of the *Brassicaceae* family (Akmal et al., 2011).

4.3 Materials and methods

4.3.1 Mesocosm experimental design

A pot experiment involving the planting of *B. juncea* seeds in a greenhouse was carried out using Pöpplemann TEKU 5 litres growing pots capable of containing 2 kg soil-based compost. The experimental design – completely randomized design (CRD) – involved the preparation of four different treatments with five replicates. The first two treatments, Treatment A and B, were the planting and remediation-study controls respectively while the last two treatments (Treatment C and D) incorporated the selected bioremediation strategies as follows:

Treatment A: 2 kg of soil-based compost. This is the John Innes No. 2 compost without the crude oil contamination - used as the planting control.

Treatment B: 2 kg of soil-based compost + 0.5% w/w crude oil. This is the remediation-study control involving the John Innes No. 2 compost with crude oil contamination but without any form of remediation strategy.

Treatment C: 2 kg of soil-based compost + 0.5% w/w crude oil + RENA application. This treatment involved the John Innes No. 2 compost, crude oil contamination and RENA application in which aeration of the treatment is provided via weekly tillage by full-depth mixing of the pot contents. It is also referred to as the RENA treatment.

Treatment D: 2 kg of soil-based compost + 0.5% w/w crude oil + bioaugmentation. In this, the bioaugmentation of the John Innes No. 2 compost is achieved by the introduction of spent *Pleurotus ostreatus* mushroom substrate via uniform full-depth mixing of the pot, followed by the crude oil contamination. This treatment is likewise referred to as the bioaugmentation treatment.

The use of 0.5% crude oil in soil is based on the need to obtain 5,000 mg/kg of total petroleum hydrocarbons (i.e. sum of alkane and aromatic hydrocarbons)

in the contaminated soil. The incubation was done for 42 d before planting *B. juncea*.

4.3.2 Growing medium and chemicals

John Innes Number 2 soil-based compost was used for the experiment while the reagents, standard chemicals and HPLC grade solvents for the experiment were obtained from Sigma-Aldrich Co Limited (UK) and Fisher Scientific UK Limited. These include: hexane, acetone, deuterated EPA 525 PAH standard solution and deuterated alkane standard solution, among others, to determine the alkanes' distribution as well as the PAHs content in the soil and the mineral contents of the plants after harvesting.

4.3.3 Initial investigations

In order to gain a useful knowledge of the distribution of the alkanes and aromatics (essentially the 16 US EPA priority PAHs), an initial investigation was carried out on the crude oil used. This involved the GC/MS analysis of the whole crude oil followed by the fractional distillation under laboratory conditions as discussed in Chapter 3. The determination of the pH, moisture content, water holding capacity, total organic carbon, total nitrogen and total phosphorus of the growing media were carried out using appropriate standard scientific methods (as explained in Chapter 3) in five replicates.

4.3.4 Spiking and the incubation process

A modified method of Brinch et al. (2002) was used for spiking the composts with crude oil as the perfect means of artificially contaminating hydrophobic compounds with soil is yet to be found (Brinch et al., 2002). For the experiment, the addition of crude oil to the composts was done manually by first shaking the crude oil and adding the required quantity to 25% of the whole volume of the compost to be used and subsequent addition of the homogeneous mixture to the remaining 75% of the bulk. 0.5 w/w "Libyan" crude oil was added to the appropriate treatments to achieve a TPH concentration of approximately 5,000 mg/kg.

For the RENA treatment, aeration via weekly tillage of the crude oil: soil mixture was provided by manual and uniform stirring for 5 min to full-depth of the pot. The bioaugmentation treatment, involving the John Innes compost and the spent *Pleurotus ostreatus* mushroom substrate, was also similarly mixed (i.e. to full-depth) in ratio 4:1 (Eggen, 1999) at the start of the experiment. The pot treatments containing crude oil were first kept in the fume cupboard for five days on plant pot saucers to make allowance for the release of volatile compounds before being transferred to the greenhouse. Water was added at regular intervals to ensure the soils were kept at the same moisture content (Wu et al., 2013) while the pots were left for the degradation of petroleum hydrocarbons over time.

10 g of the soil sample was taken at two time points at the start of the experiment - day (0) and day (7), and other two points at the end - day (28) and day (42), to assess the level of petroleum hydrocarbons degradation before planting. Soil samples were also taken after the harvesting of plant samples and were all kept at -20^{o}C prior to analysis. In addition, 5 g of soil sample from each treatment was taken for the microbial population examination.

Generally, all the treatments were incubated for 42 d before the planting of *B. juncea* seeds which were grown for 65 d. The number of days used for the incubation fall within that used by Liste and Alexander (2002) where contaminated soils were stored between 14 - 47 d before the addition of plant seeds.

4.3.5 Seed planting and germination assay

Following the experimental design involving CRD as explained in Section 4.3.1, 10 *B. juncea* seeds were planted at 3 mm depth in each of the soil treatments after the 42 d incubation. The seeds were left to germinate in the greenhouse and after 10 d of germination, thinning was carried out to remove weaker seedlings, leaving 3 seedlings in each pot. The plants were allowed to grow for 65 d though there was an initial pest attack by cabbage white caterpillars at the early growth stage.

The environmental conditions were strictly monitored and the pots re-positioned periodically to minimize variation in plant growth. Percentage seed germination and the plant heights obtained after three weeks, before the attack of cabbage white caterpillars, were recorded.

4.3.6 Hydrocarbons analysis

Changes in the alkanes and PAHs' concentration in the contaminated pot treatments during the period of experiment were assessed by taking soil samples. The sequential extraction method was used for the hydrocarbons extraction with subsequent GC/MS analysis as previously described in Chapter 3. The modified percentage of degradation expression of Bento et al. (2005) was used in calculating the percentage of alkane groups remaining at each time point using the equation below:

$$\text{Percentage (\%) of Alkanes Remaining} = \frac{\text{Alkanes concentration at time point}}{\text{Alkanes concentration at the start point}} \times 100$$

4.3.7 Statistical analysis

Microsoft Excel 2010 package was used in analysing the experimental results from the compost/growing medium's physicochemical properties carried out with five replicates and the data obtained were expressed as mean ± standard error. The normality of the distribution was assessed using the Shapiro-Wilk's test and where applicable, One-Way Analysis of Variance (ANOVA) was done to compare the means of the values obtained using the IBM SPSS Statistics (IBM Corp. Released 2012. IBM SPSS Statistics for Windows, Version 21.0. Armonk, NY: IBM Corp.). Whenever the assumption of homogeneity of variances was violated as assessed by Levene's Test of Homogeneity of Variances, Games-Howell *post-hoc* analysis was used in place of Tukey HSD *post-hoc* analysis to evaluate statistical differences among means ($p < 0.05$).

4.4 Results and discussion

4.4.1 Initial investigation findings

4.4.1.1 Crude oil characterisation

Figure 4.1 and 4.2 revealed the distribution of the alkanes (C_{10} – C_{36}) and 10 of the US EPA priority PAHs respectively as found in the whole crude oil used. From the GC/MS analytical result as shown in Figure 4.1, the crude oil contains higher concentrations of C_{10} - C_{14}, C_{15} - C_{20} and C_{21} - C_{27} alkane fractions than C_{28} - C_{36} alkanes at the instrument's highest oven operating temperature of 310^{o}C. Also, the crude oil used had a pristane/phytane (Pr/Ph) ratio of 0.98 which is within the range of 0.8 – 3.0 of most crude oils (Peters et al., 2005) and indicated that it was from an anoxic depositional environment characterised by Pr/Ph ratio < 1 (El Nady and Harb, 2011).

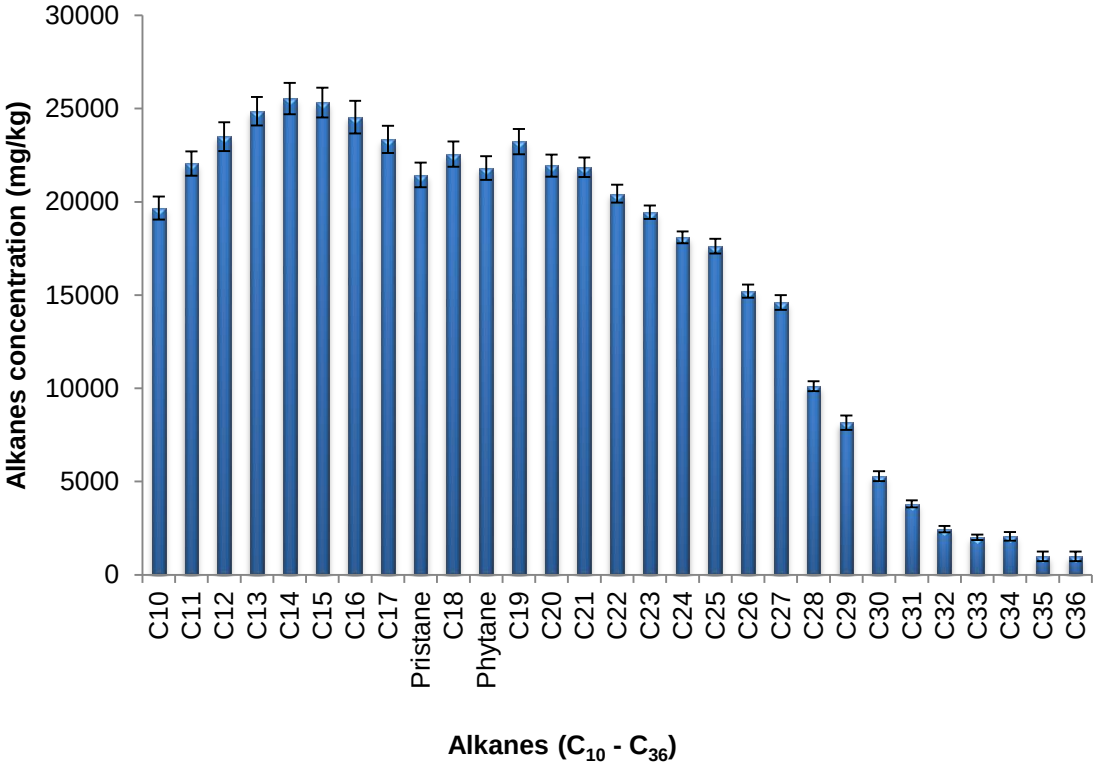


Figure 4-1: Alkanes (C_{10} - C_{36}) distribution in the whole crude oil. Values are the means of five replicates ± SE.

On the other hand, only 10 of the US EPA priority pollutant PAHs were detected and quantified in the crude oil with higher concentrations of phenanthrene and anthracene as shown in Figure 4.2.

- Acy = Acenaphtylene
- Flu = Fluorene
- Phe = Phenanthrene
- Ant = Anthracene
- Pyr = Pyrene
- BaA = Benzo(a)anthracene
- Chr = Chrysene
- BbF = Benzo(b)fluoranthene
- BkF = Benzo(k)fluoranthene
- BaP = Benzo(a)pyrene

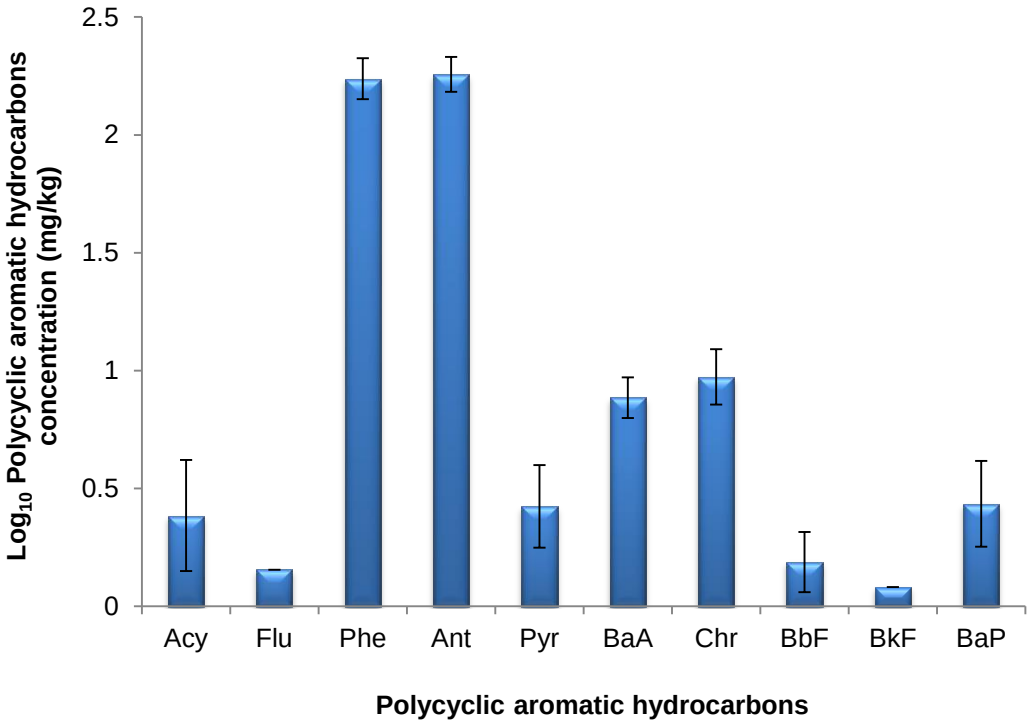


Figure 4-2: Distribution of the 10 US EPA priority pollutant PAHs in the whole crude oil. Values are the means of five replicates ± SE.

4.4.1.2 Fractional distillation

Figure 4.3 shows the weight of the different fractions of crude oil recovered from the fume cupboard fractional distillation under laboratory conditions. The colour of the different fractions obtained from the initial temperature of the fume cupboard to 200^{o}C was colourless and yellow between 200 – 270^{o}C. At

temperature > 270^{o}C, the remaining fraction was found to be light brown after the removal of the lighter fractions. This is similar to the findings of Odebunmi et al. (2002) where different colour of the fractions was obtained during the elution liquid chromatography. The deepening of the colours of these fractions (i.e. from colourless — yellow — light brown, Figure 4.4) affirms the increase in the weight and complexity of the petroleum hydrocarbon molecules as the temperature increases (Swingle and Snapp, 1931) during the fractional distillation. However, due to laboratory conditions, there were some weight losses in the fractions collected.

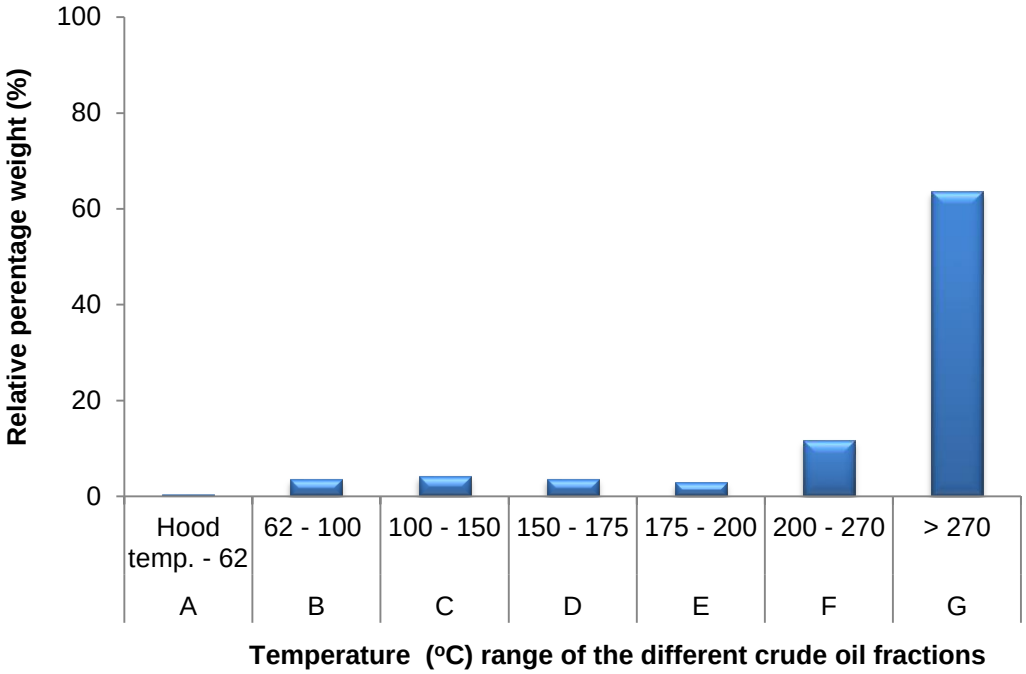


Figure 4-3: Outline of the crude oil fractions' relative percentage weight to the whole crude oil. A – G = Crude oil fractions at the respective temperature range.

Figure 4-4: Changes in colour of the different fractions of crude oil.
A – G = Crude oil fractions at the respective temperature range.

4.4.1.3 Modification of the John Innes No. 2 compost pH

In order to adjust the pH of the soil-based compost from its initial value of 5.31 ± 0.01 (as shown in Table 4.1) to that suitable for microbial activity and oil degradation as mentioned in Chapter 2, liming was carried out as explained in Chapter 3, Section 3.2.2.1. 20 g of the John Innes No. 2 compost was mixed with varying amount of lime to know the approximate amount of lime required to get the desired pH in the overall soil-based compost to be used for the experiment.

Table 4-1: Properties of the John Innes No. 2 compost before liming

Property	Soil-based compost
pH	5.31 ± 0.01
Moisture content (%)	31.47 ± 0.12
Water holding capacity (%)	63.84 ± 0.60

Values are the means of three replicates ± SE.

Table 4-2: Changes in pH of the John Innes No. 2 compost on liming

Sample	Weight of lime added (g)	pH
A	0.00	5.56 ± 0.05
B	0.10	6.09 ± 0.14
C	0.12	5.98 ± 0.18
D	0.14	6.19 ± 0.13
E	0.16	6.30 ± 0.06
F	0.18	6.33 ± 0.05
G	0.20	6.38 ± 0.02

Values are the means of three replicates ± SE.
Mass of John Innes No. 2 compost used = 20 g.

Table 4.1 and 4.2 reveal the properties of the soil-based compost before lime addition and changes recorded in the pH on liming respectively while Figure 4.5 shows the linear plot of the pH of soil-based compost against the weight of lime added.

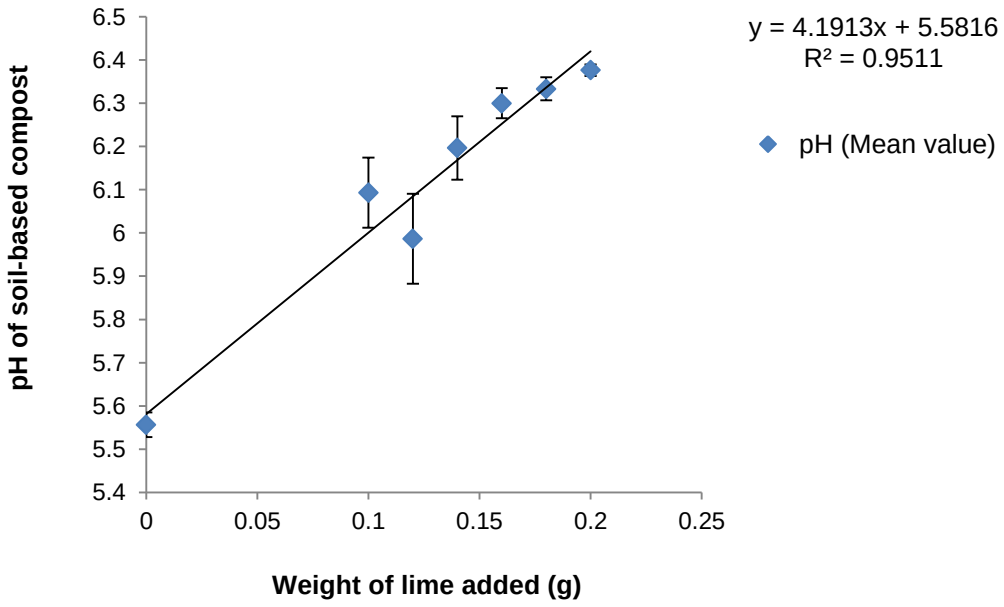


Figure 4.5: Linear plot of changes in soil-based compost pH on liming. Values are the means of three replicates ± SE.

4.4.1.4 Physiochemical properties and microbial population counts

As it is important to know the characteristics of contaminated sites prior to treatments (Bento et al., 2005), the physiochemical properties of the composts/growing media were determined as explained in Chapter 3. The result as well as that of the microbial populations as shown in Table 4.3 revealed that after liming, the pH of the soil-based compost and moisture content fell within the acceptable range for oil degradation as recommended by Vidali (2001) with higher bacterial and fungal counts. However, the spent *Pleurotus ostreatus* mushroom compost had: a pH below the organic materials' optimal range of 5.5 and 8.0 for composting (Verdonck, 1988; Kapetanios et al., 1993; Zorpas et al., 2003; Ahmed et al., 2007), higher values of electrical conductivity and carbon/nitrogen ratio. The spent mushroom compost also had a higher moisture content and water-holding capacity than the soil-based compost, indicating that it contained more organic matter, as confirmed in the higher total carbon content (Table 4.3).

Table 4-3: Composts' physicochemical properties and microbial populations

Property	Soil-based growing medium	Spent mushroom compost
pH	6.60 ± 0.03	4.75 ± 0.01
Moisture Content (%)	30.80 ± 0.97	37.60 ± 0.24
Water-Holding Capacity (%)	58.07 ± 0.60	81.17 ± 0.54
Electrical Conductivity (S/m)	0.128 ± 0.00	0.202 ± 0.00
Total Carbon (%)	9.37 ± 0.37	47.53 ± 0.25
Total Organic Carbon (%)	8.62 ± 0.16	44.95 ± 0.17
Total Nitrogen (%)	0.31 ± 0.01	0.64 ± 0.01
Total Phosphorus (mg/kg)	542.30 ± 15.81	712.05 ± 2.44
C/N Ratio	29.85 : 1.00	74.43 : 1.00
C/P Ratio	172.87 : 1.00	667.55 : 1.00
Bacterial Count (CFU/g compost)	8×10^6	1.47×10^6
Fungal Count (CFU/g compost)	3.67×10^5	2.47×10^5

Values are the means of five replicates ± SE.

According to Davidson et al. (2000), the bioaugmentation treatment involving the use of spent *Pleurotus ostreatus* mushroom compost would evolve more CO_2 as a result of its higher moisture content. Nonetheless, the water-holding capacity of the soil-based compost (within the range of 50 - 70%) would present a better support for aerobic biodegradation of simple or complex organic material (Pramer et al., 1972, cited in Dibble and Bartha, 1979, p. 736.) arising from the crude oil contamination.

Although the two composts have C/N and C/P ratios higher than the acceptable theoretical values for a mixed microbial population in soil of 10:1 (Waksman, 1924; Dibble and Bartha, 1979) and 100:1 (Thompson et al., 1954, cited in Dibble and Bartha, 1979, p. 737) respectively, these ratios are expected to be higher for petroleum biodegradation (Dibble and Bartha, 1979). Also, according to King et al. (1998, p. 93), "in theoretical terms, the C:N ratio should

be optimal when it approximates the 400:10 ‘textbook’ number”. From the results shown in Table 4.3, only the soil-based compost had: a C/N ratio close to this value, satisfies the provision of a pH range of 6.5 to 8.0 and within the soil moisture level of 50 to 80% of field capacity required for the successful application of land treatment (King et al., 1998).

In addition, only the soil-based growing medium had a C/N ratio slightly above the desired ratio of 24:1 required by soil microorganism (USDA NRCS, 2011) while the introduction of spent *Pleurotus ostreatus* mushroom substrate (with a high C/N ratio) to the bioaugmentation treatment could lead to a condition of nitrogen deficiency in the growing medium (USDA NRCS, 2011).

On the other hand, the electrical conductivity of the spent *Pleurotus ostreatus* mushroom compost also fell in the range within which the yield of sensitive crops could be restricted (Richards, 1954). However, the total heterotrophic bacteria plate counts of the two compost/growing media were suitable for this experiment as they were above the 10^6 colony-forming units per gram of soil (CFU/g) suggested by King et al. (1998).

4.4.2 Effect of compost treatments on hydrocarbons degradation

4.4.2.1 Alkane groups degradation

The modified ranking of Coulon et al. (2005): C_{10} - C_{14}, C_{15} - C_{20}, C_{21} - C_{27} and C_{28} - C_{36}, was used in assessing the rate of changes of alkane groups in the mesocosm experiment from the start to the end. At the start of the experiment after spiking (Day 0), the only statistical significant difference was recorded in the C_{10} – C_{14} alkane group where Tukey *post-hoc* analysis revealed that the increase in concentration from Treatment C to D [39.35, 95% CI (20.25 to 58.45) mg/kg] was statistically significantly different ($p < 0.05$) as shown in Figure 4.6. Although some traces of alkanes were found in an irregular pattern in the planting control (Treatment A - involving no crude oil addition) at the start of the experiment, the PAHs were completely below the limit of detection. Hence, the hydrocarbons degradation study was centred on Treatments: B, C, and D while Treatment A was re-introduced at the planting stage.

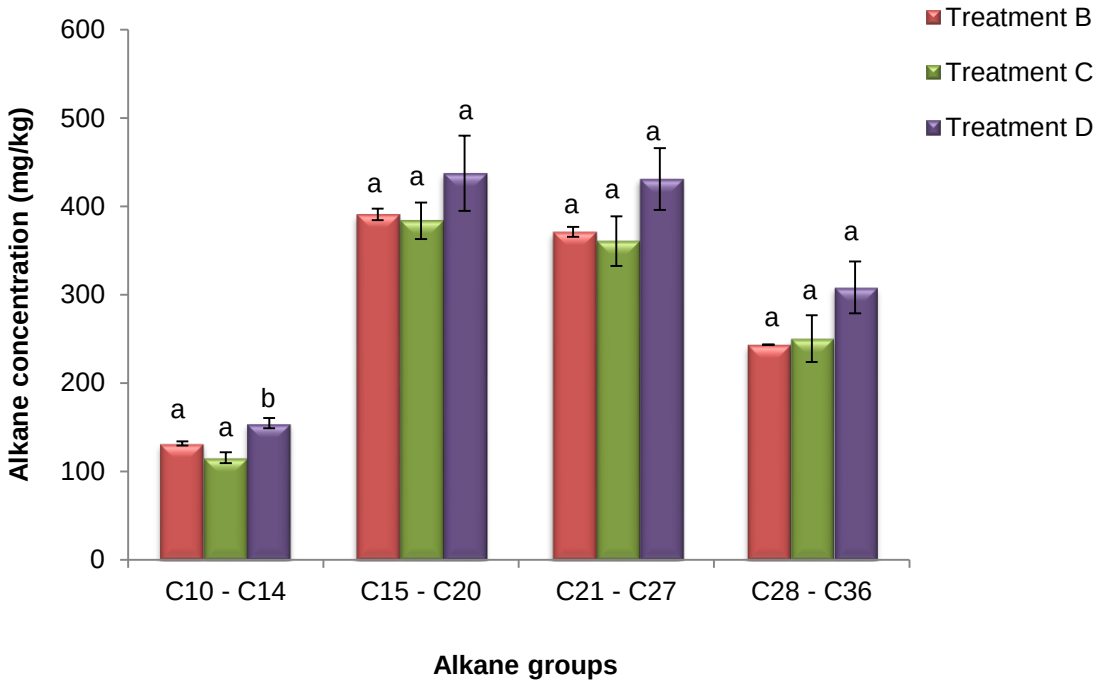


Figure 4.6: Initial alkanes' distribution in the pot treatments (Day 0). Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

GC/MS analysis of the soil samples collected at different times during the incubation period and after harvesting showed that weekly tillage as used in the RENA treatment enhanced the alkanes' degradation although the heavier alkanes (C_{28} – C_{36}) showed some level of restriction. This observation followed a similar trend recorded by Coulon et al. (2005) where alkanes C_{28} – C_{36} had the lowest rate of change among the alkane groups considered in the mesocosm experiment. This is because compounds that give the greatest amount of energy with the minimum amount of biochemical effort by the microbes are attacked and degraded first which contributes to the faster degradation of light hydrocarbons compared with the long-chain (paraffinic) hydrocarbons compounds (King et al., 1998). The planting of *B. juncea* however improved the level of degradation. Figure 4.7 illustrates the changes/degradation in the concentration of the different alkanes groups in each treatment over time.

Furthermore, as shown in Figure 4.7, the RENA treatment was more effective in the breakdown of $C_{15} – C_{20}$ and $C_{21} – C_{27}$ alkanes compared with the bioaugmentation treatment. To some extent, a related result was recorded in the experiment of Bento et al. (2005) where the light fraction ($C_{12} – C_{23}$) of total petroleum hydrocarbons present in Hong Kong soil contaminated with diesel oil gave a higher percentage of degradation upon natural attenuation and the weekly rate of degradation was two times greater under natural attenuation compared with the bioaugmentation result when a bacterial consortium isolated from another diesel oil contaminated soil was added to the Hong Kong soil. Nonetheless, in the same experiment, the best performance in diesel oil degradation was with bioaugmentation of the Long Beach diesel oil contaminated soil (Bento et al., 2005).

Generally, the improved petroleum hydrocarbons degradation ability of RENA in this experiment is supported by the findings of Wegwu et al. (2010) where RENA application ensured successful remediation of crude oil-contaminated soil samples from three sites. In the study, the application of RENA decreased the TPH content at the three sites from 14,569 mg/kg, 3,713 mg/kg and 2,156 mg/kg to 45.7mg/kg, 139 mg/kg and 103 mg/kg respectively (Wegwu et al., 2010).

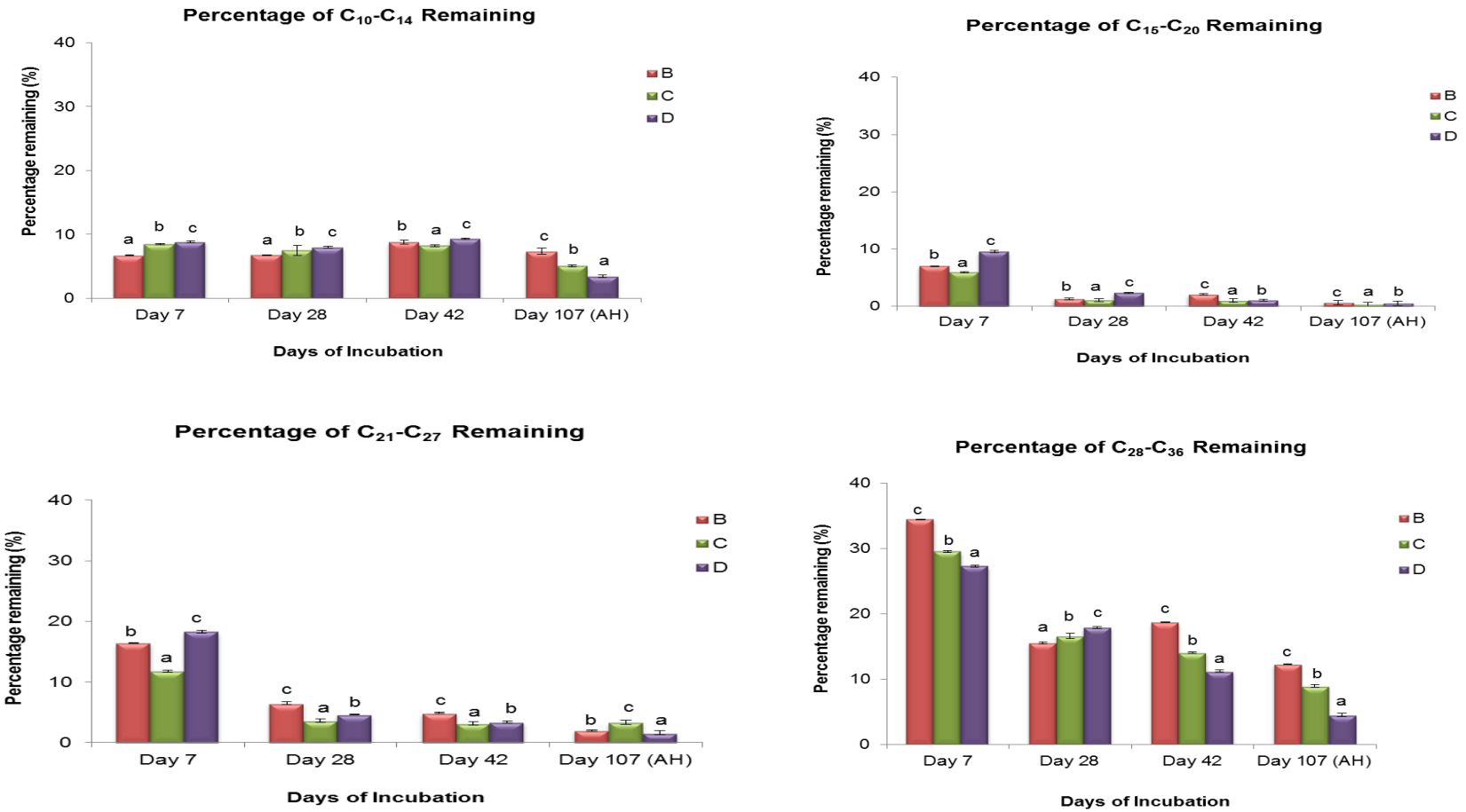


Figure 4.7: Comparison of the percentage of alkanes group remaining in each treatment during the incubation period and after harvesting. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

4.4.2.2 PAHs degradation

At the start of the experiment, only phenanthrene and anthracene concentrations in the treatments (Figure 4.8) were above the method's LOQ (see Appendices; Table A) and the LOQ value of 0.02 mg/kg commonly used by laboratories in Nigeria to assess PAHs in Nigerian soils (Okparanma, 2013). The incubation period and consequent degradation study therefore focussed on these two PAHs, which are also US EPA priority compounds. There were no statistically significant differences in the concentration of these PAHs among the pot treatments ($p > 0.05$) at the start of the experiment, as shown in the figure, which also substantiated the homogenous mixing of crude oil with the composts/growing media.

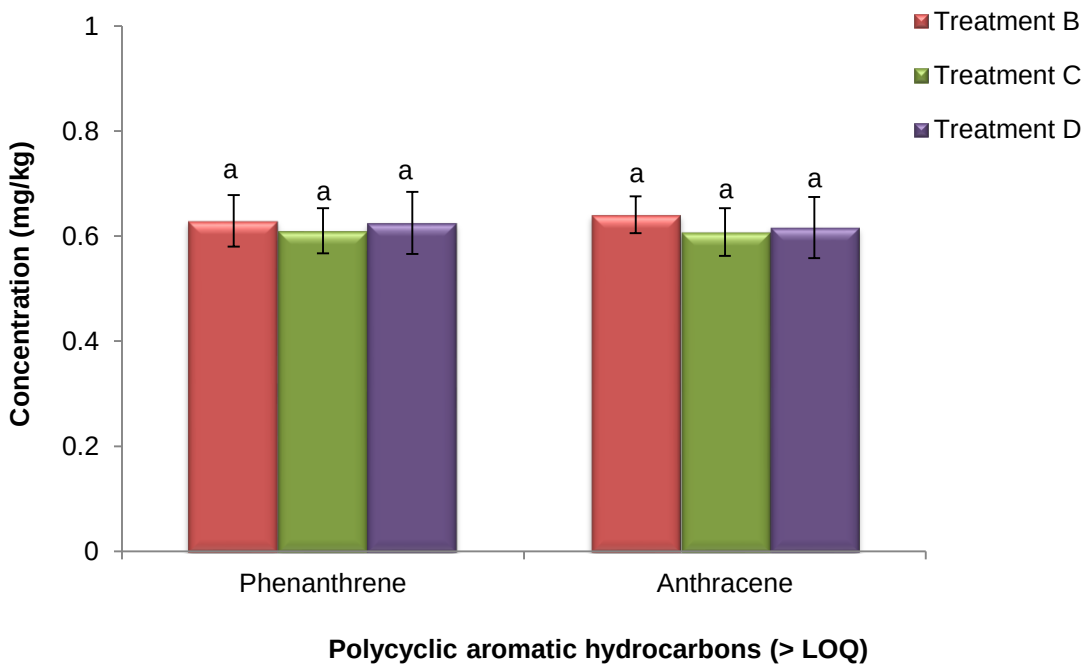


Figure 4.8: Initial PAHs' distribution (> LOQ) in the pot treatments (Day 0). Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Although anthracene was only below the LOQ following RENA treatment at the first time point (Day 7), after 42 d of incubation, the concentrations of the two priority PAHs fell below the LOD in the pot treatments where bioremediation strategies were applied (i.e. the RENA and bioaugmentation treatments)

whereas phenanthrene content in Treatment B remained quantifiable (Figure 4.9). According to Wegwu et al. (2010), the tillage of the soils in the RENA treatment may have influenced the degradation of the PAHs as observed while the planting of *B. juncea* also enhanced the removal of phenanthrene from Treatment B. The result supports the view of Lundstedt (2003) that land farming (such as used in RENA) could be a useful method for degrading low molecular weight compounds.

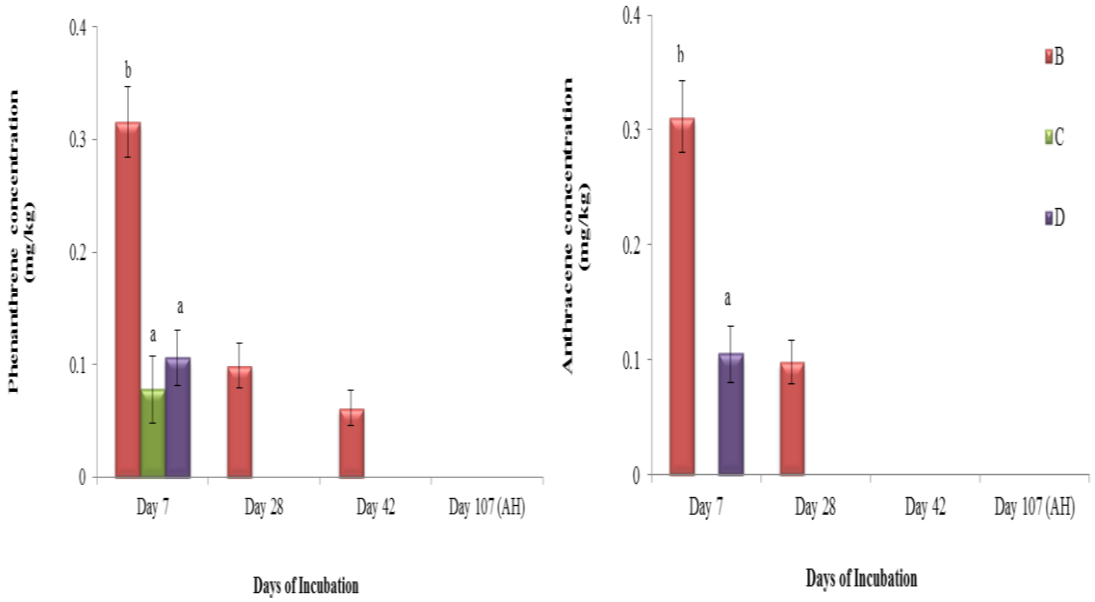


Figure 4.9: Phenanthrene and anthracene degradation over time (AH: After Harvesting). Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

4.4.3 Effect of bioremediation strategies on plant growth and nutritional composition

4.4.3.1 Effect on plant growth parameters

The effect of the selected bioremediation strategies on the germination of *B. juncea* seeds is shown in Figure 4.10 in which the lowest percentage of germinated seeds was recorded in Treatment D involving bioaugmentation with spent *Pleurotus ostreatus* mushroom compost. This is as a result of the

introduction of spent *Pleurotus ostreatus* mushroom compost (with unfavourable electrical conductivity) to the soil substrate.

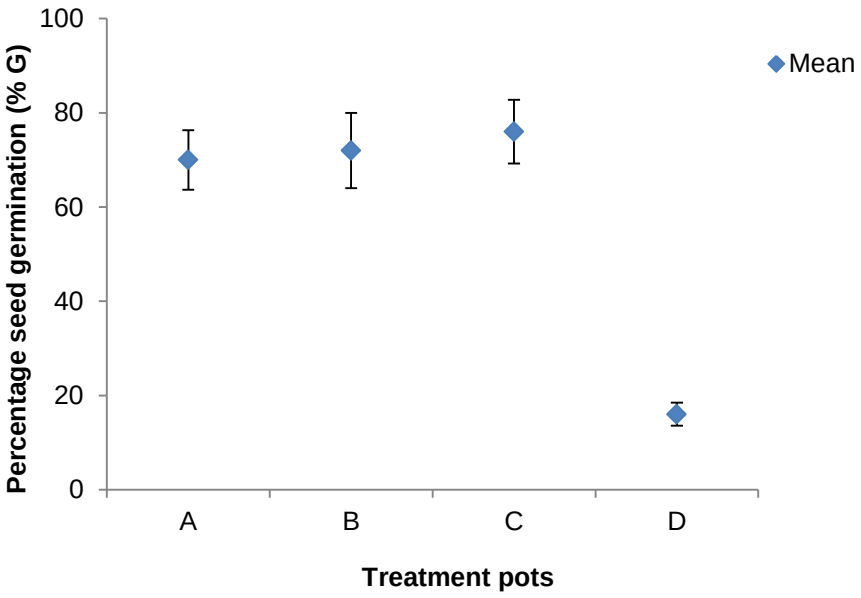


Figure 4.10: Germination of *Brassica juncea* in the different treatment pots. Values are the means of three replicates ± SE.

As shown in Figure 4.11, the plant heights on Day 10 were statistically significantly different ($p < 0.05$) while from the Tukey *post-hoc* analysis carried out, the increase from Treatment A to B [0.97, 95% CI (0.46 to 1.49) cm] was statistically significantly different ($p < 0.05$) as well as the decrease from Treatment C to D [2.22, 95% CI (1.71 to 2.73) cm].

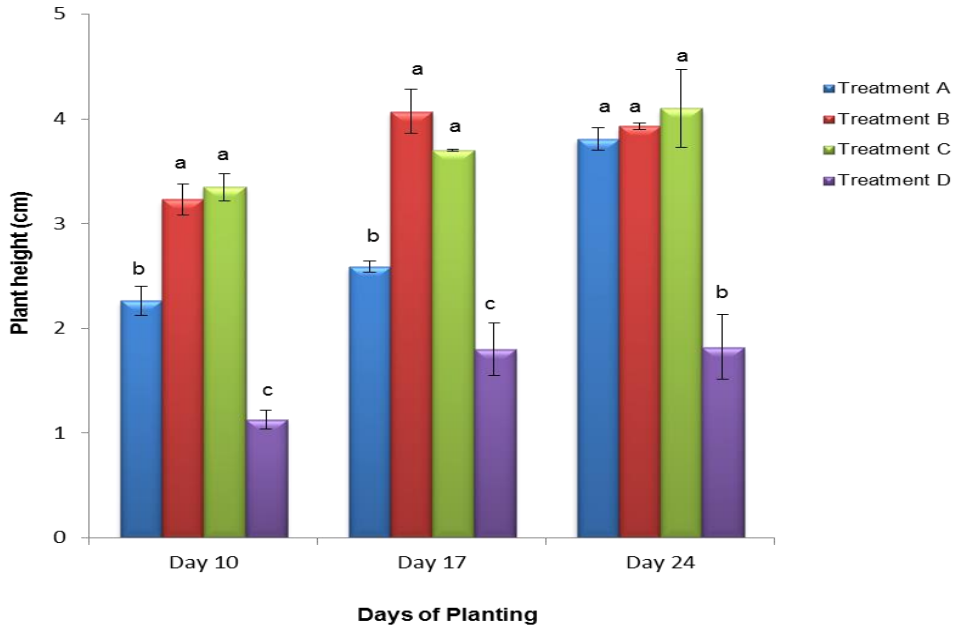


Figure 4.11: Effect of the different pot treatments on the plant heights. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The plant heights on Day 17 were also found to be statistically significantly different ($p < 0.05$); Games-Howell post-hoc analysis revealed that the increase from Treatment A to B [1.48, 95% CI (0.62 to 2.34) cm] was statistically significantly different ($p < 0.05$) as well as the decrease from Treatment C to D [1.90, 95% (0.86 to 2.94) cm]. However, in the Day 24 plant height data, the only statistical difference was in the decrease from the RENA treatment (Treatment C) to the bioaugmentation treatment (Treatment D) [2.28, 95% CI (0.74 to 3.83) cm] prior to the pest attack.

Practically, the significant increase ($p < 0.05$) in the plant height recorded in the plants samples from Treatment B and C involving crude oil contamination on Day 10 and 17, compared with those from the control treatments (with no crude oil contamination) support earlier report in the literature (e.g. Carr, 1919) of the plant-stimulation ability of low concentration of crude oil. Nevertheless, Treatment D (bioaugmentation treatment) which also involved the application of crude oil had the least plant height on Day 10, 17 and 24 as a result of the unfavourable electrical conductivity of the incorporated spent mushroom compost.

4.4.3.2 Effects on plants' nutritional (mineral) composition

The nutritional composition of the plants was only determined in the plants under Treatments A, B and C because the Treatment D plant samples were completely destroyed by juvenile caterpillars of cabbage white butterfly (details in section 4.5) without recovery. The effect of the three remaining treatments on the nutritional composition of *B. juncea*, i.e. the mineral distributions as found in the above-ground plants part after harvesting, was evaluated because green vegetables are said to be good sources of dietary minerals (Mathew, 1985, cited in Taura and Habibu, 2009, p. 1) which can be classified as major and minor minerals (Nieman et al., 1992).

The result of the mineral analysis as presented in Table 4.4, revealed that crude oil contamination in Treatment B (remediation-study control) and application of RENA in Treatment C had no significant effect ($p > 0.05$) on the samples' major dietary minerals. In other words, crude oil contamination in Treatment B and application of RENA in Treatment C had no interaction with any of the five major dietary minerals (Na, Mg, Ca, K and P) considered in this experiment.

Table 4-4: The distribution of major dietary minerals (mg/kg) in each treatment

Treatments	Distribution of Major Dietary Minerals (mg/kg)				
	Na	Mg	Ca	K	P
Treatment A	2766.6 ±	5042.92 ±	21,710.2 ±	64,696 ±	5712 ±
(Clean soil, Planting control)	138.80[a]	138.80[a]	2278.03[a]	5363.73[a]	489.89[a]
Treatment B	2949.93 ±	4579.59 ±	19,193.53 ±	65,226 ±	4798.67 ±
(Soil + Crude oil)	155.92[a]	83.53[a]	1863.7[a]	5642.18[a]	187.74[a]
Treatment C	2596.6 ±	4739.59 ±	20,073.53 ±	60,432.67 ±	4965.33 ±
(Soil + Crude oil + RENA)	300.22[a]	301.18[a]	1008.32[a]	551.04[a]	229.29[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.
No data was recorded for Treatment D plant samples as they were completely damaged by the pests.

In the same vein, the treatments had no significant effect ($p > 0.05$) on the mineral ratios of the harvested samples as shown in Table 4.5. Meanwhile,

Nieman et al. (1992) have suggested that a food source is good if the Ca/P ratio is > 1 and poor if it is < 0.5 (Aremu et al., 2006; Yusuf et al., 2007; Aremu et al., 2011; Olagbemide and Philip, 2014). Hence, the Ca/P ratios of the vegetable samples from all the pot treatments (Table 4.5) agree with the report of Nieman et al. (1992) that leafy vegetables are good nutritious type of food to be included in the human diet. In addition, the samples will not contribute to high blood pressure because the Na/K ratios were < 0.6 (Nieman et al., 1992).

Table 4-5: Mineral ratios of the harvested *Brassica juncea* samples

Treatments	Ca/P ratio	Na/K ratio
Treatment A (Clean soil, Planting control)	3.80 ± 0.05[a]	0.043 ± 0.00[a]
Treatment B (Soil + Crude oil)	3.99 ± 0.28[a]	0.045 ± 0.00[a]
Treatment C (Soil + Crude oil + RENA)	4.04 ± 0.39[a]	0.043 ± 0.00[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Among the analysed minor dietary minerals, RENA application in Treatment C limited the uptake of Cr and Zn by *B. juncea* while crude oil contamination without any form of remediation (Treatment B) also had a similar effect on the Zn content. Although Zn was the only mineral that was negatively affected by both the crude oil contamination and RENA application (Table 4.6), its concentration and that of Cu from all the treatments were within the maximum permissible concentrations for human consumption of 10 – 50 mg/kg and 50 mg/kg DW, respectively (Reilly, 1980, cited in Samara, 1992, p. 64). Statistical analysis of the Zn levels showed a significant difference ($p < 0.05$) between Treatments A and B [11.53, 95% CI (5.02 to 18.05) mg/kg] and between Treatments A and C [14.60, 95% CI (8.08 to 21.12) mg/kg].

Table 4-6: Distribution of minor dietary minerals (mg/kg) in each treatment

Treatments	Distribution of Minor Dietary Minerals (mg/kg)						
	Cr	Mn	Co	Cu	Zn	Mo	Fe
A (Clean soil, Planting control)	3.95 ± 0.49[a]	107.66 ± 19.02[a]	0.17 ± 0.00[a]	1.85 ± 0.11[a]	42.78 ± 2.78[a]	0.53 ± 0.00[a]	159.35 ± 20.64[a]
B (Soil + Crude oil)	2.54 ± 0.25[a,b]	88.22 ± 15.26[a]	0.17 ± 0.04[a]	1.70 ± 0.35[a]	31.25 ± 1.56[b]	0.57 ± 0.16[a]	167.87 ± 19.91[a]
C (Soil + Crude oil + RENA)	1.84 ± 0.38[b]	83.16 ± 4.42[a]	0.15 ± 0.03[a]	1.31 ± 0.25[a]	28.18 ± 0.69[b]	0.59 ± 0.11[a]	137.50 ± 18.99[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Among the undesirable mineral contaminants, only the Pb concentration in the *B. juncea* samples was significantly enhanced by RENA application (Table 4.7). Although this Pb concentration from the RENA treatment samples was significantly higher ($p < 0.05$) than those from the planting control and the remediation-study control (Table 4.7), it was below the FAO/WHO maximum level of 0.30 mg/kg (Codex Alimentarius, 2015) as well as Reilly (1980, cited in Samara, 1992, p. 64) indicated maximum permissible limit of the element (2.0 – 2.5 mg/kg DW) in vegetables for human consumption.

On the other hand, the Cd concentrations in all the harvested samples (control inclusive) were found above the FAO/WHO Codex maximum level (ML) of 0.20 mg/kg for leafy vegetables (Codex Alimentarius, 2015). They were, however, lower than the maximum permissible concentration of the element (1 mg/kg DW) in vegetables for human consumption as pointed out by Reilly (1980, cited in Samara, 1992, p. 64).

Table 4-7: Distribution of undesirable mineral contaminants

Treatments	Distribution of Mineral Contaminants (mg/kg)				
	Al	Sr	Ba	Cd	Pb
Treatment A (Clean soil, Planting Control)	153.36 ± 22.86[a]	57.95 ± 5.06[a]	1.59 ± 0.00[a]	0.45 ± 0.01[a]	0.08 ± 0.01[a]
Treatment B (Soil + Crude oil)	156.29 ± 33.49[a]	53.01 ± 4.07[a]	1.45 ± 0.11[a]	0.52 ± 0.05[a]	0.08 ± 0.02[a]
Treatment C (Soil + Crude oil + RENA)	107.46 ± 18.76[a]	57.35 ± 43.01[a]	1.46 ± 0.08[a]	0.48 ± 0.01[a]	0.21± 0.01[b]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The BCF results (Table 4.8) which indicate the ability of *B. juncea* used in this experiment to accumulate the mineral contaminants as a function of the metal concentration in the different treatment pots, support the report of Sharma et al. (2010) that *B. juncea* is a hyper-accumulator of Cd. This explains its high concentration in the control treatment samples as well as the observed Cd contents in all the harvested *B. juncea* above-ground plant samples above the FAO/WHO Codex maximum level (ML) of 0.20 mg/kg. Assessment of the BCF also revealed that the application of RENA involving tillage significantly promoted (Table 4.8; $p = 0.001$) the transfer of Pb from the soil treatment to the above-ground plant part as seen in Table 4.8.

Table 4-8: Bioconcentration factor on the mineral contaminants

Treatments	Bioconcentration factor (BCF)				
	Al	Sr	Ba	Cd	Pb
Treatment A	0.01 ±	1.54 ±	0.11 ±	6.01 ±	0.01 ±
(Clean soil, Planting Control)	0.00[a]	0.08[a]	0.00[a]	0.33[a]	0.00[a]
Treatment B	0.01 ±	1.41 ±	0.10 ±	6.93 ±	0.01 ±
(Soil + Crude oil)	0.00[a]	0.09[a]	0.01[a]	0.66[a]	0.00[a]
Treatment C	0.01 ±	1.53 ±	0.10 ±	6.39 ±	0.03±
(Soil + Crude oil + RENA)	0.00[a]	0.08[a]	0.01[a]	0.39[a]	0.00[b]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Generally, the factor underlying the significant reduction in the Cr and Zn concentrations (Table 4.6) (dilution or antagonistic effect) including the significant increase in Pb concentration (Table 4.7) (synergistic or concentration effect) as a result of the different treatment effects could not be ascertained as the pest attack prevented the assessment of the required actual yield of *B. juncea* samples from each of these treatments at the end of the experiment.

4.5 Pest attack on the plants

4.5.1 Effect of the caterpillar attack on the different treatments' samples

After the 24 d plant measurements, the plants were attacked by juvenile caterpillars of cabbage white butterfly with most damaging effect on the plants from: (1) the control treatment which was not contaminated with crude oil, and (2) the bioaugmentation treatment with initial poor plant growth.

The experiment was continued with the removal of the caterpillars and the eggs laid. The level of monitoring of the plants was thereafter increased to prevent further attack till the end of the experiment. There was regeneration of *B. juncea* leaves from Treatment A (planting control), B (remediation-study control) and C (RENA treatment) while Treatment D (involving bioaugmentation)

plant samples which were completely lost in the course of the pest attack did not grow again (i.e. no recovery).

4.5.2 The form of the caterpillar attack and the protective effect of crude oil

The addition of crude oil at a low concentration of 5,000 mg/kg TPH to some of the soil treatments (except the planting control) tends to have a protective effect on *B. juncea* used for this experiment against the attack of juvenile caterpillar of cabbage white butterfly which are known to have damaging effects on brassica plants (PAN UK, 2010; MAF, 2012). Although there are two species of this butterfly: the large/great cabbage white (*Pieris brassicae*) and the small cabbage white (*Pieris rapae*) (PAN UK, 2010; MAF, 2012), the plants were attacked by the latter as the caterpillars from the eggs laid on the underside of leaves (PAN UK, 2010; Wisconsin Fast Plants Program, 2015) were pale green (Figure 4.12) (PAN UK, 2010). This species of butterfly, i.e. the *Pieris rapae*, although from Europe (NSW DPI, 2003; Wisconsin Fast Plants Program, 2015), have spread to different part of the world (Wisconsin Fast Plants Program, 2015) with their greatest level of activety in the spring and autumn seasons (NSW DPI, 2003). As members of the genus *Brassica*, such as *Brassica juncea* and *Brassica napus* (rapeseed) are known to contain glucosinolates (van Doorn et al., 1998; Graser et al., 2000; Bhandari and Kwak, 2015), Graser et al., (2000) stated that the presence of these compounds in the latter crop influences its level of vulnerability to some insect and fungal pests.

On the other hand, according to Stadler and Buteler (2009, p. 169), “petroleum oils are some of the oldest and safest pesticides in use”, and have been applied in the managements of plants’ pests (Helmy et al., 2012) as well as crop protection for many years (Agnello, 2002). The production of advanced oil products via the various manufacturing, purification and refining processes (Stadler and Buteler, 2009), have helped in expanding their range of application (Cranshaw and Baxendale, 2013), for specific uses (Agnello, 2002) and with improved environmental friendliness (Stadler and Buteler, 2009).

Although there are several terminologies for describing oils such as: dormant oil, horticultural oils, mineral oil (petroleum-based oil as opposed to vegetable oils), spray oil, summer oils etc (Cranshaw and Baxendale, 2013), in the view of these authors, the oils used for plant protection may be best described as horticultural oils while there is a growing knowledge in the practicality of highly refined horticultural mineral oils (HMOs) (petroleum spray oils) and agricultural mineral oils (AMOs) (broad-range petroleum spray oils) in modern pest management practices (Agnello, 2002). Among the other advantages in the use of mineral oils in the control of pest, according to Helmy et al. (2012) as well as Beattie and Smith (1993, cited in Buteler and Stadler, 2011, p. 119), there have not been any record of pest resistance and in some cases, the oils can be mixed with other pesticides to enhance the pesticides' effectiveness (Helmy et al., 2012; Cranshaw and Baxendale, 2013) .

Generally, the effects of oils on pest insects vary and include their ability to: (1) lead to the death of insects as a result of the blockage of their breathing air holes (spiracles) (Helmy et al., 2012; Cranshaw and Baxendale, 2013), (2) act as poisons (sometimes) owing to their reactions with the fatty acids of the insect resulting in alterations to their normal metabolism (Helmy et al., 2012; Cranshaw and Baxendale, 2013), and (3) distort the feeding pattern of the insect (Cranshaw and Baxendale, 2013). Figure 4.12 shows the protective effect of crude oil against the juvenile caterpillars of cabbage white butterfly.

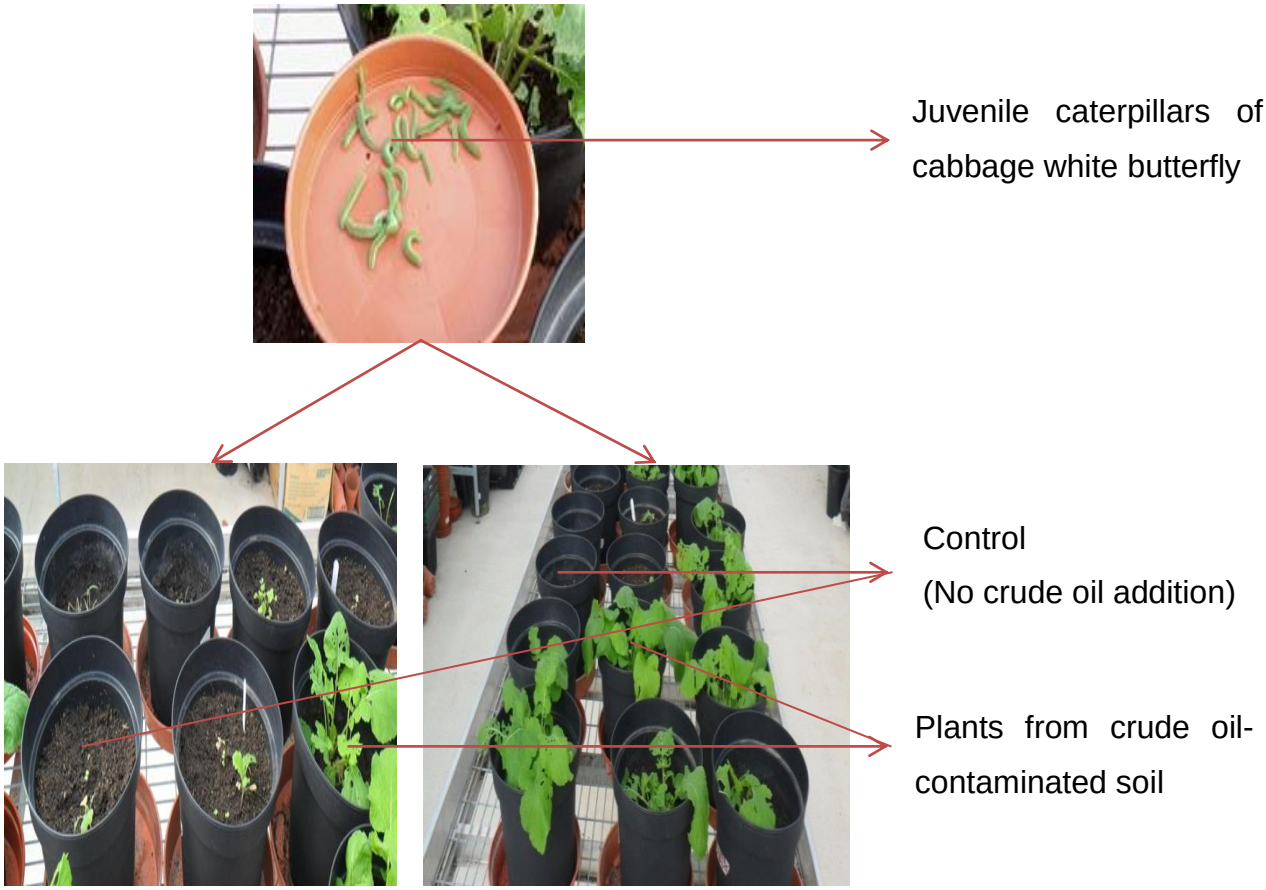


Figure 4.12: Protective effect of crude oil against juvenile caterpillars of cabbage white butterfly

The presence of high concentration of some alkanes in the crude oil used for this study such as the normal paraffin (alkane) molecules – nC_{21}, nC_{23}, nC_{24} and nC_{25}, (Figure 4.1) which depicts the range in which spray oils combine low phytotoxicity with insecticidal efficiency (Stadler and Buteler, 2009) explains the destructive effect of the cabbage white butterfly juvenile caterpillars on the plants from the control pot treatment with no crude oil contamination (Figure 4.12). The crude oil used in the mesocosm study had a repellent effect on the pest which reduced the level of damage on plants from the crude oil-containing treatments as it discourages the pest from egg-laying and feeding on the leaves (NSW DPI, 2005; Buteler and Stadler, 2011). Despite the repellent action of crude oil and as a result of the initial impaired growth of plant samples from Treatment D (bioaugmentation treatment) which also contains crude oil, Treatment D plant samples on the average experienced the most pest-damaging effect among the plants from the crude oil-containing treatments.

There is also a possibility of crude oil residual films production which according to Trammel (1965, cited in Buteler and Stadler, 2011, p. 125), will prevent the attachment of these pests to the plant surfaces (Buteler and Stadler, 2011). However, early detection and regular monitoring of the undersides of the leaves of the grown plants would have reduced the damage caused by this pest (PAN UK, 2010).

4.6 Conclusion

Results of the analysis of the experimental crude oil revealed that it contains higher concentrations of the light alkane fractions (C_{10} - C_{14}, C_{15} – C_{20} and C_{21} – C_{27}) as well as two of the US EPA priority pollutant PAHs (phenanthrene and anthracene) with low molecular weights which accounted for the fast degradation of the petroleum hydrocarbons as observed in the bioremediation experiment.

Although both RENA and bioaugmentation enhanced the degradation of the two PAHs at an initial TPH cut-off value of 5,000 mg/kg, the use of microorganisms from the growing medium itself (rather than the addition of spent mushroom substrate) improved the level of hydrocarbons degradation than that observed in the bioaugmentation treatment as indigenous microorganisms adapt better in their natural environment (Bento et al., 2005). The PAHs degradation outcome in the two treatments involving bioremediation strategies, however, support the report of McMillen et al. (2001) that human health may not be at risk from these two priority pollutant PAHs at total petroleum hydrocarbon (TPH) crude oil: soil concentration below 10,000 mg/kg.

Furthermore, the introduction of spent *Pleurotus ostreatus* mushroom compost (with unfavourable electrical conductivity) in the bioaugmentation treatment inhibited the germination of *B. juncea* seeds and also affected the growth of the plant before the pest attack. Hence, only RENA application, as a bioremediation strategy, supported the optimum growth of *B. juncea* and had no negative effect on the nutritional composition (major and minor dietary minerals) of the harvested samples with the exception of Cr and Zn.

Whereas crude oil contamination as used in Treatment B and RENA application (Treatment C) had no interaction with the analysed major dietary minerals (Na, Mg, Ca, K and P) and no significant effect on the mineral ratios, the Ca/P and Na/K ratios in the harvested vegetable samples from all the treatments indicated that they are within the recommended limit to maintain human health.

Evaluation of the mineral contaminants' BCF supports the findings that *B. juncea* can accumulate heavy metals such as Cd (Kumar et al., 1995; Nouairi et al., 2006; Sharma et al., 2010) and revealed that RENA application involving tillage significantly enhanced the uptake of Pb by the above-ground plant part. Nonetheless, the pest attack did not permit the true assessment of the overall yield of the plants from each of the treatments which would have helped in making further deductions.

Generally, the land farming treatment technology of RENA provided a better approach than the bioaugmentation method in: (a) remediating contaminated sites with low molecular weight compounds in line with Lundstedt (2003) findings, and (b) supporting the cultivation of crops in erstwhile bioremediated sites. It also indicated the suitability of RENA in treating spills arising from petroleum-derived spray oils (PDSOs). However, in order to ensure a complete human health assessment as well as safety of the populace, it will be necessary to analyse the level of bio-accumulated PAHs and discourage the cultivation of vegetables with heavy metals-accumulation tendencies, such as *B. juncea*, in RENA-treated sites.

CHAPTER 5

RESPONSES OF SELECTED GREEN LEAFY VEGETABLES TO DIFFERENT CRUDE OIL REMEDIATION INTERVENTION VALUES

5.1 Chapter Summary

Not many studies have considered the influence of crude oil contamination at remediation intervention values on agrifood production. Hence, this chapter covers the results of the experiment on three selected green leafy vegetables' responses to crude oil contamination in the range of 1,500 – 10,000 mg/kg TPH in soil. Results from the study showed that all the selected vegetables experienced reduced growth and yield at 10,000 mg/kg TPH while as a result of the growth features of the variety of *B. juncea* used, the nutritional/phytochemical analysis were on the other two vegetables. Crude oil contamination enhanced the crude protein, some minerals' contents in these two vegetables as well as sucrose and total sugars level in *L. sativa* at this CRIV. Whereas all the *B. oleracea* harvested samples had their Cd contents below the FAO/WHO Codex maximum level of 0.20 mg/kg, only *L. sativa* samples from: the control treatment (no crude oil addition) and 10,000 mg/kg TPH treatment, had their Cd levels below this recommended value. The Pb contents in *L. sativa* were also below FAO/WHO Codex Alimentarius Commission recommended level of 0.30 mg/kg. On the other hand, the phytochemical analysis showed that the total phenolics, total flavonoids, tartaric acid, malic acid, chlorophyll a and b contents of *L. sativa* were enhanced by crude oil contamination at 10,000 mg/kg TPH which also favoured the amount of tartaric acid, citric acid and chlorophyll b in *B. oleracea.* Although the assessment of bio-accumulated PAHs in the harvested vegetable samples could not be completed due to technical constraints, analysis of the volatiles from the treatment pots revealed that eleven VOCs could be emitted during plant growth in crude oil-contaminated soils at CRIV between 3,000 - 5,000 mg/kg TPH with *p*-xylene having the greatest toxic potential among the VOCs released at the highest level of crude oil contamination of 5,000 mg/kg TPH.

The research results support the findings in literature that the response of plants to crude oil contamination varies and provide beneficial information as regards the use of crude oil, at sub-lethal concentrations, as an abiotic stress factor capable of enhancing the nutritional and phytochemical composition of some green leafy vegetables.

5.2 Introduction

The agricultural sector is known to support economic growth and development in many countries. Some of these countries, including Nigeria, have also benefited from the gains from oil exploration and production processes. According to Ugwu and Kanu (2012), the Nigerian economy depended largely on agriculture between the pre- and post- independence era of 1930 to 1965. The sector contributed 65 – 70% of total exports in the 1960s but went down to less than 2% in the late 1990s as a result of increase in crude oil revenue in the early 1970s (Olajide et al., 2012).

Owing to crude oil operations, plants and animals in the environment are often exposed to toxic petroleum hydrocarbons during oil spillage. A number of oil spill incidences have been recorded (see Chapter 1) with various environmental impacts. There is a possibility that some of these petroleum hydrocarbons may become bioaccumulated in plants (Glasson et al., 1999, cited in Oghenejeboh et al., 2008, p. 605) while biochemical changes within the affected plants may also result (Achuba, 2006).

Although crude oil intervention values (CRIV) are used in some countries to determine crude oil contaminated sites requiring remediation, to date, only few studies have considered the effect of these CRIV on the yield and concentration of nutrients as well as bioactive compounds in agricultural produce. McMillen et al. (2001) also pointed out that the routine analysis of PAHs (except naphthalene) and metals at exploration and production (E & P) crude oil or condensate spill sites are unnecessary when the TPH level in soil is less than 1% (10,000 mg/kg).

In this study, an in-depth analysis of the effect of different CRIV at 1,500 - 10,000 mg/kg TPH, lower than the suggested 1.5% (i.e. 15,000 mg/kg) TPH

critical value for both animal toxicity and phytotoxicity by Tang et al. (2011), on selected green leafy vegetables [*Brassica juncea* (mustard), *Brassica oleracea* (kale) and *Lactuca sativa* (lettuce)] was carried out. This was to assess the influence of the stress caused by the presence of petroleum hydrocarbons in the soil on plant species while volatile organic compounds (VOCs) from pot treatments were also examined to provide additional information regarding the environmental impact of agrifood production at crude oil remediation intervention values.

5.3 Materials and methods

5.3.1 Mesocosm experimental design

The mesocosm pot experiment to study the effect of different CRIV on green leafy vegetables involved the planting of *Brassica juncea*, *Brassica oleracea* and *Lactuca sativa* using 1 kg of John Innes No 2 compost in Plastecnic Perego 1.5 litres diameter pots (Plastecnic SPA, Perego, Italy) with the following treatments in a Randomized Complete Block Design (RCBD):

Treatment 1: 1 kg of growing medium (0 mg/kg TPH – Control)

Treatment 2: 1 kg of growing medium + 0.15% w/w crude oil (1,500 mg/kg TPH)

Treatment 3: 1 kg of growing medium + 0.3% w/w crude oil (3,000 mg/kg TPH)

Treatment 4: 1 kg of growing medium + 0.5% w/w crude oil (5,000 mg/kg TPH)

Treatment 5: 1 kg of growing medium + 1.0% w/w crude oil (10,000 mg/kg TPH)

The use of the varying crude oil: soil TPH concentrations between 1,500 – 10,000 mg/kg TPH were based on the different CRIV employed in different national environmental regulations.

After thorough mixing, appropriate volume of water was added to adjust the moisture to 50% field capacity and to ensure that all the treatments have the same moisture content (Coulon et al., 2012). This same percentage of water was used in the experiment of Tang et al. (2011) involving plant growth at different TPH concentrations with respect to International Organization for Standardization (ISO) and OECD method. Soil samples were taken and stored

at -20^{o}C to assess the distribution of the alkanes and PAHs in each of the treatment at the start of the experiment prior to planting. Soil samples were also collected for the physiochemical properties analysis. Figure 5.1 shows the leafy vegetables arrangement at the glasshouse in RCBD at different experimental growth stages.

Figure 5-1: Selected leafy vegetables in pots arranged in RCBD at different experimental growth stages: (a) start, (b) mid, and (c) end of experiment.

The pot treatments were arranged in Randomized Complete Block Design (RCBD) (Figure 5.2) with the use of random numbers (Random.org, 2014).

Experimental Design: **Randomized Complete Block Design (RCBD)**					
Selected Plants: ***Brassica juncea*, *Brassica oleracea* and *Lactuca sativa***					
Block 1	●	●	●	●	●
	●	●	●	●	●
	●	●	●	●	●
Block 2	●	●	●	●	●
	●	●	●	●	●
	●	●	●	●	●
Block 3	●	●	●	●	●
	●	●	●	●	●
	●	●	●	●	●

Key:

Pots' Colour Codes	Bench Assigned (Treatment) Number	Crude oil : Soil Concentration Used for the Leafy Vegetables Experiment
●	1	0 mg/kg TPH (Control)
●	2	1,500 mg/kg TPH
●	3	3,000 mg/kg TPH
●	4	5,000 mg/kg TPH
●	5	10,000 mg/kg TPH

Figure 5-2: A typical RCBD pots set-up on the bench at the glasshouse using random numbers.

5.3.2 Plant materials, growth conditions and measurements

The growing medium and seeds were obtained from recognised and registered companies in the UK as discussed in Chapter 3. The planting was carried out at Compartment 3 of the Cranfield University Research Glasshouse Facility for 60 d with an average temperature of 24^{o}C and 58.21% relative humidity.

The dormancy of the seeds was broken before planting by soaking them for 24 h in distilled water (Oyedeji et al., 2012). Ten seeds were selected randomly and sown in each of the pots on 7th May, 2014. The evaluation of the percentage seed germination and thinning to one plant per pot were carried out after 10 d of planting. Thereafter, the plant heights and number of leaves ≥ 2.0 cm were assessed weekly starting from the 10th and 20th day respectively. Stomatal conductance (mmol/m^{2}s) prior to harvesting as well as other postharvest measurements was also recorded. At the end of the experiment, the agricultural produce obtained were transferred to the laboratory for processing (freeze-drying inclusive) and proper storage prior to analysis. The growth of the selected leafy vegetables in the treatment pots at the different CRIV is shown in Figure 5.3.

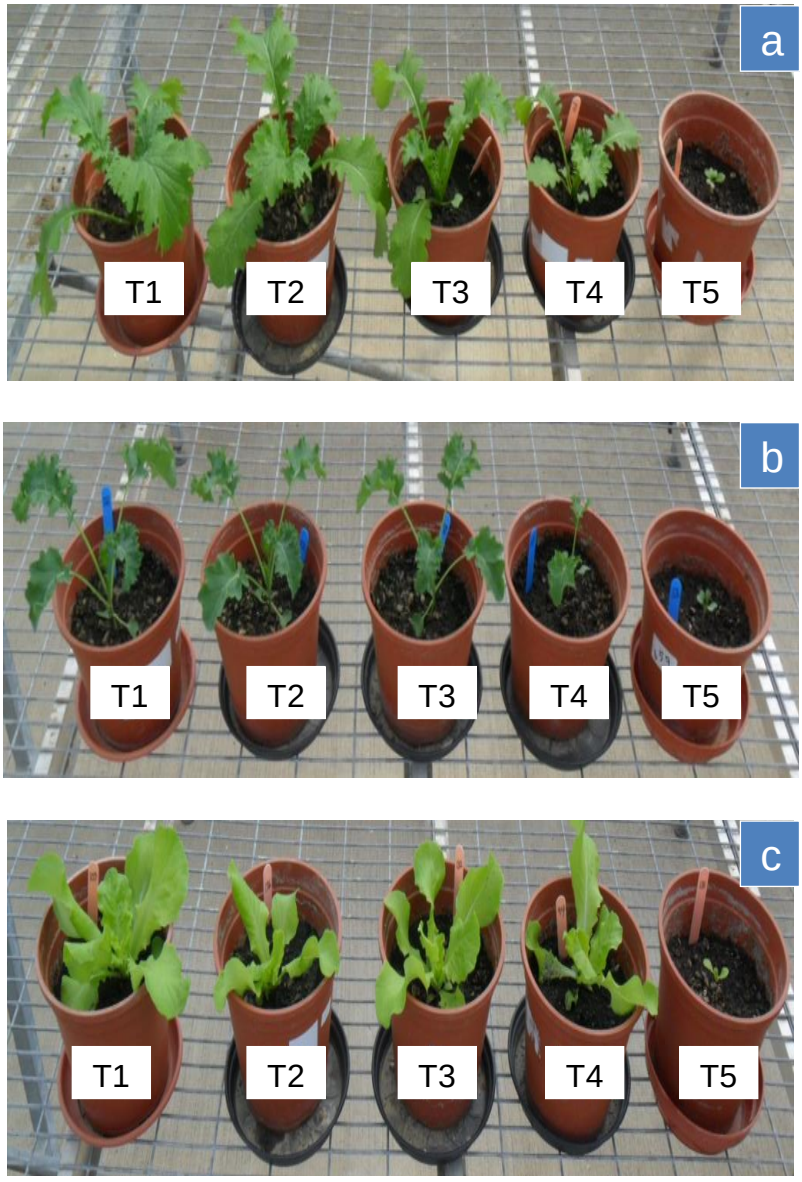


Figure 5-3: The growth of the selected leafy vegetables (a = *B. juncea*, b = *B. oleracea* and c = *L. sativa*) at the glasshouse involving different CRIV (Treatments - T1, T2, T3, T4 and T5 = CRIV at 0 mg/kg TPH - Control, 1,500 mg/kg TPH, 3,000 mg/kg TPH, 5,000 mg/kg TPH and 10,000 mg/kg TPH respectively).

5.3.3 Sample preparation and analysis

The sample preparation were as discussed in Chapter 3 while the nutritional and biochemical composition analysis of the harvested agricultural products proceeded with only two of the three selected green leafy vegetables (*Brassica oleracea* and *Lactuca sativa*) as most of the *Brassica juncea* leaves were lost

as a result of seed production by the plant in the course of the experiment after about 45 d of growth. Figure 5.4 illustrates the flowers and seeds produced by the *Brassica juncea* plant.

Figure 5-4: *Brassica juncea* flower and seed production

5.3.4 Statistical analysis

The effect of the different crude oil: soil treatments on the yield and chemical compositions of the selected leafy vegetables were assessed using IBM SPSS Statistics (IBM Corp. Released 2013. IBM Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.). The Shapiro-Wilk's test was used to determine the normality of the distribution with the application of appropriate data transformation technique where necessary. One-Way ANOVA was employed to compare the means; the Levene's test was applied to check the homogeneity of variance followed by Tukey *post-hoc* test whenever a statistical significant difference is detected among the means ($p < 0.05$). SigmaPlot for Windows Version 11.0 (Systat Software, Inc., 2008) and Microsoft Excel package, 2010 version (Microsoft corporation) were also used when appropriate.

5.4 Results and discussion

5.4.1 Physicochemical properties result

Although the moisture content of the John Innes growing medium (16.12%) (prior to water addition for the experiment) was below the optimum value for oil degradation (Vidali, 2001), the pH (6.51) was found to satisfy the desired requirement. The electrical conductivity was also within the range in which there is almost negligible salinity effects (Richards, 1954) on the selected leafy vegetables. However, the pH is slightly above the desired range of pH 6.0 – 6.2 by lettuce plants (Seaman, 2015).

On the other hand, the C/N ratio is above the acceptable theoretical value of 10:1 required for a mixed microbial population in soil (Waksman, 1924; Dibble and Bartha, 1979) whose optimal value is expected to be higher for petroleum biodegradation (Dibble and Bartha, 1979). The C/P was however found to be lower than the accepted theoretical values of 100:1 given by Thompson et al. (1954, cited in Dibble and Bartha, 1979, p. 737). The results of the physicochemical properties of the John Innes growing medium are shown in Table 5.1.

Table 5-1: Physicochemical properties of the John Innes growing medium

Property	Value
pH	6.51 ± 0.03
Moisture Content (%)	16.12 ± 0.46
Electrical Conductivity (S/m)	0.143 ± 0.00
Total Carbon (%)	11.02 ± 0.43
Total Organic Carbon (%)	10.66 ± 0.29
Total Nitrogen (%)	0.54 ± 0.03
Total Phosphorus (mg/kg)	1880.58 ± 40.58
C/N Ratio	20.37 : 1.00
C/P Ratio	58.59 : 1.00

Values are the means of three replicates ± SE.

5.4.2 Soil hydrocarbons results

Figure 5.5 reveals the distribution of the alkane groups in each of the crude oil-containing treatments used for the leafy vegetables experiment at the start of the experiment in the glasshouse (day 0); the control (0 mg/kg TPH) had no crude oil contamination. The result confirmed the progressive increase in the amount of crude oil added to the growing medium from 1,500 mg/kg to 10,000 mg/kg TPH. However, comparison of the alkanes concentration at 5,000 mg/kg TPH in this experiment and that obtained in the earlier study (Chapter 4, Figure 4.6), showed that the alkane contents were lower. This could be as a result of the use of fume cupboard at the start of the experiment in the previous study as well as the additional storage period of the crude oil used. Hence, the two alkane distributions' results support the importance of environmental conditions evaluation in crude oil remediation studies.

Notably, there is a possibility for interferences arising from human and technical factors during the extraction process as well as GC/MS analysis of the soil extracts to cause a difference in the input levels of the petroleum hydrocarbons at the point of crude oil contamination and the measured concentrations.

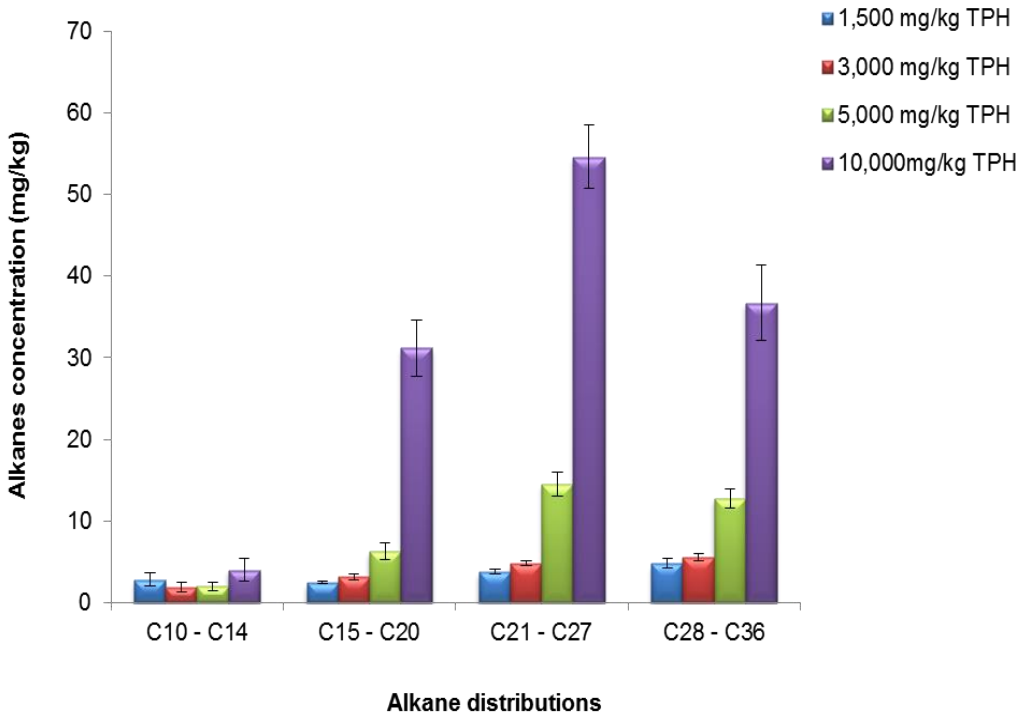


Figure 5-5: Initial alkanes' distribution in the John Innes crude oil-containing treatments at the start of the experiment in the glasshouse (Day 0). Values are the means of three replicates ± SE.

5.4.3 Results and discussions from the glasshouse experiment and analysis of agricultural produce

5.4.3.1 Growth parameters and postharvest measurements

5.4.3.1.1 Percentage seed germination

Among the three selected leafy vegetables (*B. juncea*, *B. oleracea* and *L. sativa*), only the percentage seed germination of *B. juncea* was significantly affected ($p < 0.05$) by the different CRIV used in this experiment. Compared with the control treatment samples, crude oil contamination between 3,000 – 10,000 mg/kg TPH significantly reduced the percentage seed germination of *B. juncea* as shown in Figure 5.6.

The reduction in the percentage seed germination of *B. juncea* at these CRIV (3,000 – 10,000 mg/kg TPH) can be linked to: (1) poor aeration (Ekpo and Ebeagwu, 2009; Al-Moaikal et al., 2012), (2) absorption of oil by the seeds (Al-

Moaikal et al., 2012), (3) inhibition of the uptake of required oxygen and water for germination by the oil coating the seed (Baker, 1970), (4) toxic effect of the oil, and (5) unfavourable soil moisture conditions (Merkl et al., 2004).

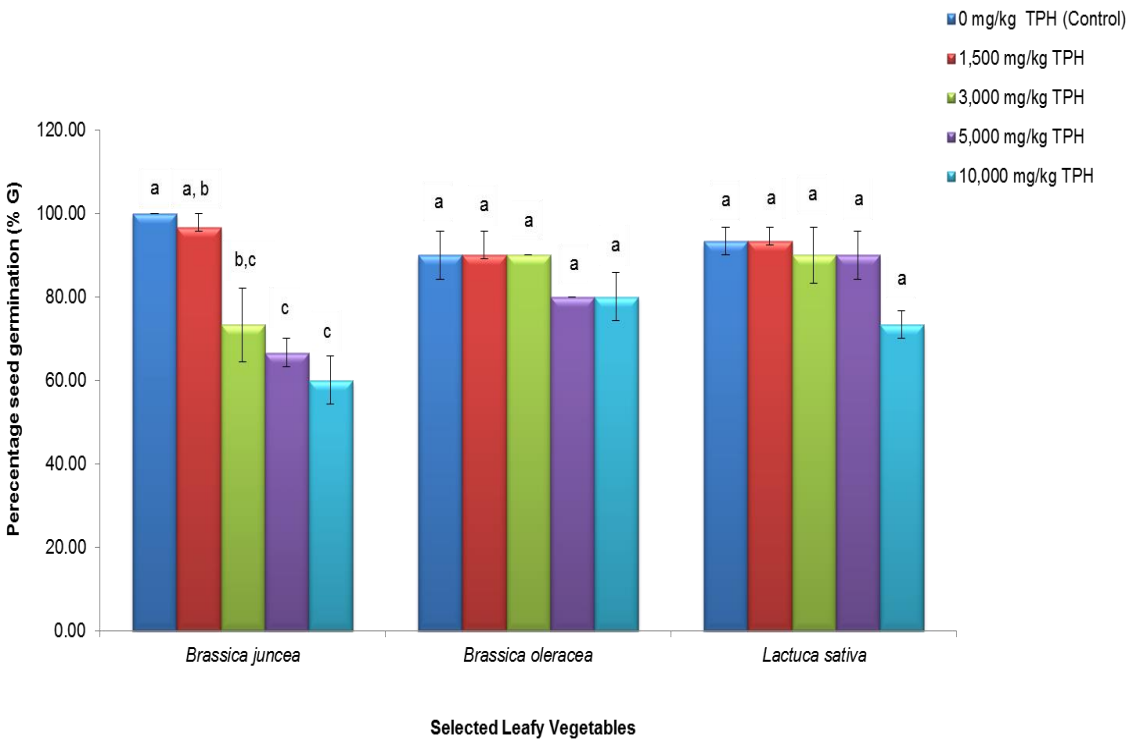


Figure 5-6: Selected leafy vegetables percentage seed germination responses to different CRIV. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

On the other hand and in line with Inckot et al. (2011), the non-significant effect ($p > 0.05$) of CRIV in the range of 1,500 – 10,000 mg/kg TPH on the percentage seed germination of *B. oleracea* and *L. sativa* samples, compared with the control treatment sample, is associated with TPH concentration that is lower than the phytotoxic level and the kind of petroleum used. The former suggestion agrees with that of Tang et al. (2011) in which TPH content of 1.5% was given as the critical value for both animal toxicity and phytotoxicity; this is higher that the range of crude oil concentrations used in the present study.

5.4.3.1.2 Variation in average plant heights and number of leaves

As shown in Figure 5.7 and 5.8, crude oil contamination at 10,000 mg/kg TPH, compared with the control treatment samples, had the most reducing effect on the leafy vegetables' average plant height and average number of leaves respectively. The experiment of Ogbo et al. (2009) and Baruah et al. (2014) gave a similar result in which crude oil contamination led to the reduction in the measured plant growth parameters. This reduced growth of plants under petroleum hydrocarbons contamination can be attributed to the: (1) presence of toxic compounds of petroleum hydrocarbons (Bossert and Bartha, 1985, cited in Merkl et al., 2004, p. 182), (2) hindrance caused by the oil-polluted soil on transpiration and photosynthesis (Amakiri and Onofeghara, 1983; Akapo et al., 2011; Baruah et al., 2014), (3) impact of petroleum hydrocarbons on soil physicochemistry leading to poor wetting and aeration of the soil (Nwazue, 2011) i.e. unsatisfactory soil conditions (De Jong, 1980), and (4) the tendency of hydrocarbons to coat plant roots thereby altering water and nutrient absorption (Xu and Johnson, 1995).

There was however an increased level in the average plant height of *B. juncea* from all the treatments after 45 d of growth due to flower and seed production by the plant.

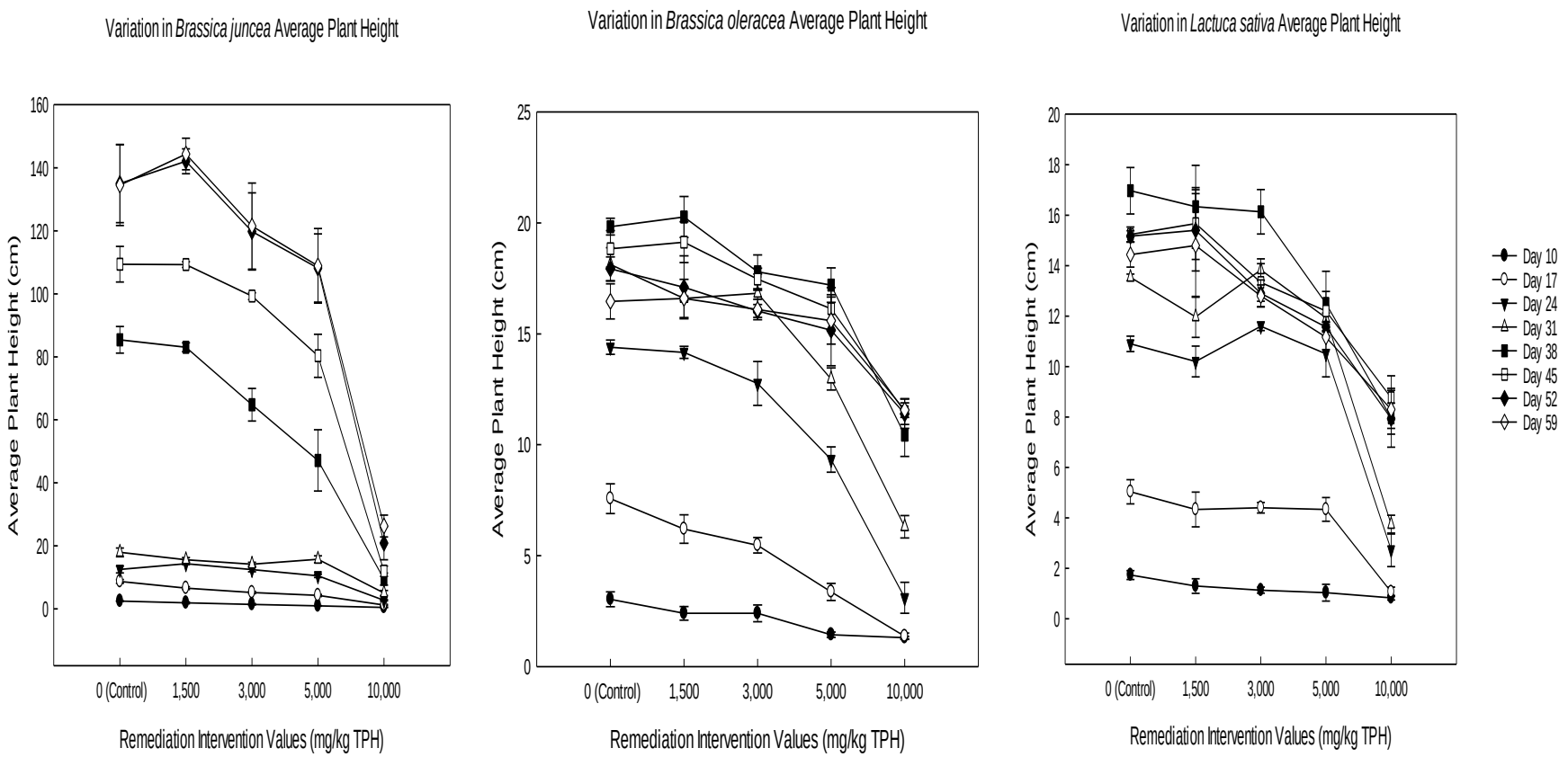


Figure 5-7: Effect of different CRIV on the selected leafy vegetables plant heights – (a) *B. juncea*, (b) *B. oleracea*, and (c) *L. sativa*. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

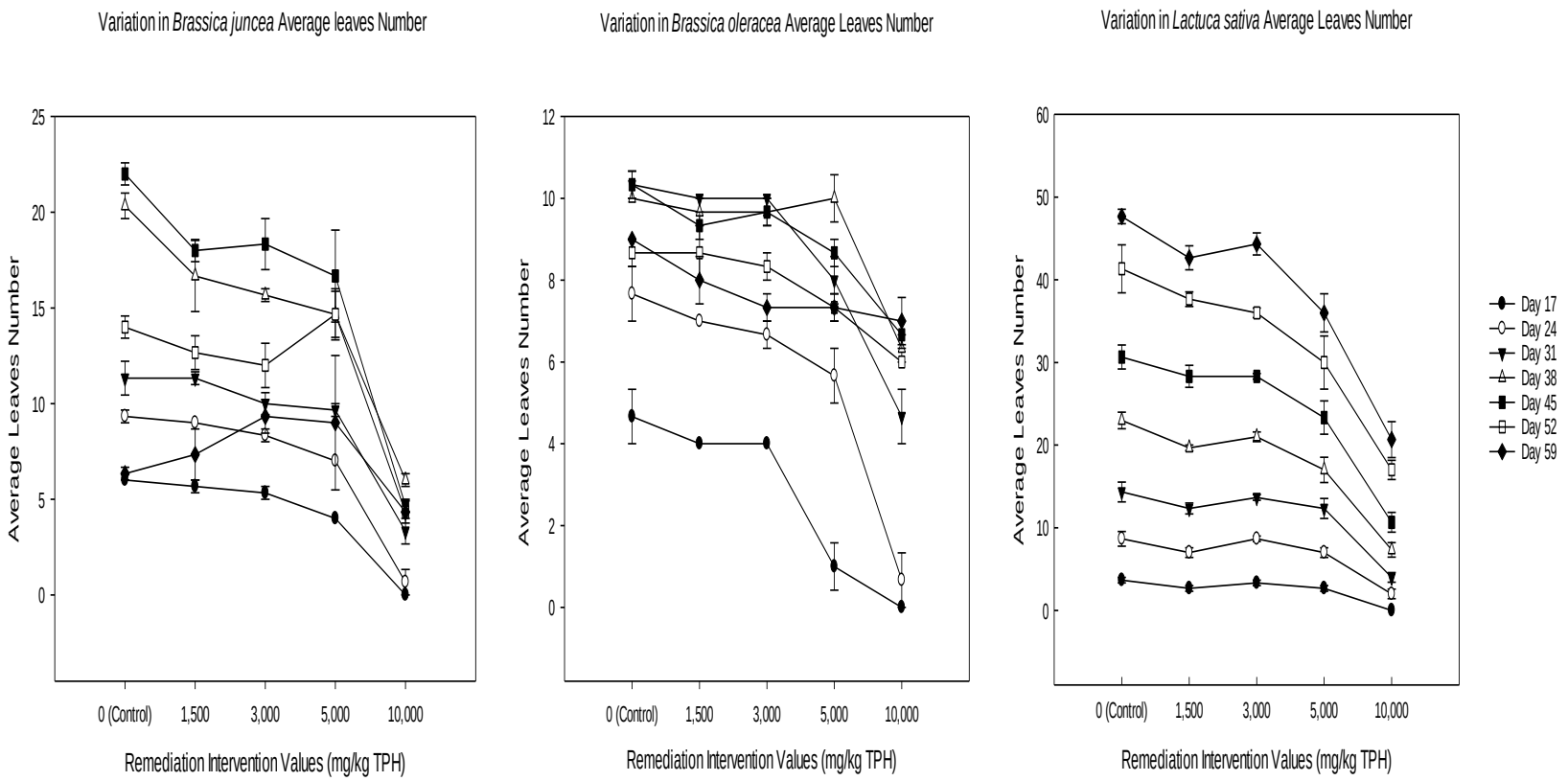


Figure 5-8: Effect of different CRIV on the selected leafy vegetables average number of leaves - (a) *B. juncea*, (b) *B. oleracea*, and (c) *L. sativa*. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

5.4.3.1.3 Changes in stomatal conductance

Figure 5.9 illustrates the changes in the effect of crude oil contamination on the stomatal conductance (mmol/m^{2}s) of the three selected leafy vegetables and provides a general overview of their response under the different CRIV treatments. The assessment of the stomatal conductance of these leafy vegetables, which is a measure of the rate of diffusion of carbon dioxide (CO_2) into the leaves for photosynthesis as well as the rate of water loss in transpiration (Rebetzke et al., 2000), showed that the greatest negative impact of the crude oil contamination was on *B. juncea*. This explains the highest level of impairment caused by crude oil contamination, particularly at 10,000 mg/kg TPH, on the growth parameters of *B. juncea* [e.g. in the average plant height (Figure 5.7) and average number of leaves (Figure 5.8)], when compared with the effect on the other two leafy vegetables at this CRIV.

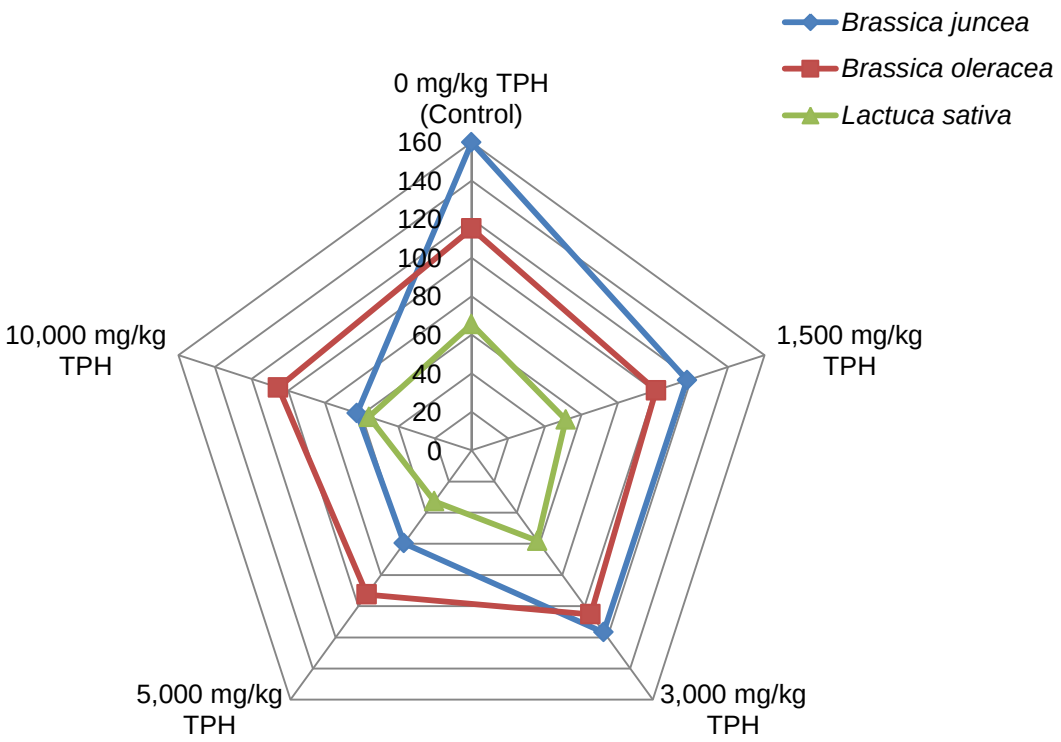


Figure 5-9: Effect of the different CRIV on the stomatal conductance (mmol/m^{2}s) of the selected leafy vegetables prior to harvesting.
Values are the means of three replicates.

Lin and Mendelssohn (1996) indicated the effect of crude oil contamination to include reduced photosynthesis which according to these authors can be as a result of oil penetration to the soil affecting below-ground processes and consequently photosynthesis.

5.4.3.1.4 Variation in *Brassica juncea* flower production

Following the effect of crude oil contamination at 10,000 mg/kg TPH on the stomatal conductance of *B. juncea* as pointed out in Section 5.4.3.1.3, experimental result on the effect of the different CRIV on *B. juncea* flower production also revealed that crude oil contamination at 10,000 mg/kg TPH delayed the flowering and seed production of the plant (see Figure 5.10). This is consistent with the view that crude oil contamination can affect plant development (Merkl et al., 2005, cited in Oyedeji et al., 2013, p. 149) and causes delay in the species' life cycle (Oyedeji et al., 2013).

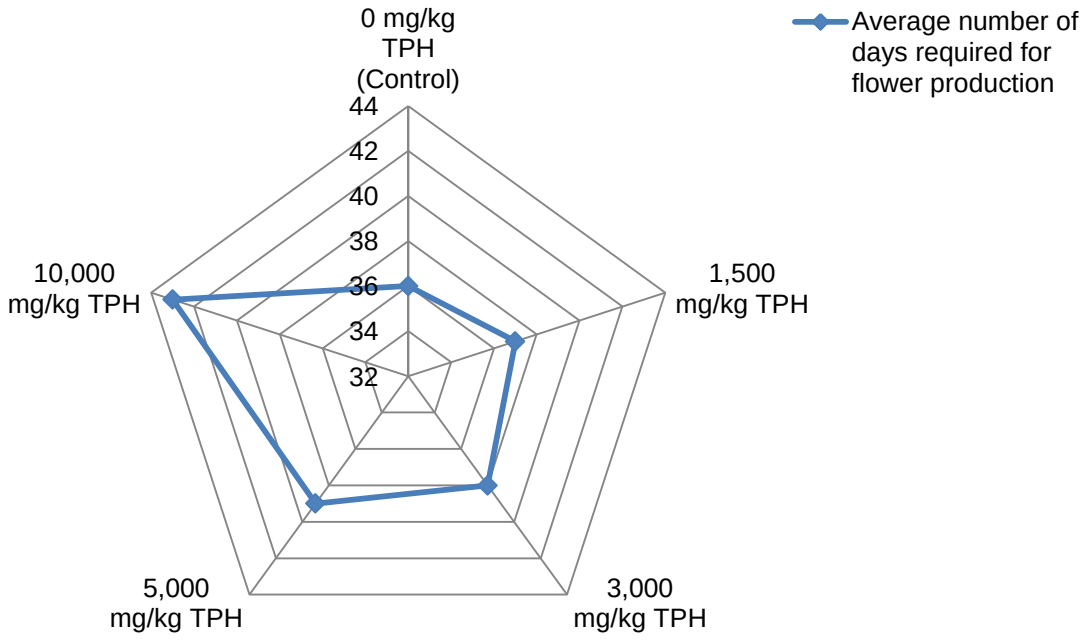


Figure 5-10: Effect of different CRIV on *Brassica juncea* flower production. Values are the means of three replicates.

5.4.3.1.5 Variation in the above-ground plant parts and fresh root yield

The experimental results on the above-ground plant part yield of the leafy vegetables agree with the report of Oyedeji et al. (2013) that the presence of contaminants in the environment can significantly affect shoot growth. Compared with the control treatment samples, crude oil contamination at CRIV between 5,000 – 10,000 mg/kg TPH had a significant reducing effect ($p < 0.05$) on the above-ground plant part yield of *B. juncea* and *B. oleracea* (Figure 5.11). Meanwhile, the yield of the above-ground plant parts of *L. sativa* was the most sensitive among the selected green leafy vegetables to the increase in crude oil contamination as they were significantly affected at CRIV between 3,000 – 10,000 mg/kg TPH.

Although Inckot et al. (2011) indicated that most studies reported reduced growth and plant biomass production in soil contaminated with petroleum, in the view of Acosta-Gallegos and Shibata (1989), poor development of shoots can be an effort of the plant towards reducing the rate of water use and delaying the onset of more severe stress, hence, ensuring survival.

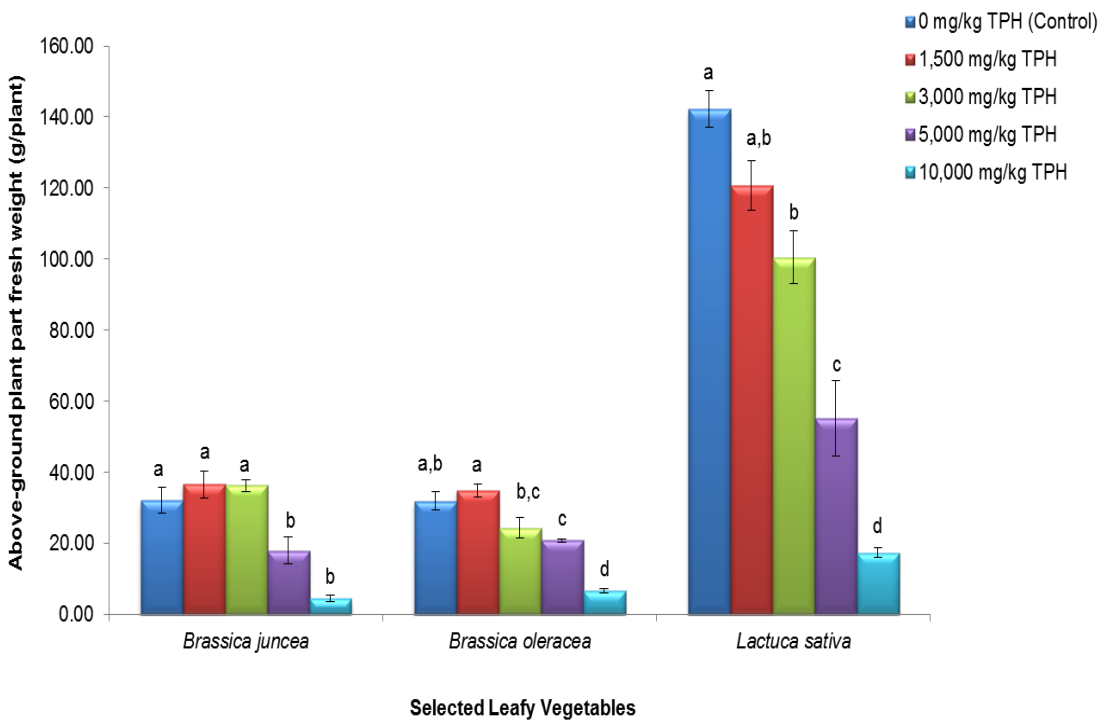


Figure 5-11: Yield of the selected leafy vegetables above-ground plant part at different CRIV. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

In contrast to the observed effect of the different levels of crude oil contamination on the above-ground plant parts yield, the root yields of *B. juncea*, *B. oleracea* and *L. sativa*, compared with the control treatment sample, were significantly affected ($p < 0.05$) by crude oil contamination in the range of: 5,000 – 10,000 mg/kg TPH, 10,000 mg/kg TPH, and 3,000 – 10,000 mg/kg TPH, respectively (see Figure 5.12).

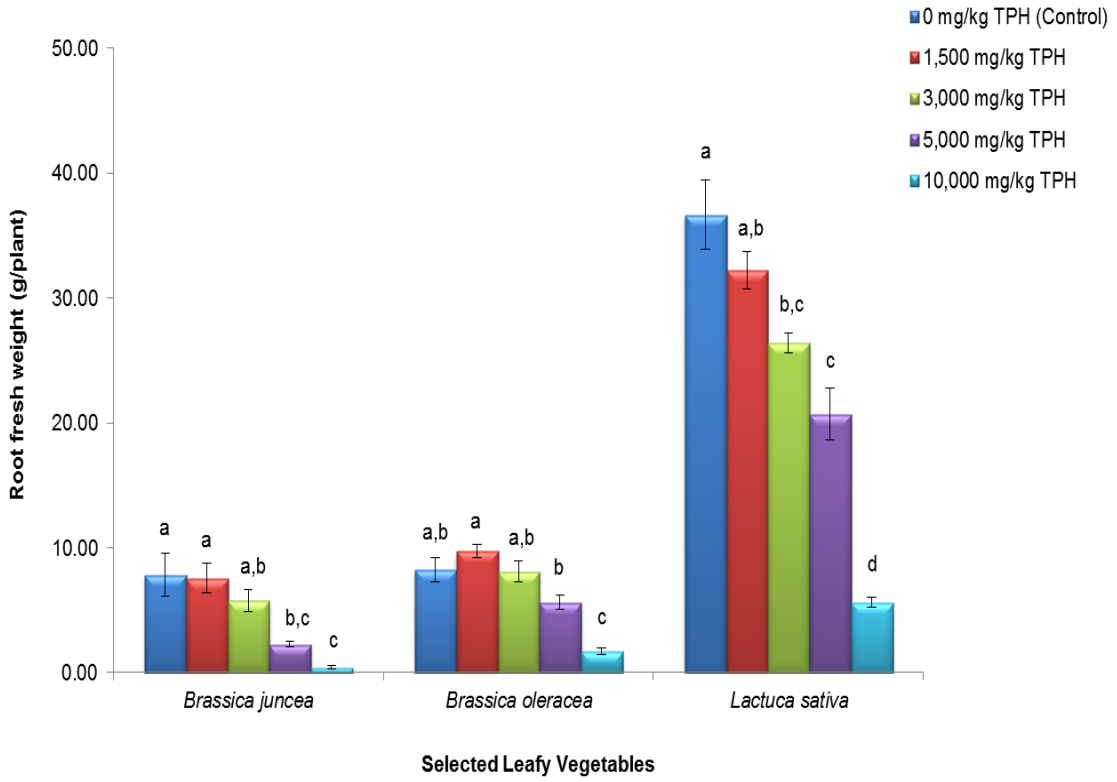


Figure 5-12: Yield of the selected leafy vegetables roots at different CRIV. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Generally and following the report of Jarrell and Beverly (1981), the recorded reduced yield (a function of dry matter accumulation) of these plants at some specific CRIV indicates: (1) blockage of metabolic pathway by 'toxic' concentrations of the crude oil components, (2) decline in photosynthesis, (3) poor photosynthate translocation, (4) water limiting-lowered turgor pressure, (5) hormonal imbalance, and (6) accelerated senescence. Zelitch (1971, cited in Jarrell and Beverly, 1981, p. 202) added that increase in respiration can also give rise to this phenomenon.

5.4.3.2 Nutritional composition analysis results

5.4.3.2.1 Non-structural carbohydrates (sugars) distribution

The statistical analysis of the experimental results revealed that crude oil contamination in the range of 1,500 – 10,000 mg/kg TPH as used in this

experiment, had no significant effect ($p > 0.05$) on the total sugar, fructose and sucrose contents of *B. oleracea* harvested above-ground plant part samples. However, there was a significant reduction ($p = 0.026$) in the glucose concentration following additional increase in crude oil stress from the 5,000 mg/kg TPH treatment to the 10,000 mg/kg TPH treatment as shown in Figure 5.13. This decrease in glucose content agrees with the view of Cramer et al. (2011) that, abiotic stress (which in this case was from crude oil contamination), may affect the synthesis, storage and composition of sugars. Figure 5.14 shows some of the chromatograms obtained in the analysis of the sugars in the leafy vegetables using the HPLC.

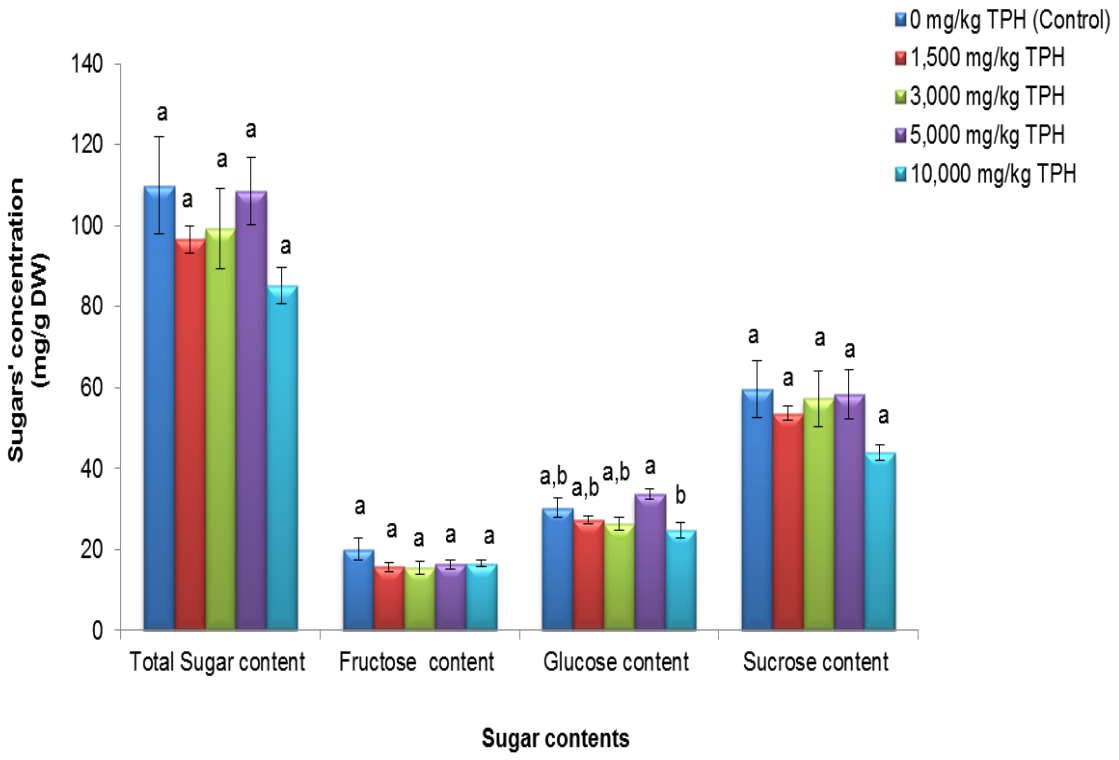


Figure 5-13: Effect of different CRIV on the sugar distribution in *Brassica oleracea.* Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

On the other hand and unlike the sugars' distribution in *B. oleracea*, CRIV of 3,000 – 5,000 mg/kg TPH resulted to a significant reduction ($p < 0.05$) in the total sugar content of the *L. sativa* samples from these treatments compared

with that found in the control treatment samples while increased level of the crude oil stress at 10,000 mg/kg TPH, brought about a significant increase in the total sugars' concentration (Figure 5.15). CRIV of 3,000 – 5,000 mg/kg TPH also significantly reduced the concentration of fructose and glucose in the *L. sativa* samples when compared with the amounts found in the control treatment samples. Meanwhile, only the concentration of sucrose in *L. sativa* samples from the 10,000 mg/kg TPH treatment was significantly different from the amount of this sugar in the control treatment samples.

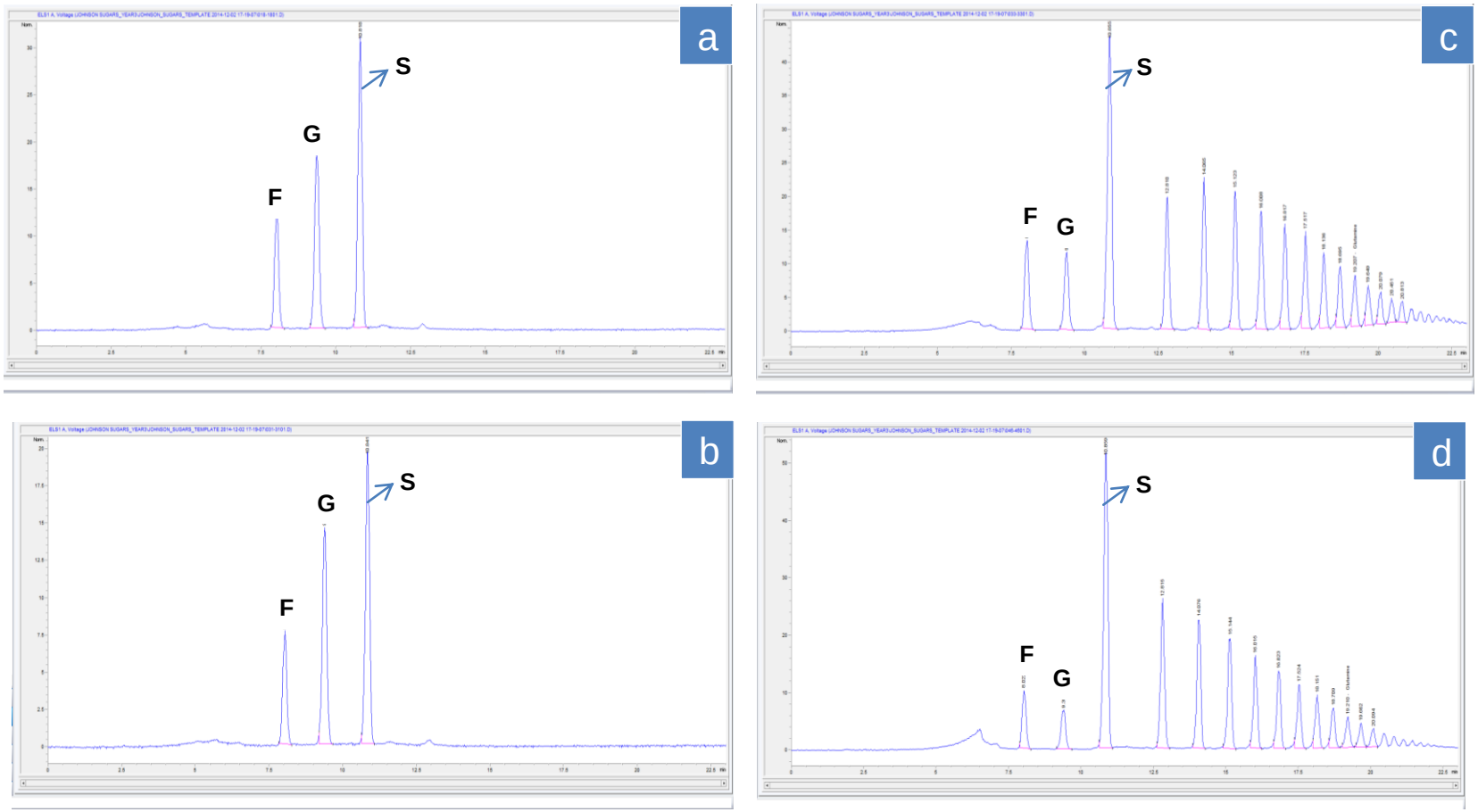


Figure 5-14: Sugars chromatograms in the: *Brassica oleracea* – (a) and (b); *Lactuca sativa* – (c) and (d) at the 0 mg/kg TPH (Control) and 10,000 mg/kg TPH respectively [Peak F, G and S = Fructose, Glucose and Sucrose (Retention times = 8.064, 9.418 and 10.882 mins correspondingly)]

As earlier highlighted and following the report of Cramer et al. (2011), the significant decrease ($p < 0.05$) in the total sugar, fructose and glucose contents of *L. sativa* samples from the 3,000 – 5,000 mg/kg TPH treatments compared to the amount recorded in the control treatment (without crude oil contamination) samples is as a result of the impact of crude oil, an abiotic stress factor (Noori et al., 2012), on the synthesis, storage as well as composition of these sugars.

Contrarily and in line with Wang and Frei (2011), the significant increase in sucrose content of the *L. sativa* samples from the 10,000 mg/kg TPH treatment with respect to the control treatment samples, is as a result of the effect of crude oil stress at this CRIV on the physiology of the plant. Following the report of Wingler et al. (2012) and Muller et al. (2011), the reduced growth of *L. sativa* under the influence of crude oil stress at 10,000 mg/kg TPH (see Figure 5.7) which induced soil water deficit, led to carbon accumulation in the harvested samples from this CRIV treatment.

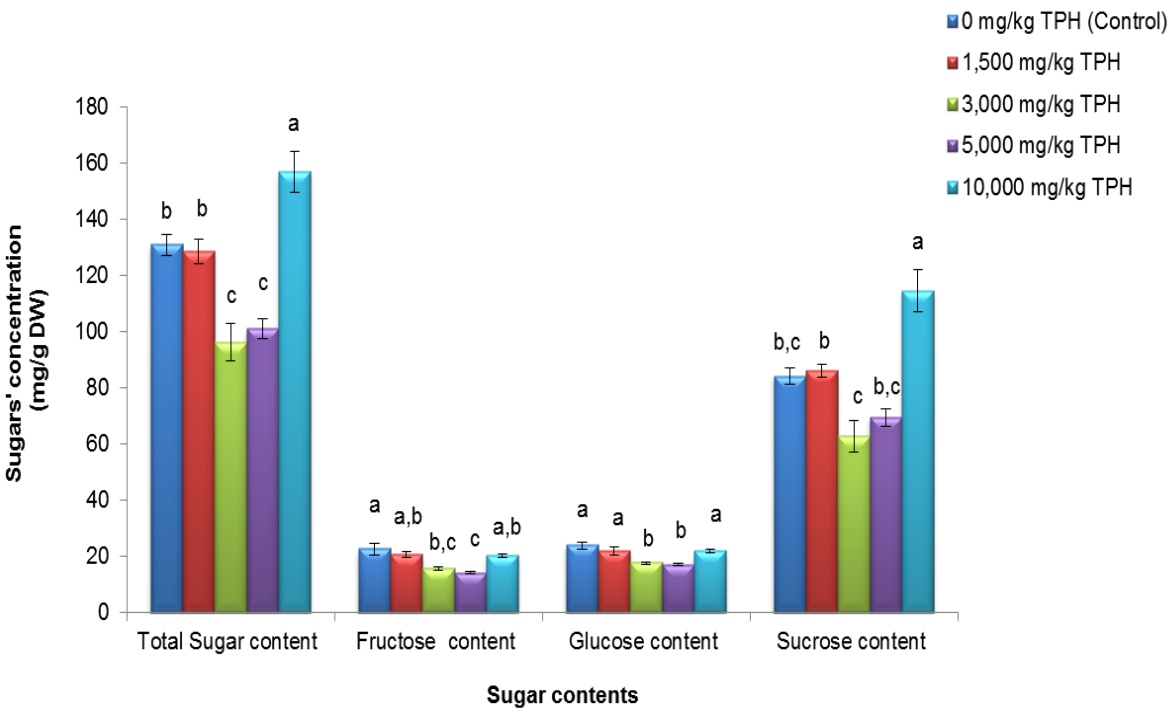


Figure 5-15: Effect of different CRIV on the sugars' distribution in *Lactuca sativa*. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The increase in carbon concentration at 10,000 mg/kg TPH according to Muller et al. (2011) may be linked to organ expansion being affected earlier and more intensively than photosynthesis and metabolism. In addition, the elevated level of sucrose in the *L. sativa* above-ground plant part from the 10,000 mg/kg TPH treatment, suggests that these treatments' plants experienced water stress as Kameli (1990, cited in Akinci and Losel, 2012, p. 21) noted that sugars accumulate in plants under water stress condition. This contributed to the total sugar content, defined as the sum of the fructose, glucose and sucrose content (Ayaz et al., 2006), having the highest concentration of 156.73 mg/g DW in the *L. sativa* samples from the 10,000 mg/kg TPH treatment as shown in Figure 5.15.

Generally, Achuba (2006) considers significant increase in sugar content in plants growing in hydrocarbon-contaminated soil, with respect to the control samples, as an indication of environmental stress.

Sugar ratios

The consumption of fructose has been linked with the possibility of inducing functional gut symptoms (Barrett and GIbson, 2007), hence, according to Food Intolerance Diagnostics (2014) the desired fructose/glucose (F/G) ratio should be less than unity as the presence of glucose in food helps the tolerance level of fructose. The result of the F/G ratio in the leafy vegetables is illustrated in Table 5.2.

Although all the harvested *B. oleracea* samples have F/G ratio < 1, consumption of samples from the 5,000 mg/kg TPH treatment would have the highest tolerance level of fructose as crude oil contamination at this CRIV had a significant reduction ($p < 0.05$) on the F/G ratio of this treatment samples (0.48) compared with that found in the control treatment samples. Meanwhile, following similar comparison with the control treatment samples, crude oil contamination in the range used in this experiment (1,500 – 10,000 mg/kg TPH), had no significant effect ($p > 0.05$) on the F/G ratio (< 1) of the *L. sativa* samples and, hence, no effect on their tolerance level of fructose.

Table 5-2: Sugar ratios in the selected leafy vegetables

Vegetable samples	Treatments	F/G ratio	(G + F)/S
Brassica oleracea	0 mg/kg TPH (Control)	0.66 ± 0.04^{a}	$0.85 \pm 0.02^{a,b}$
	1,500 mg/kg TPH	$0.58 \pm 0.04^{a,b}$	$0.80 \pm 0.01^{a,b}$
	3,000 mg/kg TPH	$0.59 \pm 0.02^{a,b}$	0.73 ± 0.04^{b}
	5,000 mg/kg TPH	0.48 ± 0.01^{b}	$0.86 \pm 0.05^{a,b}$
	10,000 mg/kg TPH	0.66 ± 0.02^{a}	0.94 ± 0.03^{a}
Lactuca sativa	0 mg/kg TPH (Control)	0.95 ± 0.04^{a}	0.56 ± 0.05^{a}
	1,500 mg/kg TPH	0.94 ± 0.04^{a}	$0.50 \pm 0.01^{a,b}$
	3,000 mg/kg TPH	0.89 ± 0.02^{a}	0.53 ± 0.04^{a}
	5,000 mg/kg TPH	0.84 ± 0.01^{a}	$0.45 \pm 0.01^{a,b}$
	10,000 mg/kg TPH	0.92 ± 0.03^{a}	0.37 ± 0.03^{b}

F/G = Fructose/Glucose ratio.
(G + F)/S = (Glucose + Fructose)/Sucrose.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

On the other hand, the only significant difference in the monosaccharide/disaccharide [(G + F)/S] ratio in the *B. oleracea* samples was found between the values obtained in the 3,000 mg/kg TPH and 10,000 mg/kg TPH treatments' samples. In the *L. sativa*, crude oil contamination at 10,000 mg/kg TPH significantly reduced the [(G + F)/S] ratio when compared with the value of this ratio in the control and 3,000 mg/kg TPH treatments' samples.

5.4.3.2.2 Crude protein contents

Tukey *post-hoc* analysis revealed that crude oil contamination at 10,000 mg/kg TPH significantly increased ($p < 0.05$) the level of crude protein content in *B. oleracea* (Figure 5.16). Meanwhile, for the *L. sativa* harvested samples, there was an initial significant reduction ($p < 0.05$) at 5,000 mg/kg TPH before the observed increase at 10,000 mg/kg TPH.

This decrease in crude protein content of *L. sativa* samples from the 5,000 mg/kg TPH treatment can be attributed to the: (1) effect of induced water stress by the crude oil contamination at this CRIV on the plant metabolism leading to a reduction

in protein synthesis (Akinci and Losel, 2012), or (2) formation of soluble and insoluble protein-polyphenol complexes arising from the binding affinity of polyphenols for proteins (Papadopoulou and Frazier, 2003). Haslam (1966, cited in McManus et al., 1985; p. 1429) also indicated the ability of polyphenols to associate with proteins.

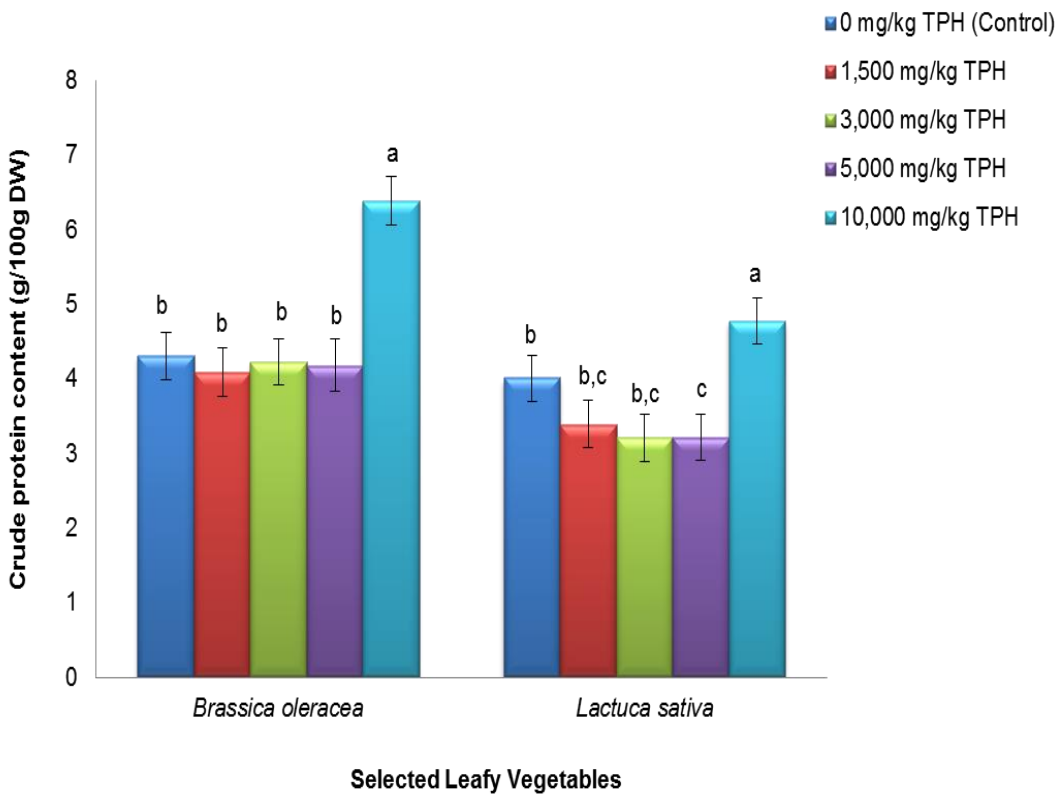


Figure 5-16: Crude protein distribution in the selected leafy vegetables. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

On the other hand and in line with the report of Achuba (2006), the elevated concentration of crude protein in *B. oleracea* and *L. sativa* samples at 10,000 mg/kg TPH, is an indication of induced environmental stress by the crude oil contamination. This view is also supported by the experiment of Al-Hawas et al. (2012) while free proline has been identified by Hare and Cress (1997) to accumulate in some plants under environmental stresses such as water deficit where it serves as a defence mechanism against osmotic stress by acting as a compatible solute. Rhodes et al. (1999) also acknowledged proline accumulation as a common metabolic response of

higher plants to water deficit which was noted by Bates et al. (1973) to have a faster proportionate increase than other amino acids in plants under water stress.

Furthermore, the accumulation of this amino acid (proline) is reported as a response of plants under nutrient deficiency (Delauney and Verma, 1993) while (Smirnoff and Cumbes, 1989; Rhodes et al. 1999) noted that proline can be involved in the scavenging of hydroxyl radical in the stressed plants. In view of the anaerobic stress caused by crude oil contamination, Rhodes et al. (1999) as well as Streeter and Thompson (1972) indicated that amino acids such as alanine and γ-aminobutyric acid (GAB) accumulate in plants under anaerobic conditions.

Also, as stated in Pinto et al. (2014), K plays a key role in protein synthesis, enzyme activation and osmotic function. Hence, the maximum level of K observed at 10,000 mg/kg TPH in the above-ground plant part of the selected vegetables - *B. oleracea* (17,567.27 mg/kg DW) (Figure 5.17) and *L. sativa* (38,643.93 mg/kg DW) (Figure 5.18), contributed to the observed elevated protein content at this CRIV.

Basically, the increase in crude protein content in the two assessed leafy vegetables at 10,000 mg/kg TPH, showed that some amino acids accumulated in the plant samples from the treatments involving this CRIV as a result of the induced water (osmotic) stress, anaerobic stress as well as nutrient deficiency. This is in line with previous reports as environmental stress has been found to lead to increase in protein content of harvested plants (Wang and Frei, 2011) which also points to a less severe impact of crude oil stress at this CRIV on the nitrogen harvest index compared to the dry matter harvest index of the two leafy vegetables as the dry matter accumulation of crops is usually negatively affected by stresses (Gooding et al., 2003; Wang and Frei, 2011).

5.4.3.2.3 Mineral analysis results

Validation of the minerals analytical method

The analysis of the NIST 1515 Apple Leaves SRM using the ICP-MS, and AAS where appropriate showed that the obtained/achieved values for the mineral contents in the SRM following the analytical method employed in assessing the distribution of the minerals in the samples, were consistent with the provided certified values (Table 5.3).

Table 5-3: Results of the mineral analysis of NIST 1515 Apple Leaves SRM

Metals	SRM 1515 Apple Leaves Certified Value (mg/kg DW)	ICP-MS Achieved Value (mg/kg DW)
Na	24.40 ± 1.20	21.00 ± 3.30
Mg	0.271 ± 0.008[a]	0.2536 ± 0.00[a]
Ca	1.526 ± 0.015[a]	1.4987 ± 0.01[a]
K	1.61 ± 0.02[a]	1.4424 ± 0.00[a,b]
P	0.159 ± 0.011[a]	0.1632 ± 0.00[a]
Cr	(0.30)[c]	1.11 ± 0.08
Mn	54.00 ± 3.00	51.83 ± 0.53
Co	(0.09)[c]	0.08 ± 0.00
Cu	5.64 ± 0.24	4.45 ± 0.10
Zn	12.50 ± 0.30	12.56 ± 0.19
Mo	0.094 ± 0.013	0.08 ± 0.01
Fe	83.00 ± 5.00	63.53 ± 1.14[b]
Se	0.05 ± 0.009	0.22 ± 0.01
V	0.26 ± 0.03	0.11 ± 0.00
Ni	0.91 ± 0.12	1.31 ± 0.29
Al	286.00 ± 9.00	138.40 ± 2.47
As	0.038 ± 0.007	0.14 ± 0.00
Sr	25.00 ± 2.00	23.83 ± 0.24
Ba	49.00 ± 2.00	43.20 ± 0.27
Cd	0.013 ± 0.002	0.01 ± 0.00
Pb	0.47 ± 0.024	0.31 ± 0.01

[a]Values in weight percent
[b]Values obtained via the use of the AAS
[c]Non-certified values

Minerals distribution in *Brassica oleracea* L. acephala group

As highlighted in Section 5.4.3.1.5 (Figure 5.11), crude oil application at 5,000 mg/kg TPH and 10,000 mg/kg TPH had a significant reducing effect on the yield of *B. oleracea* compared with that obtained from the control treatment with no crude oil

contamination. Hence, the factors responsible for the observed high and significant concentration ($p < 0.05$) of Ca, K, Mn, Sr, Ba and detectable amount of Ni (Table 5.4) as well as contributing to this response pattern (concentration effect owing to reduced yield) of *B. oleracea* samples at 10,000 mg/kg TPH are: (1) faster rate of movement to the roots (Nye and Tinker, 1977, cited in Jarrell and Beverly, 1981, p. 200) by these minerals, (2) enhanced root activity (Pearson and Steer, 1977, cited in Jarrell and Beverly, 1981, p. 200) at this CRIV, (3) increased transport of these minerals to the above-ground plant part (Pitman, 1975, cited in Jarrell and Beverly, 1981, p. 200), (4) greater demand for these minerals (Jarrell and Beverly, 1981) by the plant, and (5) positive impact on these minerals uptake mechanisms (van Steveninck, 1965, cited in Jarrell and Beverly, 1981, p. 200) by the crude oil contamination at this CRIV.

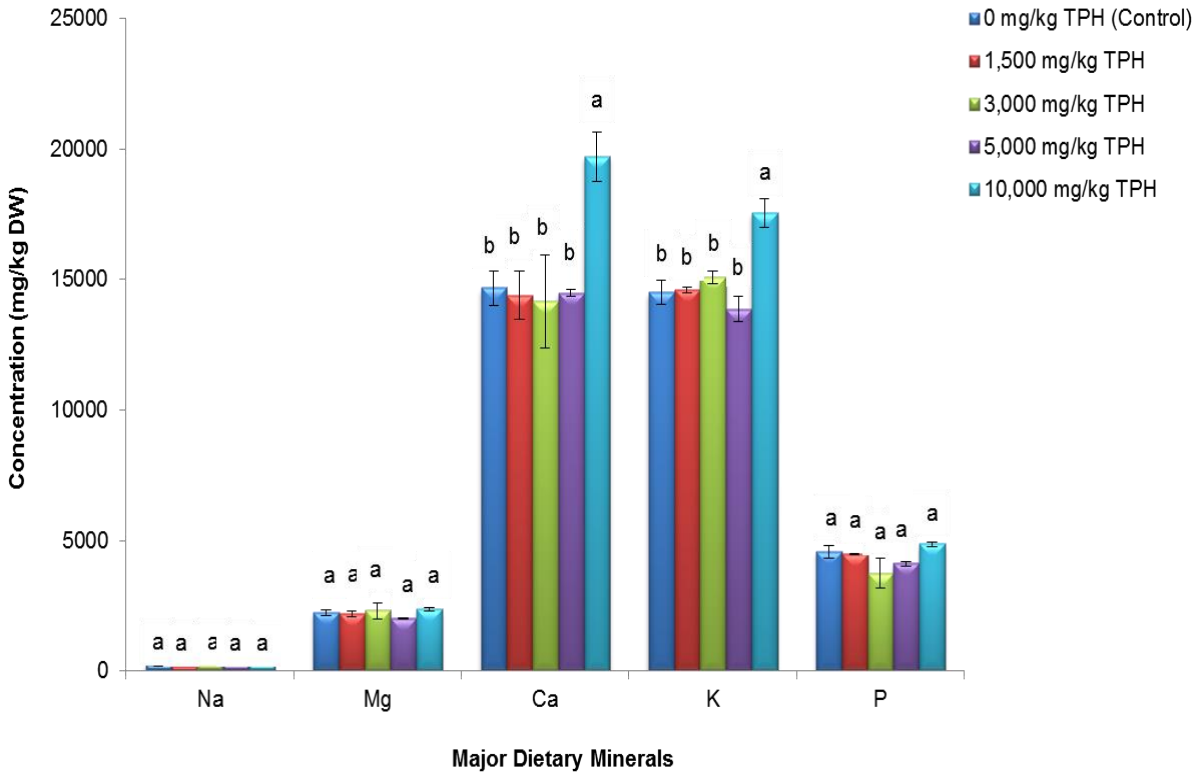


Figure 5-17: The distribution of major dietary minerals in *Brassica oleracea* above-ground plant parts. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Meanwhile, the non-significant effect ($p > 0.05$) of the 5,000 mg/kg TPH treatment, despite the reduced yield of *B. oleracea* at this CRIV, on the mineral

contents of the samples (with the exception of Fe and V contents, Table 5.4) compared with the control treatment samples, indicates that there is no direct interaction between crude oil contamination at 5,000 mg/kg TPH and the analysed minerals (Jarrell and Beverly, 1981).

In addition, results of the mineral analysis of the *B. oleracea* samples revealed an antagonistic effect (Jarrell and Beverly, 1981) of crude oil contamination in the range used in this experiment on the Fe and V content of *B. oleracea* as the control samples (with no crude oil contamination) had significantly higher concentrations ($p < 0.05$) of Fe and V than the amount found in samples from the crude oil-containing treatments (i.e. samples from CRIV between 1,500 – 10,000 mg/kg TPH).

Table 5-4: Distribution of minor dietary minerals (mg/kg DW) in *Brassica oleracea* above-ground plant parts

Treatments	Distribution of Minor Dietary Minerals (mg/kg DW)									
	Cr	Mn	Co	Cu	Zn	Mo	Fe	Se	V	Ni
Treatment 1 (0 mg/kg TPH, Control)	1.23 ± 0.36^{a}	86.93 ± 12.43^{b}	0.32 ± 0.08^{a}	1.79 ± 0.14^{a}	19.86 ± 2.69^{a}	1.14 ± 0.13^{a,b}	48.10 ± 3.75^{a}	0.28 ± 0.02^{a}	0.06 ± 0.01	ND
Treatment 2 (1,500 mg/kg TPH)	1.12 ± 0.39^{a}	80.73 ± 8.48^{b}	0.24 ± 0.01^{a}	1.77 ± 0.15^{a}	16.79 ± 0.37^{a}	0.91 ± 0.45^{b}	27.73 ± 0.96^{b}	0.27 ± 0.03^{a}	ND	ND
Treatment 3 (3,000 mg/kg TPH)	0.71 ± 0.06^{a}	92.13 ± 5.67^{b}	0.30 ± 0.10^{a}	1.79 ± 0.34^{a}	17.55 ± 0.33^{a}	1.19 ± 0.04^{a,b}	27.60 ± 0.95^{b}	0.32 ± 0.02^{a}	ND	ND
Treatment 4 (5,000 mg/kg TPH)	0.74 ± 0.07^{a}	98.19 ± 9.65^{b}	0.31 ± 0.02^{a}	1.86 ± 0.08^{a}	17.79 ± 0.72^{a}	1.23 ± 0.05^{a,b}	34.70 ± 2.59^{b}	0.33 ± 0.06^{a}	ND	ND
Treatment 5 (10,000 mg/kg TPH)	0.97 ± 0.08^{a}	152.26 ± 7.74^{a}	0.52 ± 0.04^{a}	2.13 ± 0.33^{a}	21.31 ± 2.51^{a}	2.12 ± 0.12^{a}	36.87 ± 1.38^{b}	0.37 ± 0.02^{a}	ND	1.75 ± 0.36

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.
ND = Not Detected.

Assessment of the Cd contents showed that the levels of this mineral contaminant in all the harvested above-ground plant part of *B. oleracea* samples were below the FAO/WHO Codex maximum level of 0.20 mg/kg for leafy vegetables (Codex Alimentarius, 2015) while the Pb contents of these samples were also below the limit of detection (Table 5.5). Consequently, the consumption of the harvested samples would not contribute to any human health risk arising from the intake of these mineral contaminants.

Practically, the general variations in the *B. oleracea* samples' minerals distribution is associated with crude oil stress interference on carbon assimilation by the plant thereby resulting to more reducing effect on the total biomass accumulation than the uptake of some of these minerals and/or as a result of changes in the distribution of the assimilated nutrients by the plant (Pikki et al., 2007). Also, the observed variations in the minerals contents within this same vegetable type is linked to the difference in soil and environmental conditions (Szefer and Nriagu, 2007; Martinez-Ballesta et al., 2010).

Table 5-5: Distribution of undesirable mineral contaminants (mg/kg DW) in *Brassica oleracea* above-ground plant parts

Treatments	Distribution of Mineral Contaminants (mg/kg DW)					
	Al	As	Sr	Ba	Cd	Pb
Treatment 1 (0 mg/kg TPH, Control)	7.46 ± 2.32[a]	0.16 ± 0.00[a]	39.04 ± 1.75[a]	4.58 ± 0.13[a]	0.18 ± 0.02[a]	ND
Treatment 2 (1,500 mg/kg TPH)	2.37 ± 0.84[a]	0.16 ± 0.04[a]	38.11 ± 5.10[a]	5.02 ± 0.39[a]	0.18 ± 0.01[a]	ND
Treatment 3 (3,000 mg/kg TPH)	2.59 ± 0.60[a]	0.19 ± 0.06[a]	39.37 ± 2.43[a]	5.06 ± 0.56[a,b]	0.18 ± 0.01[a]	ND
Treatment 4 (5,000 mg/kg TPH)	2.81 ± 1.24[a]	0.19 ± 0.02[a]	39.64 ± 0.47[a]	5.17 ± 1.25[a,b]	0.18 ± 0.00[a]	ND
Treatment 5 (10,000 mg/kg TPH)	6.65 ± 0.56[a]	0.23 ± 0.04[a]	53.77 ± 2.43[b]	8.10 ± 0.35[b]	0.19 ± 0.01[a]	ND

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.
ND = Not Detected.

Minerals distribution in *Lactuca sativa*

Crude oil contamination in the range used in this experiment (1,500 – 10,000 mg/kg TPH) enhanced the Na contents in the harvested *L. sativa* samples (Figure 5.18). The significant reduced yield ($p < 0.05$) of the samples at CRIV between 3,000 – 10,000 mg/kg TPH (Figure 5.11) led to the observed concentration effect in the treatments' samples. This response pattern (i.e. concentration effect) was, however, more pronounced in samples from the 10,000 mg/kg TPH treatment (with the least yield, Figure 5.11) as they contain significant higher levels ($p < 0.05$) of Na, K, Cr, Mn, Co, Fe, Se, V, Al, As, Sr and Ba compared with the amount of these minerals in the control treatment samples. The enhanced concentration of these minerals at this CRIV is as a result of the similar factors attributed to the concentration effect in *B. oleracea* samples obtained from the 10,000 mg/kg TPH treatment earlier mentioned.

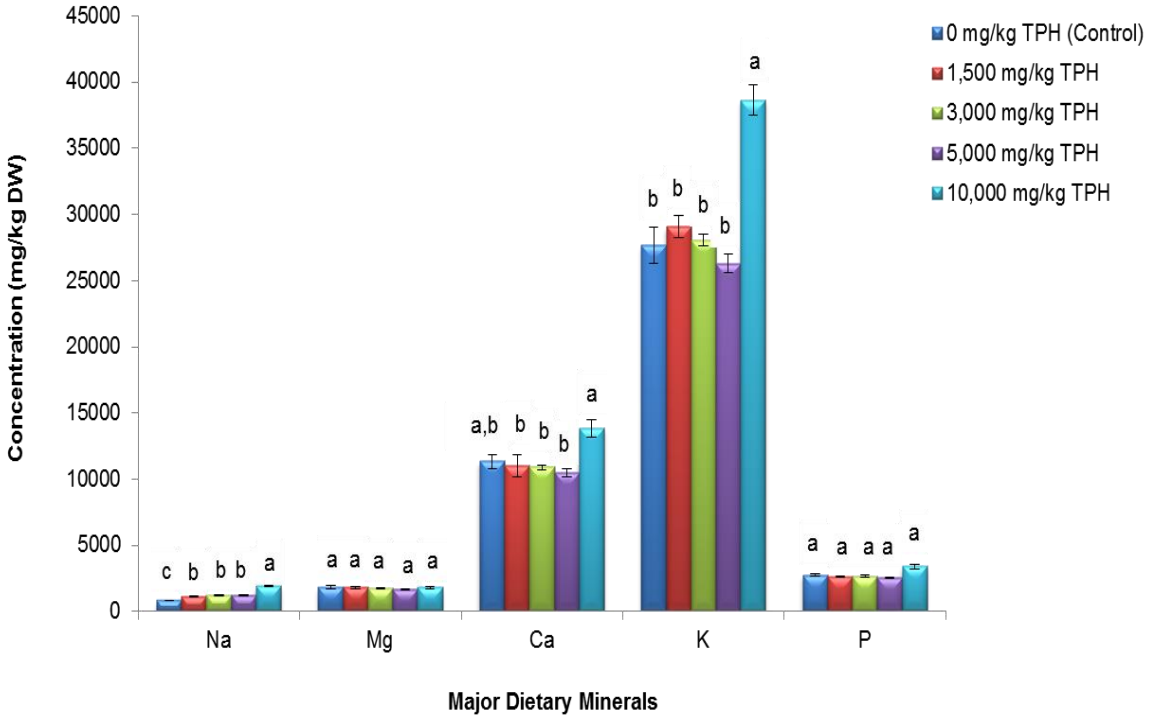


Figure 5-18: The distribution of major dietary minerals (mg/kg) in *Lactuca sativa* above-ground plant parts. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Compared with the control treatment samples, the reduced yield at the 5,000 mg/kg TPH treatment also significantly increased the As concentration of the *L. sativa* samples harvested at this CRIV while the reduced yield at the 3,000 mg/kg TPH treatment only favoured the Na content. Hence, with the exception of Na and As contents, there is no direct interaction (Jarrell and Beverly, 1981) between crude oil application at 3,000 – 5,000 mg/kg TPH and most of the analysed minerals in *L. sativa* samples from these treatments despite the reduced yield recorded. Meanwhile, Ni was not detected in all the harvested *L. sativa* samples (Table 5.6).

Table 5-6: Distribution of minor dietary minerals (mg/kg) in *Lactuca sativa* above-ground plant parts

Treatments	Distribution of Minor Dietary Minerals (mg/kg)									
	Cr	Mn	Co	Cu	Zn	Mo	Fe	Se	V	Ni
Treatment 1 (0 mg/kg TPH, Control)	0.84 ± 0.00^{b,c}	77.33 ± 5.07^{b}	0.09 ± 0.00^{b}	0.90 ± 0.14^{a}	17.81 ± 0.81^{a}	0.03 ± 0.01^{a}	94.87 ± 1.62^{b}	0.47 ± 0.06^{b}	0.15 ± 0.03^{b}	ND
Treatment 2 (1,500 mg/kg TPH)	0.71 ± 0.04^{c}	71.39 ± 6.04^{b}	0.10 ± 0.01^{b}	0.79 ± 0.12^{a}	18.76 ± 1.15^{a}	0.01 ± 0.00^{a}	72.60 ± 7.78^{b}	0.63 ± 0.04^{b}	0.17 ± 0.02^{b}	ND
Treatment 3 (3,000 mg/kg TPH)	0.84 ± 0.04^{b,c}	85.79 ± 4.37^{b}	0.10 ± 0.00^{b}	1.03 ± 0.23^{a}	18.11 ± 0.55^{a}	0.03 ± 0.00^{a}	83.80 ± 10.08^{b}	0.61 ± 0.03^{b}	0.19 ± 0.02^{b}	ND
Treatment 4 (5,000 mg/kg TPH)	1.01 ± 0.03^{b}	96.99 ± 9.45^{b}	0.12 ± 0.02^{b}	1.29 ± 0.04^{a}	17.29 ± 0.38^{a}	0.04 ± 0.01^{a}	85.07 ± 14.05^{b}	0.63 ± 0.04^{b}	0.19 ± 0.02^{b}	ND
Treatment 5 (10,000 mg/kg TPH)	1.46 ± 0.09^{a}	172.53 ± 3.03^{a}	0.22 ± 0.01^{a}	1.67 ± 0.69^{a}	17.27 ± 0.58^{a}	0.07 ± 0.04^{a}	259.13 ± 12.61^{a}	1.47 ± 0.06^{a}	0.49 ± 0.02^{a}	ND

Values are the mean of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.
ND = Not Detected.

Furthermore, the high amount of Al in the *L. sativa* harvested above-ground plant part at CRIV of 10,000 mg/kg TPH (206.65 mg/kg DW), imply exposure to Al stress or toxicity for the plant at this remediation intervention value in which the secretion of organic acids may occur internally, especially in the roots as the plant struggles under this condition (Ondo et al., 2013). Consequently, this Al stress or toxicity may have contributed to the high amount of malic acid and detectable tartaric acid in the *L. sativa* above-ground plant part at this remediation intervention value of 10,000 mg/kg TPH (Figure 5.22).

Although, only the Cd contents in the control (no crude oil addition) and 10,000 mg/kg TPH treatment samples were below the FAO/WHO Codex maximum level of 0.20 mg/kg (Codex Alimentarius, 2015), the Cd contents of all the harvested *L. sativa* samples were lower than the maximum permissible concentration of the element (1 mg/kg DW) in vegetables for human consumption as indicated by Reilly (1980, cited in Samara, 1992, p. 64). In addition, the Pb contents in all the harvested above-ground plant parts of the vegetable (Table 5.7) were below the recommended and permitted maximum concentration of 0.30 mg/kg for Pb in leafy vegetables (Codex Alimentarius, 2015).

Generally, according to Martínez-Ballesta et al. (2010), the mineral nutrient composition of crops change (as observed in the two green leafy vegetables) when: (1) external conditions (such as environmental stress) affect plant growth, and (2) there is a reduction in plant growth as well as biomass at harvest. The reduction in growth of the leafy vegetables from the 10,000 mg/kg TPH treatment, in particular, support the view of Onwurah et al. (2007) that the effect of crude oil presence in the soil, lead to enhanced accumulation of Mn (as in *B. oleracea*, Table 5.4; *L. sativa*, Table 5.6) and Al (as in *L. sativa*, Table 5.7) which are toxic to plant growth as observed in this experiment.

Table 5-7: Distribution of undesirable mineral contaminants (mg/kg) in *Lactuca sativa* above-ground plant parts

Treatments	Distribution of Mineral Contaminants (mg/kg)					
	Al	As	Sr	Ba	Cd	Pb
Treatment 1 (0 mg/kg TPH, Control)	63.79 ± 12.08^{a}	0.09 ± 0.01^{a}	28.91 ± 1.31^{a}	2.68 ± 0.02^{a}	0.19 ± 0.01^{a,b}	0.06 ± 0.01^{a}
Treatment 2 (1,500 mg/kg TPH)	54.45 ± 9.94^{a}	0.13 ± 0.01^{a,b}	28.77 ± 2.25^{a}	2.70 ± 0.25^{a}	0.21 ± 0.01^{a,b}	0.04 ± 0.00^{a}
Treatment 3 (3,000 mg/kg TPH)	60.05 ± 10.22^{a}	0.13 ± 0.01^{a,b}	28.31 ± 0.90^{a}	2.74 ± 0.17^{a}	0.22 ± 0.01^{b}	0.05 ± 0.01^{a}
Treatment 4 (5,000 mg/kg TPH)	64.92 ± 11.11^{a}	0.16 ± 0.00^{b}	27.84 ± 0.77^{a}	2.76 ± 0.01^{a}	0.22 ± 0.01^{b}	0.09 ± 0.01^{a}
Treatment 5 (10,000 mg/kg TPH)	206.65 ± 13.87^{b}	0.34 ± 0.02^{c}	37.64 ± 1.77^{b}	4.37 ± 0.23^{b}	0.17 ± 0.02^{a}	0.14 ± 0.02^{a}

Values are the mean of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Leafy vegetables mineral ratios

Although CRIV in the range of 1,500 – 10,000 mg/kg TPH had no significant effect ($p > 0.05$) on the Ca/P ratio and Na/K ratio of the *L. sativa* and *B. oleracea* harvested samples respectively (Table 5.8), crude oil contamination at 10,000 mg/kg TPH significantly increased ($p < 0.05$) the Ca/P ratio in the *B. oleracea* samples. This revealed that *B. oleracea* samples from the 10,000 mg/kg TPH treatment, compared with the control and 1,500 mg/kg TPH treatments' samples, will present lower calcium-binding effect of oxalic acid; according to Nieman et al. (1992, p. 281), "when the diet contains sufficient calcium, the calcium-binding effect of oxalic acid does not appear to be a cause for concern".

On the other hand, crude oil contamination in the range of 3,000 – 10,000 mg/kg TPH elevated the Na/K ratio in the *L. sativa* samples as shown in Table 5.8. Despite this significant increase in Na/K ratio, the consumption of these two leafy vegetables (*B. oleracea* and *L. sativa*) samples with Na/K ratios < 0.6 would not contribute to high blood pressure (Nieman et al., 1992).

Table 5-8: Mineral ratios of the harvested leafy vegetables samples

Treatments	Ca/P[1] ratio		Na/K[2] ratio	
	B. oleracea	*L. sativa*	*B. oleracea*	*L. sativa*
Treatment 1 (0 mg/kg TPH, Control)	3.20 ± 0.09^{b}	4.13 ± 0.13^{a}	0.02 ± 0.00^{a}	0.03 ± 0.00^{a}
Treatment 2 (1,500 mg/kg TPH)	3.21 ± 0.19^{b}	4.19 ± 0.32^{a}	0.01 ± 0.00^{a}	0.04 ± 0.00^{a,b}
Treatment 3 (3,000 mg/kg TPH)	3.78 ± 0.12^{a,b}	4.11 ± 0.12^{a}	0.02 ± 0.00^{a}	0.04 ± 0.00^{b,c}
Treatment 4 (5,000 mg/kg TPH)	3.52 ± 0.07^{a,b}	4.05 ± 0.08^{a}	0.02 ± 0.00^{a}	0.05 ± 0.00^{b,c}
Treatment 5 (10,000 mg/kg TPH)	4.05 ± 0.17^{a}	4.07 ± 0.28^{a}	0.01 ± 0.00^{a}	0.05 ± 0.00^{c}

[1]Ca/P ratio = Calcium : Phosporus ratio.
[2]Na/K ratio = Sodium : Potassium ratio.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Leafy vegetables Bioconcentration factor (BCF) and Transportation index (*Ti*)

In order to assess the degree to which the contaminant minerals accumulate in the harvested vegetable samples, the bioconcentration factors (BCF) (Van den Berg, 1994) of three of the contaminant minerals (Al, Cd and Pb) are presented. The transportation index (*Ti*) of these contaminant minerals are also evaluated alongside the BCF in the above-ground plant parts (BCF-A) and BCF in the roots (BCF-R).

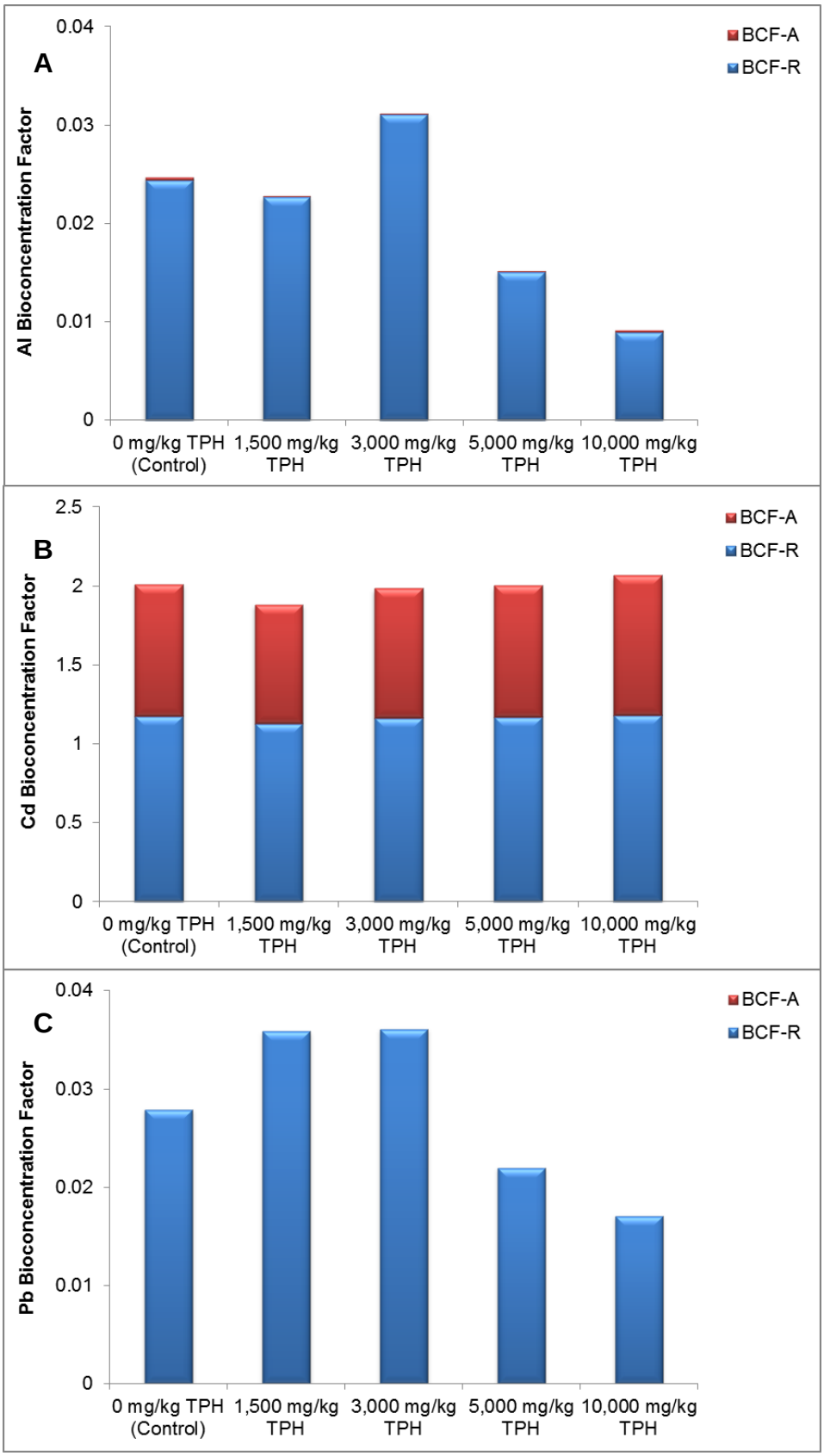
Although crude oil contamination in the range of 1,500 – 10,000 mg/kg TPH had no significant effect ($p > 0.05$) on the ability of *B. oleracea* to translocate Cd from the roots to the leaves (see Table 5.9), there was a significant difference ($p < 0.05$) between the transportation index of *B. oleracea* for Al at 1,500 – 3,000 mg/kg TPH and the highest level of crude oil contamination (10,000 mg/kg TPH) used in this experiment. The recorded transportation index of Pb in *B. oleracea* is as a result of the non-detectable level of this mineral contaminant in all the harvested *B. oleracea* above-ground plant parts as shown in Table 5.5.

Furthermore, the experimental results illustrated in Figure 5.19A revealed that the maximum amount of Al transferred from the soil to the root of *B. oleracea* was at 3,000 mg/kg TPH after which further increase in the concentration of crude oil in the growing medium led to the gradual decrease in the level of Al absorbed by the roots. *B. oleracea* root samples from the 10,000 mg/kg TPH treatment also had the least amount of Pb content. Meanwhile, and with respect to the concentration of the three considered mineral contaminants in the growing medium, Cd was the most accumulated by the *B. oleracea* above-ground plant parts.

Table 5-9: Leafy vegetables transportation index (*Ti*)

Treatments	Transportation index in the leafy vegetables					
	Al		Cd		Pb	
	B. oleracea	*L. sativa*	*B. oleracea*	*L. sativa*	*B. oleracea*	*L. sativa*
Treatment 1 (0 mg/kg TPH, Control)	1.18 ± 0.73^{a,b}	14.30 ± 1.07^{a}	70.92 ± 5.03^{a}	155.12 ± 11.11^{b}	0.00	7.49 ± 2.30^{a}
Treatment 2 (1,500 mg/kg TPH)	0.30 ± 0.13^{a}	11.72 ± 2.27^{a}	67.04 ± 9.38^{a}	147.94 ± 15.50^{b}	0.00	5.59 ± 0.75^{a}
Treatment 3 (3,000 mg/kg TPH)	0.24 ± 0.08^{a}	9.89 ± 1.67^{a}	70.73 ± 9.83^{a}	145.80 ± 7.68^{b}	0.00	5.90 ± 2.98^{a}
Treatment 4 (5,000 mg/kg TPH)	0.59 ± 0.24^{a,b}	16.12 ± 1.83^{a}	70.66 ± 0.95^{a}	115.89 ± 12.47^{a,b}	0.00	14.54 ± 2.36^{a,b}
Treatment 5 (10,000 mg/kg TPH)	2.36 ± 0.58^{b}	47.43 ± 9.39^{b}	74.52 ± 12.34^{a}	70.36 ± 4.28^{a}	0.00	21.65 ± 3.59^{b}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.



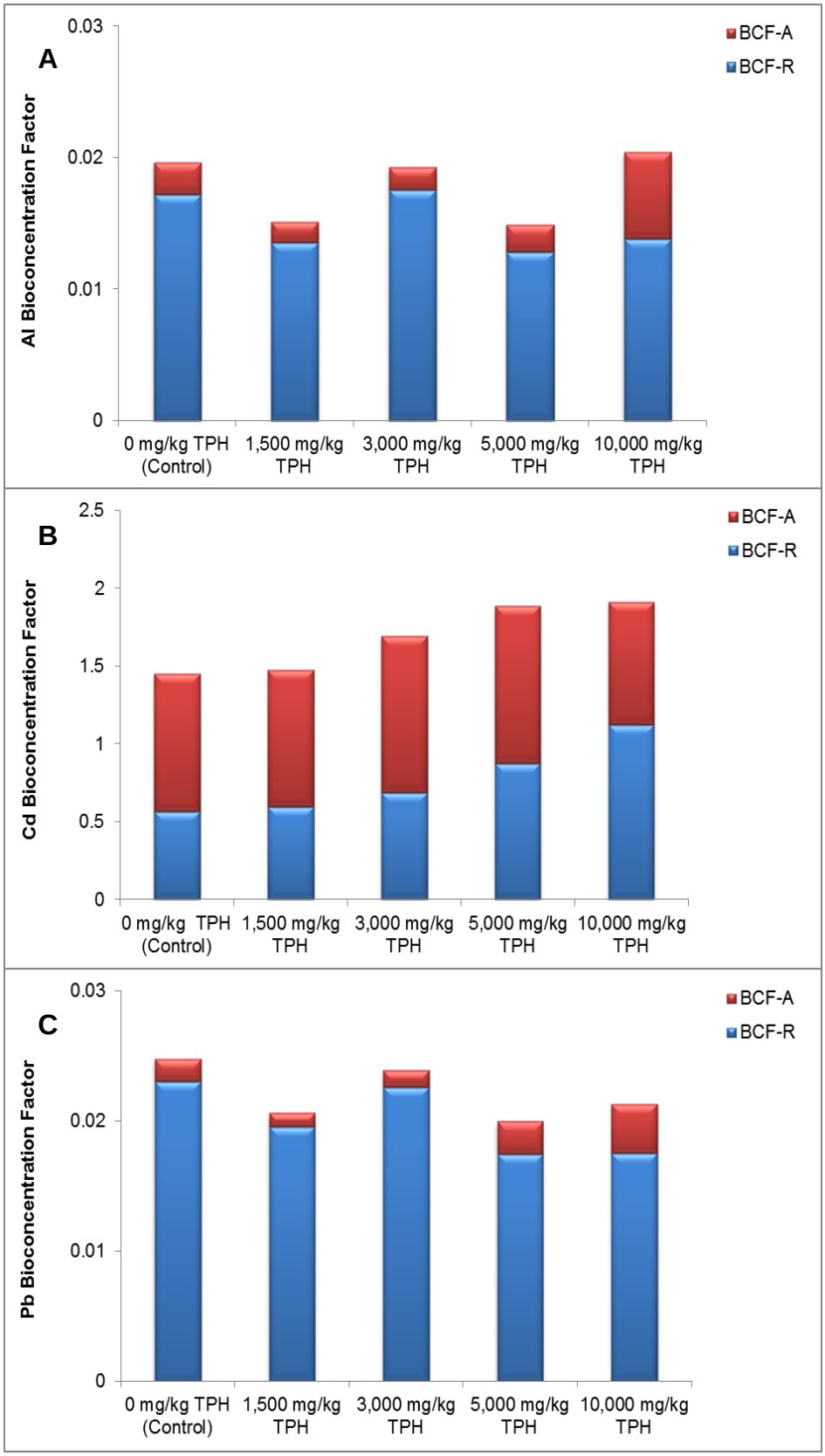
BCF-A = Bioconcentration factor in the above-ground plant part.
BCF-R = Bioconcentration factor in the root.

Figure 5-19: Bioconcentration Factor (BCF) distributions in *Brassica oleracea*

On the other hand, crude oil application at 10,000 mg/kg TPH, compared with the control treatment samples, significantly enhanced the translocation of Al and Pb from the roots of *L. sativa* to the above-ground plant parts. The *Ti* of Cd was however significantly reduced ($p < 0.05$) at this CRIV (Table 5.9). Crude oil contamination increased the accumulation of Cd in the root of *L. sativa* (Figure 5.20) while among the harvested *L. sativa* above-ground plant parts, the highest accumulation of Al and Pb was in the samples from the 10,000 mg/kg TPH.

With the exception of the bioconcentration factor in the root and above-ground plant parts of the *L. sativa* samples from the control treatment – 5,000 mg/kg TPH treatment (Figure 5.20B), the BCF results (Figure 5.19 and 5.20) showed that with respect to the concentration of the contaminants in the growing medium, the three mineral contaminants considered had higher concentrations in the roots than the above-ground plant parts of the plants which agree with the view of Cobb et al. (2000) that plant roots usually contain the highest amounts of metals among plant tissues.

Generally, the *Ti* values recorded for the two leafy vegetables in Table 5.9, to a large extent, indicate the ability of *L. sativa* to accumulate metals as previously reported in the literature (e.g. Cobb et al., 2000; Peijnenburg et al., 2000; Ramos et al., 2002).



BCF-A = Bioconcentration factor in the above-ground plant part.
BCF-R = Bioconcentration factor in the root.

Figure 5-20: Bioconcentration Factor (BCF) distributions in *Lactuca sativa*

5.4.3.3 Phytochemicals composition analysis results

5.4.3.3.1 Total phenolics and total flavonoids contents

There was no statistical significant difference in the distribution of total phenolics (p = 0.104) and total flavonoids (p = 0.260) in the *B. oleracea* harvested plant samples (Table 5.10). This showed that crude oil contamination in the range of 1,500 – 10,000 mg/kg TPH and its induced abiotic stresses had no significant effect on the total phenolics and total flavonoids contents of *B. oleracea*.

Table 5-10: Phenolics distribution in *Brassica oleracea*

Treatments	Phenolics distribution	
	Total phenolics content (mg GAE/g DW)*[1]	Total Flavonoids content (mg QE/g DW)*[2]
Treatment 1 (0 mg/kg TPH, Control)	19.18 ± 0.62[a]	27.89 ± 0.98[a]
Treatment 2 (1,500 mg/kg TPH)	17.71 ± 0.63[a]	27.45 ± 0.60[a]
Treatment 3 (3,000 mg/kg TPH)	19.48 ± 0.48[a]	28.31 ± 0.38[a]
Treatment 4 (5,000 mg/kg TPH)	19.80 ± 0.82[a]	28.72 ± 0.68[a]
Treatment 5 (10,000 mg/kg TPH)	20.41 ± 0.56[a]	29.72 ± 0.69[a]

*[1]Total phenolics content (TPC) values were expressed as mg of gallic acid equivalents (GAE) per g of dry weight extract.
*[2]Total flavonoids content values were expressed as mg of quercetin equivalents (QE) per g of dry weight extract.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different (p < 0.05) according to Tukey *post-hoc* test.

Meanwhile, crude oil contamination at CRIV of 10,000 mg/kg TPH had a statistical significant effect on the TPC (p = 0.001) and total flavonoids content (p = 0.002) of the lettuce type (Lettuce Butterhead Analena) used in this study (Table 5.11). The highest and significant level of total phenolics (25.84 mg GAE/g DW) and total flavonoids (40.78 mg QE/g DW) found in the *L. sativa* samples from the 10,000 mg/kg TPH treatment, showed that crude oil contamination at this CRIV as well as its induced nutrient deficiency in the growing media enhanced the level of the flavonoids (a type of stress-induced

phenylpropanoids) present in these samples (Dixon and Paiva, 1995; Solecka, 1997; Chishaki and Horiguchi, 1997).

The experiment of Noori et al. (2012), also indicated the ability of crude oil stress to alter plants' phenolics and flavonoids contents. According to these authors (p. 3059), "tolerant plant species modify their metabolic processes to alter production of phenolics and flavonoids and reduce oxidative damage to their cells".

Table 5-11: Phenolics distribution in *Lactuca sativa*

Treatments	Phenolics distribution	
	Total phenolics content (mg GAE/g DW)*[1]	Total Flavonoids content (mg QE/g DW)*[2]
Treatment 1 (0 mg/kg TPH, Control)	20.34 ± 1.03[b]	34.92 ± 0.49[b]
Treatment 2 (1,500 mg/kg TPH)	20.15 ± 0.53[b]	32.74 ± 1.01[b]
Treatment 3 (3,000 mg/kg TPH)	20.79 ± 0.89[b]	33.48 ± 0.82[b]
Treatment 4 (5,000 mg/kg TPH)	21.04 ± 0.12[b]	35.22 ± 1.59[b]
Treatment 5 (10,000 mg/kg TPH)	25.84 ± 0.12[a]	40.78 ± 1.01[a]

*[1]Total phenolics content (TPC) values were expressed as mg of gallic acid equivalents (GAE) per g of dry weight extract.
*[2]Total flavonoids content values were expressed as mg of quercetin equivalents (QE) per g of dry weight extract.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.
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Furthermore, the outcome of these phytochemicals distribution in the *L. sativa* harvested samples, were consistent with the report of Wang and Frei (2011) in which most studies pointed to increase in phenolic content when crops are under environmental stress conditions which is as a result of the strong stimulation of the principal enzymes of the phenylpropanoid pathway – phenylalanine ammonia-lyase (PAL).

On the other hand, the highest level of total flavonoids in samples from the 10,000 mg/kg TPH treatment (Table 5.11) as well as significant increase in

sucrose concentration at this CRIV of 10,000 mg/kg TPH in *L. sativa* (Figure 5.15), suggest the presence of higher amounts of flavonols and flavones (two of the three main subclasses of flavonoids), to which sugars bind in plants (Hollman and Arts, 2000).

Largely, the results of this analysis showed that, in addition to the view of Liu et al. (2007) that cultivar, type and colour may affect the TPC of lettuce, environmental factors such as crude oil contamination > 5,000 mg/kg TPH, can affect the concentration of phenolic compounds in lettuce.

5.4.3.3.2 Non-volatile organic acids distribution

On the basis that malic and citric acids are the two principal organic acids in plants (Fennema et al., 1996) and L-ascorbic acid (vitamin C) being the most essential vitamin via fruits and vegetables in human diets (Lee and Kader, 2000), the evaluation of organic acids in the harvested agricultural produce was focused on these three organic acids (see Chapter 3, section 3.2.7.2.3), although traces of tartaric acid was found in Treatment 5 (CRIV of 10,000 mg/kg TPH) of the two leafy vegetables samples.

Experimental analysis of the organic acids distribution in the *B. oleracea* samples showed that crude oil contamination within the CRIV of 1,500 - 10,000 mg/kg TPH had no significant effect ($p > 0.05$) on the malic acid content of the harvested samples. However, CRIV of 3,000 and 5,000 mg/kg TPH led to a significant reduction ($p < 0.05$) in the ascorbic acid content compared with those found in samples obtained from the 10,000 mg/kg TPH treatment. Crude oil contamination also gave rise to a significant increase in the citric acid content in the *B. oleracea* samples obtained from the 5,000 and 10,000 mg/kg TPH treatments. Remarkably, crude oil stress at 10,000 mg/kg TPH significantly enhanced the level of two of the four organic acids (tartaric and citric acids) analysed in the harvested *B. oleracea* samples compared with the levels of these organic acids found in the control treatment samples without crude oil contamination.

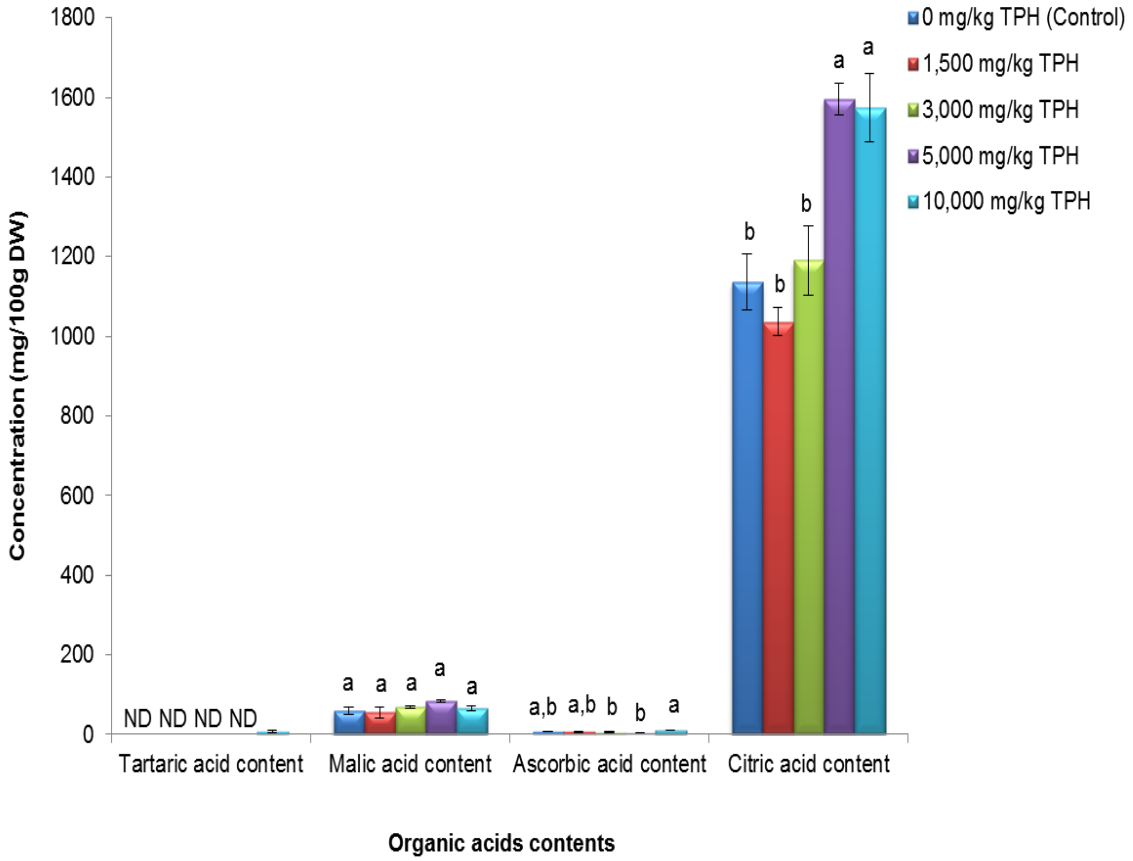

Figure 5-21: Organic acids distribution in *Brassica oleracea.* Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test. ND = Not Detected.

Similar to the observations in the *B. oleracea* samples, tartaric acid was only detected in the *L. sativa* samples from the 10,000 mg/kg TPH treatment (Figure 5.22). Crude oil contamination at 5,000 – 10,000 mg/kg TPH reduced the ascorbic acid contents of the *L. sativa* samples which were statistically significantly different ($p < 0.05$) from the level of this organic acid found in the control treatment samples. Interestingly, crude oil contamination in the range of 3,000 – 10,000 mg/kg TPH significantly enhanced the malic acid concentration in the *L. sativa* samples.

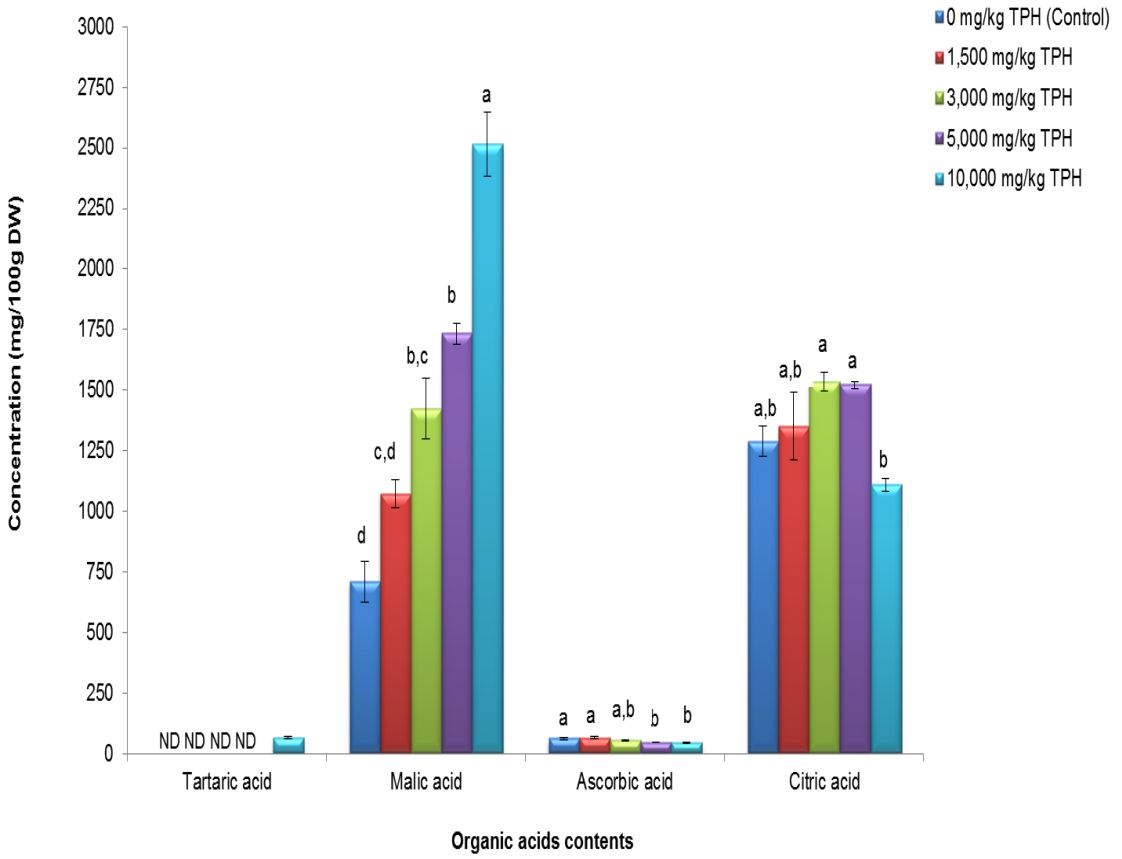

Figure 5-22: Organic acids distribution in *Lactuca sativa.* Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test. ND = Not Detected.

The few studies on the effect of petroleum hydrocarbons on organic acids distribution in plants (e.g. Nwaogu and Ujowundu, 2010; Nwazue, 2011) were majorly on ascorbic acid in which these authors associated the decrease in ascorbic acid content of plant samples under petroleum hydrocarbon stress with the antioxidant/free radical scavenging ability of this organic acid (i.e. ascorbic acid) in mopping up reactive oxygen species and free radicals. This according to Nwaogu and Ujowundu (2010) consequently reduces ascorbic acid concentration.

Generally, this research outcome supports the view of Noctor and Foyer (1998, cited in Gaur and Sharma, 2014, p. 133) that “stress can also result in the losses of nutrient constituents in the fruit or vegetable, with vitamin C loss being the most sensitive indicator of stress exposure”.

Malic acid/Citric acid ratio

Although crude oil contamination between the CRIV of 1,500 – 10,000 mg/kg TPH had no significant effect ($p > 0.05$) on the malic acid/citric acid ratios in the *B. oleracea* harvested samples (Table 5.12), CRIV of 5,000 mg/kg TPH and 10,000 mg/kg TPH significantly increased ($p < 0.05$) the malic acid/citric acid ratio in the *L. sativa* samples when compared with that found in the control treatment samples.

Table 5-12: Malic acid/Citric acid ratios in the leafy vegetables

Treatments	*B. oleracea*	*L. sativa*
0 mg/kg TPH (Control)	0.05 ± 0.01^{a}	0.55 ± 0.08^{a}
1,500 mg/kg TPH	0.05 ± 0.02^{a}	$0.79 \pm 0.07^{a,b}$
3,000 mg/kg TPH	0.06 ± 0.01^{a}	$0.93 \pm 0.09^{a,b}$
5,000 mg/kg TPH	0.05 ± 0.00^{a}	1.14 ± 0.03^{b}
10,000 mg/kg TPH	0.04 ± 0.00^{a}	2.27 ± 0.16^{c}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post- hoc* test.

Following the report of Meredith et al. (1989), this result as presented in Table 5.12 revealed that, among the two leafy vegetables, only the maturity of *L. sativa* samples was enhanced by crude oil contamination in the range of 5,000 - 10,000 mg/kg TPH.

5.4.3.3.3 Chlorophyll and carotenoids contents

In agreement with the view of Onwurah et al. (2007, cited in Baruah et al., 2014, p. 12530) that environmental stress arising from crude oil pollution and nutrient availability can affect the chlorophyll content in leaf tissues, crude oil contamination at CRIV of 10,000 mg/kg TPH resulted to a significant increase ($p < 0.05$) in the: chlorophyll b content (74.51 mg/100g DW) as well as chlorophyll a and b (78.59 mg/100g DW and 57.84 mg/100g DW) contents of *B. oleracea* (Figure 5.23) and *L. sativa* (Figure 5.24) harvested samples respectively.

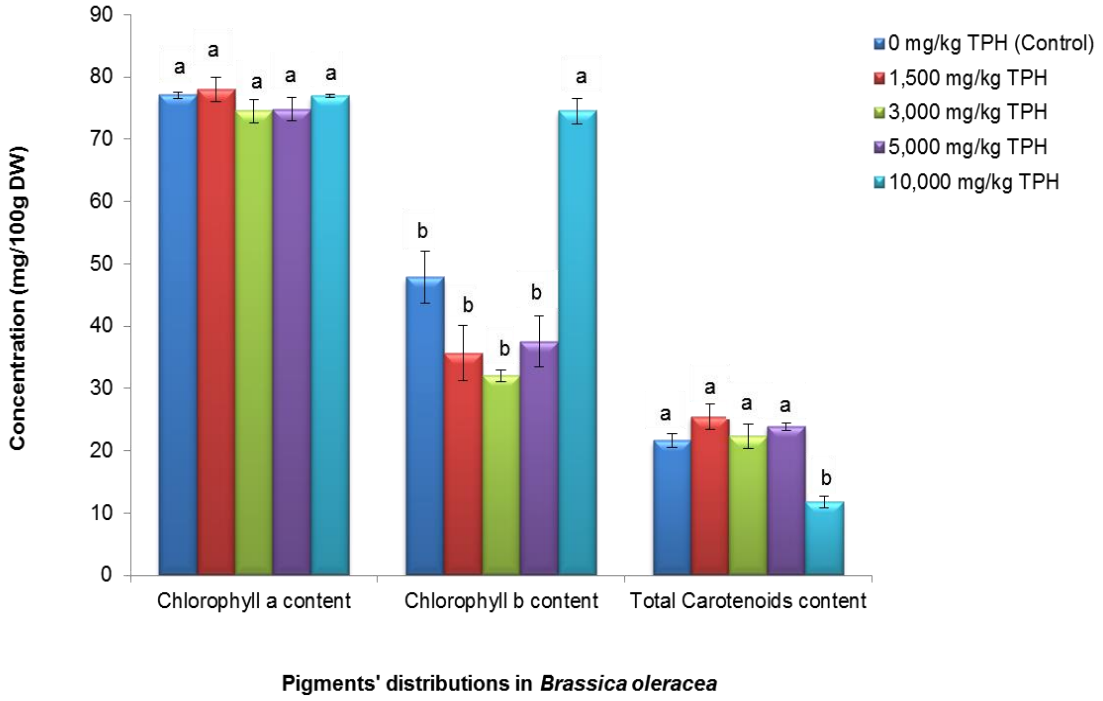

Figure 5-23: Distribution of the different pigments in *Brassica oleracea*. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Following the account of Akujobi et al. (2011, cited in Baruah et al., 2014, p. 12531), this significant difference ($p < 0.05$) in the chlorophyll content of these leafy vegetables at 10,000 mg/kg TPH compared with the amount found in the control treatment samples, substantiate the effect of crude oil stress at this CRIV on the physiology and biochemical behaviour of these plants earlier reported in the preceding sections. The significant reduction ($p < 0.05$) in *B. oleracea* total carotenoids content at 10,000 mg/kg TPH compared with the amount in the control treatment samples (Figure 5.23) as well as that involving the 5,000 mg/kg TPH and 10,000 mg/kg TPH treatments' samples in the *L. sativa* (Figure 5.24), also support the view of Gross (1991), that environmental factors (which in this case was from crude oil contamination) affect carotenoid distribution in plants.

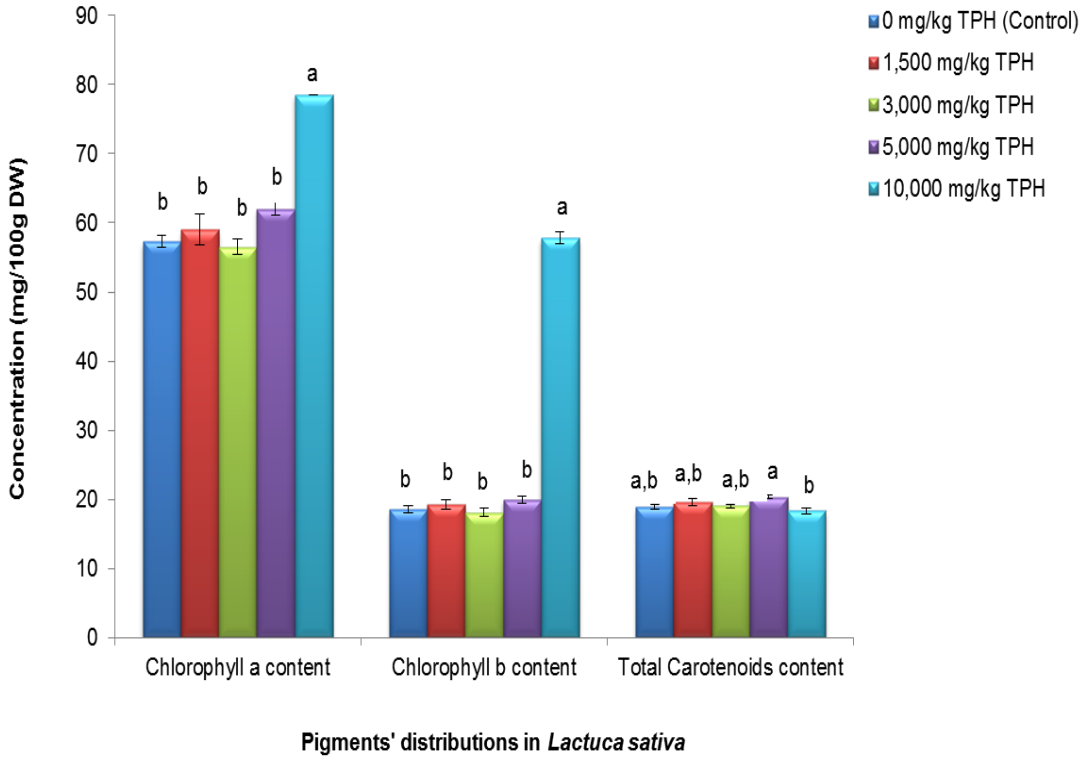

Figure 5-24: Distribution of the different pigments in *Lactuca sativa*. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Although the chlorophyll a contents in the two leafy vegetables (*B. oleracea* and *L. sativa*) harvested samples were higher than those of chlorophyll b which agrees with Gross (1991) that chlorophyll a is the major pigment in higher plants, the increase in chlorophyll content at the highest level of crude oil contamination in this experiment (i.e. 10,000 mg/kg TPH) contradicts the report of several authors (e.g. Achuba, 2006; Ibemesim, 2010; Al-Hawas et al., 2012; Baruah et al., 2014) in which environmental contamination was associated with reduced chlorophyll content. This contradiction suggests that the two leafy vegetables (*B. oleracea* and *L. sativa*) are resistant cultivars as Homayoun et al. (2011) noted that environmental stress increase the chlorophyll content of resistant cultivars. Lin and Mendelssohn (1996) also indicated that plant resistance to oil depends on the species involved.

Pigment ratio

Tukey *post-hoc* analysis revealed that the only statistical difference in the *B. oleracea* harvested samples Chl *a/b* ratio was between that in the 10,000 mg/kg TPH treatment sample and those of the other crude oil-containing treatments (i.e. samples from the 1,500 – 5,000 mg/kg TPH treatments) as shown in Table 5.13. Crude oil contamination at 10,000 mg/kg TPH also significantly reduced ($p < 0.05$) the Chl *a/b* ratio in the *L. sativa* sample from this treatment compared with the pigment ratio found in samples from the other treatments (control inclusive).

Table 5-13: The Leafy vegetables pigment ratio

Selected Plants	Treatments	Chl *a/b* ratio
Brassica oleracea	0 mg/kg TPH (Control)	$1.61 \pm 0.13^{a,b}$
	1,500 mg/kg TPH	2.19 ± 0.35^{a}
	3,000 mg/kg TPH	2.33 ± 0.07^{a}
	5,000 mg/kg TPH	1.99 ± 0.18^{a}
	10,000 mg/kg TPH	1.03 ± 0.03^{b}
Lactuca sativa	0 mg/kg TPH (Control)	3.09 ± 0.05^{a}
	1,500 mg/kg TPH	3.06 ± 0.01^{a}
	3,000 mg/kg TPH	3.12 ± 0.04^{a}
	5,000 mg/kg TPH	3.11 ± 0.05^{a}
	10,000 mg/kg TPH	1.36 ± 0.02^{b}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The difference in environmental growing conditions (Gross, 1991; Lefsrud et al., 2007) arising from the crude oil contamination resulted to the observed alterations in the pigment ratios of the harvested (*B. oleracea* and *L. sativa*) samples. Meanwhile, the significantly reduced Chl *a/b* ratio in the *L. sativa* samples from the 10,000 mg/kg TPH treatment, with respect to the control treatment samples' pigment ratio (Table 5.13), indicates accelerated leaf senescence at this remediation intervention value as Chl *a/b* ratio shifts to lower

values during leaf senescence (Gross, 1991). According to this author (i.e. Gross, 1991), Chl *a/b* ratio is a physiological status indicator.

5.4.3.4 Bio-accumulated PAHs content

As a result of technical constraint and low sensitivity of the MS of the GC/MS used, the bio-accumulated PAHs in the harvested above-ground plants part samples could not be completely assessed.

5.4.4 VOCs analysis result

5.4.4.1 Qualitative analysis result and discussion

As there is a tendency for typical crude oils to lose up to 45% of their volume within a few days (Fingas, 2013; Wang et al., 2015) in which most are VOCs (Wang et al., 2015), according to these latter authors, that is, Wang et al. (2015, p. 7735), “the prediction of crude oil volatile organic compound (VOC) evaporation and diffusion behaviour is vital for remote emission detection and risk assessment of contaminated sites”. VOCs emitted at fresh oil spill site pose a threat to the human environment, including nearby residents and responders working on the spills (NOAA, 2016). Based on the dearth of information in the literature on VOCs emission at remediation intervention values and limited amount of the experimental crude oil, analysis of the VOCs associated with CRIV between 3,000 - 5,000 mg/kg TPH were investigated in this experiment to understand the potential threat of VOCs emission emanating from oil spills on agricultural lands.

The results of the qualitative analysis of the VOCs emitted at CRIV between 3,000 – 5,000 mg/kg TPH and the control treatment (with no crude oil contamination), revealed that only 11 VOCS (see Table 5.14) had their match quality ≥ 90 on comparison of the mass spectra of the eluted compounds to the reference spectral in the NIST library. The corresponding retention times (RT) of these identified compounds are shown in Table 5.14 with some of their properties in Table 5.15.

The whole-air sampling by the TD/GC/TOF-MS as discussed in Chapter 3 (Section 3.2.5) showed that benzaldehyde, phenol, acetophenone and

tetradecane were emitted from all the treatment pots including the control treatment. The experimental analysis also revealed that the increase in the level of crude oil contamination, with the exception of the control treatment without crude oil contamination, led to the differences in the observed peak areas of the identified compounds and determined the number as well as amount of emitted VOCs.

The 5,000 mg/kg TPH treatment, involving the highest level of crude oil contamination, emitted the highest number of VOCs and was the only treatment that gave out *p*-xylene (25.5 min), bicyclo[4.2.0]octa-1,3,5-triene (26.8 min), cyclooctane (38.9 min) and biphenyl (42.9 min). Meanwhile, benzoic acid (37.7 min), phthalic anhydride (41.6 min) and 1,1'-biphenyl, 2-methyl- (43.2 min) were only obtained in the crude oil-containing treatments (i.e. 3,000 and 5,000 mg/kg TPH treatments). The control treatment (without crude oil contamination), in contrast, produced the least number and amount of VOCs.

Table 5-14: Result of the emitted VOCs with respect to the 5,000 mg/kg TPH treatment

Tentative Assignment (Compound)	Retention time (min)*	CAS Number	Molecular Weight	Molecular Formula	Ratio of peak area of compound/ peak area of internal standard		
					0 mg/kg TPH (Control)	3,000 mg/kg TPH	5,000 mg/kg TPH
p-Xylene	25.489	000106-42-3	106.8	C_8H_{10}	ND	ND	0.005
Bicyclo[4.2.0]octa-1,3,5-triene	26.776	000694-87-1	104.06	C_8H_8	ND	ND	0.005
Benzaldehyde	30.501	000100-52-7	106.04	C_7H_6O	0.029	0.105	0.114
Phenol	31.099	000108-95-2	94.04	C_6H_6O	0.062	0.151	0.136
Acetophenone	35.118	000098-86-2	120.06	C_8H_8O	0.012	0.033	0.043
Benzoic acid	37.699	000065-85-0	122.04	$C_7H_6O_2$	ND	0.035	0.146
Cyclooctane	38.985	000292-64-8	112.13	C_8H_{16}	ND	ND	0.019
Phthalic anhydride	41.642	000085-44-9	148.02	$C_8H_4O_3$	ND	0.021	0.041
Tetradecane	42.687	000629-59-4	198.24	$C_{14}H_{30}$	0.016	0.034	0.015
Biphenyl	42.861	000092-52-4	154.08	$C_{12}H_{10}$	ND	ND	0.018
1,1’-Biphenyl, 2-methyl-	43.155	000643-58-3	168.09	$C_{13}H_{12}$	ND	0.004	0.008

*Retention times in the 5,000 mg/kg TPH treatment.
ND = Not Detected.

Table 5-15: Some of the properties of the identified VOCs

	Boiling point	Melting point	Vapour pressure	Density	Solubility in water	log (K_{ow})	References
p-Xylene	138.3^{o}C	13.2^{o}C	8.84 mmHg at 25^{o}C		162 mg/L at 25^{o}C	3.15	(U.S. National Library of Medicine, 2014a)
Benzaldehyde	179^{o}C	-26^{o}C	0.127 mm Hg at 25^{o}C	1.050 at 15^{o}C	6570 mg/L at 25^{o}C	1.48	(Prager, 1995; U.S. National Library of Medicine, 2014h)
Phenol	181.75^{o}C at 760 mmHg	43^{o}C	0.3513 mmHg at 25^{o}C	1.0576 at 20^{o}C	1 g/15ml water	1.46	(Prager, 1995)
Acetophenone	202^{o}C	20.5^{o}C	0.4 mmHg at 25^{o}C	1.033 at 15^{o}C	6,130 mg/L at 25^{o}C	1.58	(Prager, 1995)
Benzoic acid	249.2^{o}C at 760mmHg	122.4^{o}C	1 mmHg at 96^{o}C	1.2659 at 15^{o}C	1 g/300 ml water	1.87 at 20^{o}C	(Prager, 1995)
Cyclooctane	149^{o}C	14.8^{o}C			7.9 mg/L at 25^{o}C	4.45	(U.S. National Library of Medicine, 2014b)
Phthalic anhydride	295^{o}C	130.8^{o}C	1 mmHg at 96.5^{o}C	1.20 at 135^{o}C (liquid), 1.53 at	6200 mg/L at 25^{o}C	1.60	(Prager, 1995; U.S. National Library of

				20°C (solid)			Medicine, 2014i)
n-Tetradecane	253.5°C	5.8	0.0116 mmHg at 25°C		0.0022 mg/L at 25°C	7.2	(U.S. National Library of Medicine, 2014c)
Biphenyl	256.1°C	69°C	0.00893 mmHg at 25°C		6.94 mg/L at 25°C	3.98	(U.S. National Library of Medicine, 2014d)
1,1'-Biphenyl, 2-methyl-	255.5°C	-0.2°C	0.020 mmHg at 25°C		18.1 mg/L at 25°C	4.3	(U.S. National Library of Medicine, 2014e)

The results of the volatiles' analysis affirm that crude oil contains VOCs as reported by UNEP (2011) and NOAA (2016) which can impact human health negatively (NOAA, 2016).

In line with Di Toro et al. (2007), the toxicity of oil components increases as log (K_{ow}) increases [(K_{ow}) = octanol/water partition coefficient], but with an inverse relationship with solubility. Hence, the toxic potential, a ratio of solubility to toxicity, decreases (Di Toro et al., 2007) which indicates that lower log (K_{ow}) compounds are more toxically potent that those with higher log (K_{ow}) values (Di Toro et al., 2007; Tang et al., 2011). Following from this and the available log (K_{ow}) values provided in Table 5.15, phenol with log (K_{ow}) of 1.46 (Prager, 1995) has the highest toxic potential among all the identified VOCs. However, with respect to the VOCs produced at the highest level of crude oil used in this experiment (5,000 mg/kg TPH), *p*-xylene, log (K_{ow}) = 3.15 (U.S. National Library of Medicine, 2014a), has the highest toxic potential.

Aside from the possibility of these VOCs to have a negative impact on human health, Lollar (2005) indicated that VOCs can also undergo photochemical reactions with nitrous oxides in the atmosphere, leading to ground-level ozone, or photochemical smog. Further details on the origin/fate as well as potential toxicity of the identified VOCs are provided in Section 5.4.4.2.

5.4.4.2 Origin/fate and potential toxicity of the identified VOCs

p-Xylene

p-Xylene is one of the three isomers of xylene which according to Ayres and Hellier (1998) are classified as dangerous under the EC directive (76/464/EEC) and produced industrially by the reforming of crude oil (b.p. 75 - 140^{o}C): after the separation of other products, such as benzene and toluene. This compound may be separated from *m*-xylene by cooling at -65^{o}C but also flammable with a sweet odour (Ayres and Hellier, 1998).

Xylenes cause irritation of the eyes, nose and throat at a level of 200 ppm (Ayres and Hellier, 1998) while higher levels have been identified to lead to headache, nausea, anorexia and flatulence in which there may also be injury to the liver, kidneys and nervous system (Riihimaki and Hanninen, 1990, cited in

Ayers, 1998, p. 318). In addition, according to Prager (1995), *p*-xylene has a mild irritation to the skin and can be absorbed through skin at slightly hazardous chronic levels with inhalation limit of 435 mg/m^3. In very low concentrations, the compound is harmful to aquatic life and produce odour in water while the waterfowl threshold concentration for harm to common crops is between 800 – 2,400 ppm (Prager, 1995). Generally, in relation to public health and from this author point of view, the compound is a mild irritant and has slight hazard with acute or chronic inhalation.

Bicyclo[4.2.0]octa-1,3,5-triene

The U.S. National Library of Medicine (2015a) identified bicyclo[4.2.0]octa-1,3,5-triene as an aromatic hydrocarbon capable of causing irritation.

Benzaldehyde

According to Prager (1995), although benzaldehyde biodegrades fairly readily, it is a major threat to benthic organisms; in relation to public health and toxicity, the compound is a slight allergen with moderate hazard. Also, the inhalation of the concentrated vapour of this organic compound may result to eyes, nose and throat irritation while prolonged contact with the skin may lead to irritation (Prager, 1995).

Phenol

Phenol is one of the constituents of coal tar with its key industrial route from cumene; the compound is also present in petroleum refinery effluent and classified as a group D substance by USEPA as a result of insufficient data to assess its carcinogenic potential (Ayres and Hellier, 1998).

This organic compound is a strong irritant and moderately toxic when there is any chronic exposure by any route (Prager, 1995). In the view of this author, phenol may have direct contact with the skin, eyes, throat etc and capable of causing eyes and skin burns. In addition, it is easily absorbed by the skin which can lead to increase in heart rate, convulsions, and death (Prager, 1995).

Acetophenone

Prager (1995) highlighted the possibility of acetophenone to choke benthic life with a slight degree of hazard to public health. With the exception of slight narcotic effect, there is likely not to be any toxic effect on inhalation or ingestion of the compound while contact with the liquid may result to eye and skin irritation (Prager, 1995).

Benzoic acid

According to Ayres and Hellier (1998), benzoic acid is obtained industrially by the cobalt catalysed oxidation of liquid toluene and it is the product of autoxidation of benzaldehyde. Although benzoic acid is moderately toxic by ingestion, these authors added that it is a severe eye irritant. In the view of Prager (1995), the compound is slightly hazardous but poses a major threat to aquatic microlife while it also presents a mild irritation to skin, eyes, and mucous membrane. With respect to its concentration in the food chain, free benzoic acid can be stored in the tissues of various organisms. The dust may also be irritating to nose and eyes while at elevated temperatures, fumes may cause irritation of eyes, respiratory system, and skin (Prager, 1995).

Cyclooctane

The U.S. National Library of Medicine (2015b) described cyclooctane as an irritant whose inhalation may result to drowsiness, fatigue, muscle weakness, etc. It is also indicated by this source to be harmful if ingested which could lead to lung damage by aspiration.

Phthalic anhydride

In line with Prager (1995), although phthalic anhydride is a mild irritant with slight ingestive and inhalative as well as slight chronic inhalative hazards, repeated exposure to the compound may cause dermatitis or sensitization while oral doses of 20 mg/kg body weight over a 120-day period may lead to blood changes. The solid form irritates skin and eyes which would lead to coughing, and sneezing while the liquid is linked with severe thermal burns (Prager, 1995).

Tetradecane

According to the U.S. National Library of Medicine (2014f), n-tetradecane may be released into the environment via: (1) fugitive emission during its production and use, (2) the exhaust of motor vehicles, and (3) the effluent of landfills and industrial processes. The compound has been indentified to have the tendency to bioconcentrate in fish and aquatic organisms while members of the public may become exposed to the compound via inhalation of motor vehicle exhaust in which it is present or the ingestion of contaminated foods/drinking water containing the compound (U.S. National Library of Medicine, 2014f).

Biphenyl

Biphenyl, one of the most thermally stable of all organic compounds (Prager, 1995), was first reported in 1862 by Fittig and is the major product obtained from the pyrolysis of benzene at 700^{o}C while it is also produced commercially from the product of hydrodealkylation of toluene (Ayres and Hellier, 1998). Although the compound is found naturally in coal tar, crude oil and natural gas, it is also synthesized in certain plants and in algae (U.S. National Library of Medicine, 2014g).

This organic compound is not toxic by ingestion but irritates the eyes while human exposure to the compound in air exceeding 35 mg/m^3 may lead to central nervous system (CNS) and liver damage (Ayres and Hellier, 1998). Generally, biphenyl causes irritation to the eyes, skin, mucous membrane and upper respiratory tract while chronic exposure is mostly characterized by central nervous system symptoms, fatigue, headache, tremor, insomnia, sensory impairment, and mood changes; these symptoms are however uncommon (Prager, 1995).

1,1’-Biphenyl, 2-methyl-

The U.S. National Library of Medicine (2015c) considered 1,1’-Biphenyl, 2-methyl- as an aromatic hydrocarbon which can cause irritation.

5.5 Conclusion

Among the three selected leafy vegetables used in this experiment, *B. juncea* (mustard) had the least tolerance to crude oil contamination while the least yield was recorded for the three vegetables at CRIV of 10,000 mg/kg TPH. Interestingly, crude oil contamination in the experimental range (1,500 – 10,000 mg/kg TPH) had no significant effect on the Na/K ratio of *B. oleracea* as well as the F/G ratio and Ca/P ratio of *L. sativa*. Meanwhile, crude oil stress/crude oil-induced abiotic stresses at 10,000 mg/kg TPH enhanced the crude protein concentration in the *B. oleracea* and *L. sativa* samples, including the total phenolics and total flavonoids content in the latter vegetable. Although crude oil contamination at some CRIV resulted to reduction in ascorbic acid content in the leafy vegetables - indicating exposure to stress, tartaric acid was only detected in samples from the 10,000 mg/kg TPH treatment.

The environmental growing conditions arising from the crude oil contamination particularly at 10,000 mg/kg TPH affected the physiology and biochemical composition of the harvested leafy vegetable samples and also promoted leaf senescence in *L. sativa*. Among the VOCs emitted at CRIV of 5,000 mg/kg TPH, *p*-xylene [log (K_{ow}) = 3.15] presented the greatest toxic potential.

On the whole, the research findings in this Chapter provide an understanding of the: (1) effect of different CRIV on the selected leafy vegetables production, (2) beneficial role of crude oil stress at 10,000 mg/kg TPH towards enhancing the level of some essential nutrients and bioactive compounds in green leafy vegetables, as well as (3) VOCs that could be emitted as a result of oil spills on farmlands.

CHAPTER 6
YIELD AND QUALITY OF TOMATO (*Solanum lycopersicum*) AS INFLUENCED BY CRUDE OIL CONTAMINATION

6.1 Chapter Summary

As a follow-up from Chapter 5 where most of the significant impact of crude oil-contaminated soil on the green leafy vegetables considered were at CRIV of 10,000 mg/kg TPH and owing to the insufficient amount of the experimental crude oil, a study on the effect of lower CRIV (750 - 5,000 mg/kg TPH) on agricultural produce, using a sensitive fruit vegetable (tomato), was conducted in this chapter. The experimental results showed that the pH (5.69) and electrical conductivity (0.138 S/m) of the growing medium were suitable for producing the two selected tomato cultivars (Micro-Tom and M82). Although crude oil stress only affected the fruit and root yield as well as firmness of the Micro-Tom tomato plant, all the harvested tomato samples were very firm (firmness > 1.46 N/mm). Interestingly, crude oil contamination in the experimental range had no significant effect ($p > 0.05$) on the: colour (lightness, chroma and hue angle), total soluble solids, sugars (fructose, glucose and sucrose), crude protein, total phenolics, total flavonoids, lycopene and β-carotene contents of all the harvested tomato fruits. However, there were changes in the: Mo, Sr, Ba, malic and ascorbic acids (vitamin C) distribution in the Micro-Tom tomato fruits, as well as Se content in the M82 tomato fruits. Tartaric acid was also only detected (> LOD) in the M82 tomato samples from the 5,000 mg/kg TPH treatment. All the harvested tomato fruits have their Pb content below the FAO/WHO Codex maximum level of 0.05 mg/kg for fruit vegetables except the Micro-Tom tomato fruits from the control treatment (with no crude oil contamination). Overall, the data from this experiment further support the report in literature that plants respond differently to crude oil contamination as well as provide insights into the effect of sub-lethal concentration of crude oil in the range of 750 - 5,000 mg/kg TPH on one of the world's most consumed vegetables and sensitive plant.

6.2 Introduction

Tomato, a fruit vegetable (Taura and Habibu, 2009), is one of the world's most important and consumed vegetable crops (Singh et al., 2013) with different cultivars (Suárez et al., 2015) such as the Micro-Tom and M82 cultivars. Micro-Tom, a dwarf tomato cultivar, according to Scott and Harbaugh (1989, cited in Gonzalez et al., 2015, p. 63) was originally cultivated for domestic gardening purposes. The small size of the plant which enables its growth at a high density (1,357 plants m^{-2}), short life cycle that makes harvesting of mature fruit within 70 – 90 days after sowing possible (Sun et al., 2006) with relatively high yield and fruit production, even under normal fluorescent lighting (Matsukura et al., 2008), are some of the unique characteristics that differentiate this tomato cultivar from others. The M82 tomato is a determinate processing cultivar (Stevens, 1982, cited in Cater et al., 2013, p. 146) whose benefits, among others, include its suitability for: mature plant size greenhouse cultivation, research purposes and production of high number of seeds (Eshed and Zamir, 1995; Menda et al., 2004; Carter et al., 2013).

On the other hand and as regards to the management of petroleum hydrocarbons in the environment, several European as well as national regulations have declared soils to be seriously contaminated at an intervention value of 5,000 mg/kg TPH (Soil Remediation Circular, 2009; Pinedo et al., 2013; Pinedo et al., 2014). Meanwhile, current clean-up standard at the Nigerian Niger Delta also require soil contamination to be less than this value as impact on plants persists even when remediation to this level is achieved (UNEP, 2011).

Consequently and following from Chapter 5 findings where most of the significant effect on the selected green leafy vegetables were at 10,000 mg/kg TPH, this chapter assessed the impact of sub-lethal (Tang et al., 2011) CRIV at 750 - 5,000 mg/kg TPH in the environment on the yield, nutritional and phytochemical distribution of crops using a sensitive plant - tomato (Kuhn et al., 1998).

6.3 Materials and methods

6.3.1 Tomato culture and mesocosm experimental design

The protocol used for the germination of the selected tomato cultivars' seeds: Micro-Tom (WSS1219 Summer 1999) and M82, was designed to prevent the spread of disease. The seeds were first picked out into sterile 50-ml centrifuge tube after which surface sterilization was carried out for 30 min to inactivate the plant viruses with freshly diluted 10% (v/v) "Domestos" household bleach (Unilever UK Ltd., Leatherhead, UK) certified by the Royal Society for Public Health (RSPH). The seeds/bleach mixture was poured through a tea strainer and tap water was run over the seeds for about 1 min until there was no sign of detergent bubbles. The seeds from the tea strainer were then "tapped out" onto Whatman^{TM} Grade 1 filter paper (size 8.5 cm) previously moistened in tap water and placed in sterile Petri-dishes (Figure 6.1).

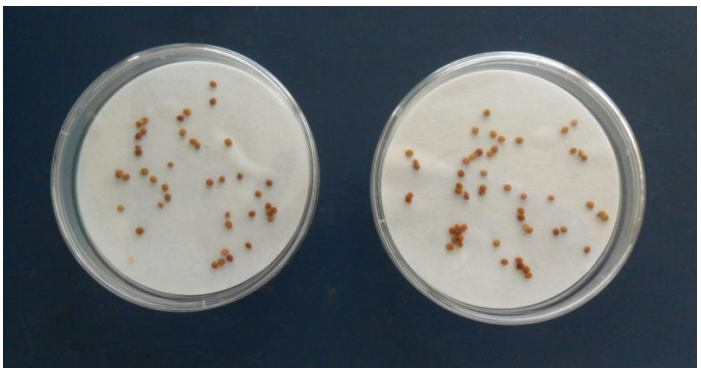

Figure 6-1: Tomato seeds germination plates

The Petri-dishes were later incubated inside a large plastic box containing damp tissue paper (to maintain a humid atmosphere and prevent the filter paper in the Petri dishes from drying out) in the dark at 25^{o}C and inspected daily. On the third day (day of radicle emergence), the germinated tomato seeds were sown in 30-ml modules filled with Sinclair all-purpose growing medium (William Sinclair Horticulture Ltd., Lincoln, England) and transferred to the glasshouse. The Micro-Tom seeds initially used had a very low germination rate as a result

of prolong storage and were subsequently changed to Micro-Tom accession WSS1414.

The established seedlings in the 30-ml modules were thereafter transferred into the respective pots containing different crude oil: soil concentrations as required. Different quantities of the growing medium were used for the selected tomato cultivars and the details of the composition of each treatment are shown in Table 6.1.

Table 6-1: Composition of the selected tomato cultivars' growing medium

Pot Treatments	Amount of Growing Medium Used		Eventual Crude oil: soil concentrations
	Micro-Tom	M82	
Treatment 1	200 g	4 kg	0 mg/kg TPH (Control)
Treatment 2	200 g	4 kg	750 mg/kg TPH
Treatment 3	200 g	4 kg	1,500 mg/kg TPH
Treatment 4	200 g	4 kg	3,000 mg/kg TPH
Treatment 5	200 g	4 kg	5,000 mg/kg TPH

Similar to the experimental set-up in Chapter 5, the randomized complete block design arrangement was adopted involving the use of 11 cm diameter Optipots and KPG-29cm-10 litres pots (Kreuwel Plastics Almelo BV, Netherlands) for the Micro-Tom and M82 tomato cultivars respectively. However, as a result of the insufficient amount of the experimental crude oil, and observation in the previous (Chapter 5) experiment in which most of the impact of crude oil stress on the leafy vegetables were at 10,000 mg/kg TPH, the CRIV used in this experiment were in the range of 750 – 5,000 mg/kg TPH. Lack of adequate space in the glasshouse also made the investigation of the effect on the M82 tomato cultivar to be done in duplicate.

At the start of the experiment and to achieve the same moisture content for the growing media in the pots, watering of the soil to 50% field capacity was carried out (Coulon et al., 2012) while soil samples were collected for the hydrocarbon analysis as well as physiochemical properties determination.

The whole experiment (after transplanting) which started on the 7th May, 2014 lasted for 3 months and was done at the Cranfield University research glasshouse facility with an average temperature of 25°C and 55.93% relative humidity. Figure 6.2 shows the stages involved in the tomato fruit production.

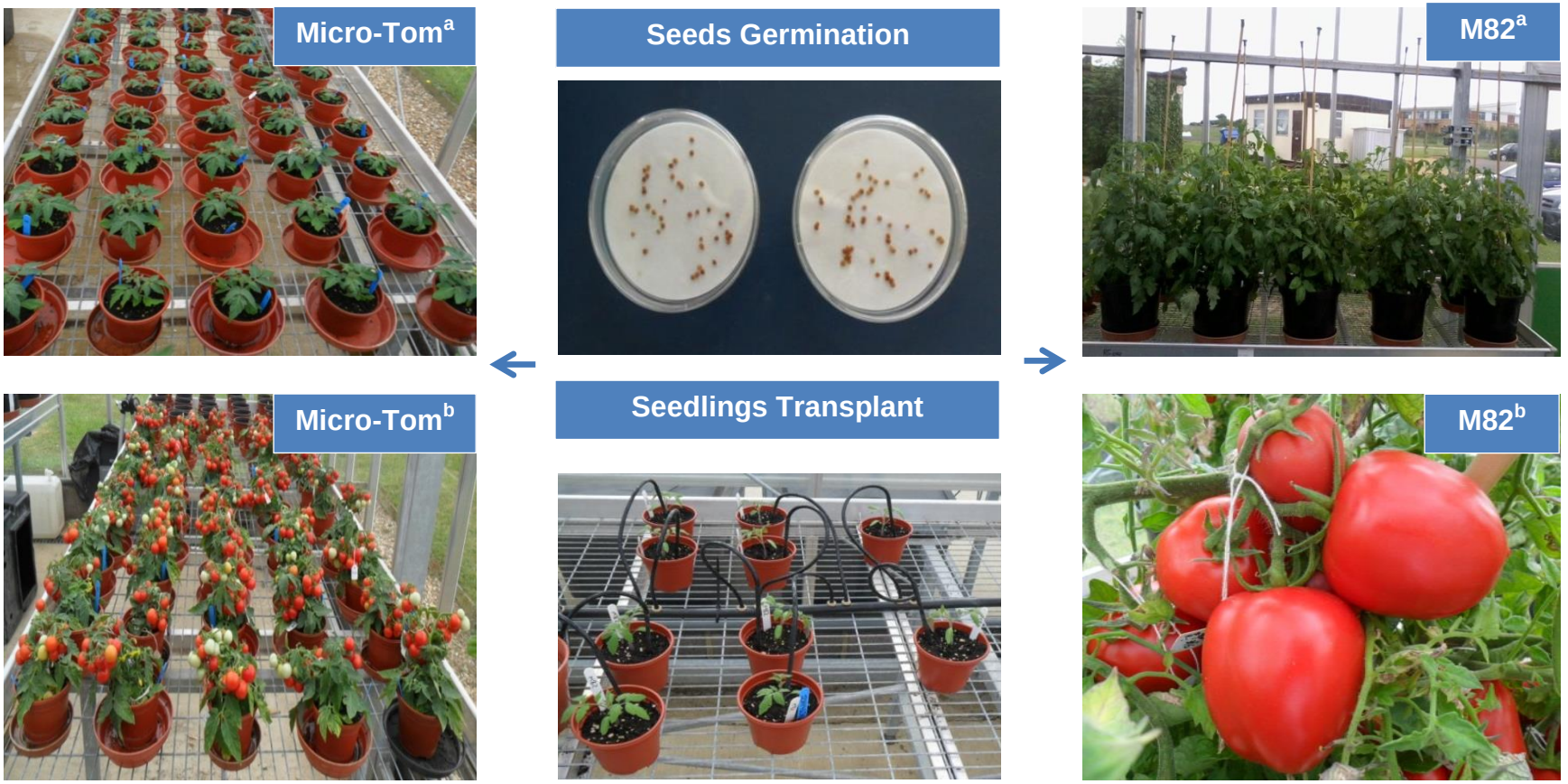

Figure 6-2: Stages in the tomato fruits production: (a) Plant growth before fruit production, (b) Ripened tomato fruits, and (Middle) – Seed germination plates in the laboratory and Seedlings transplant

6.3.2 Growth parameters and post-harvest measurements

The growth characteristics of the two tomato cultivars were monitored and recorded throughout the duration of the experiment while Levington TOMORITE® liquid tomato fertiliser (The Scotts Miracle-Gro Company, Surrey) was used when required.

Growth parameters (such as the Micro-Tom tomato plants' average plant height, average number of leaves ≥ 2.0 cm and average number of fruits produced prior to harvesting) as well as post-harvest measurements involving total mass of fruits per plant, root fresh weight, fruits colour, fruits total soluble solids (TSS) and firmness determination were carried out. Figure 6.3 illustrates the growth of the Micro-Tom tomatoes in the glasshouse at the different CRIV used.

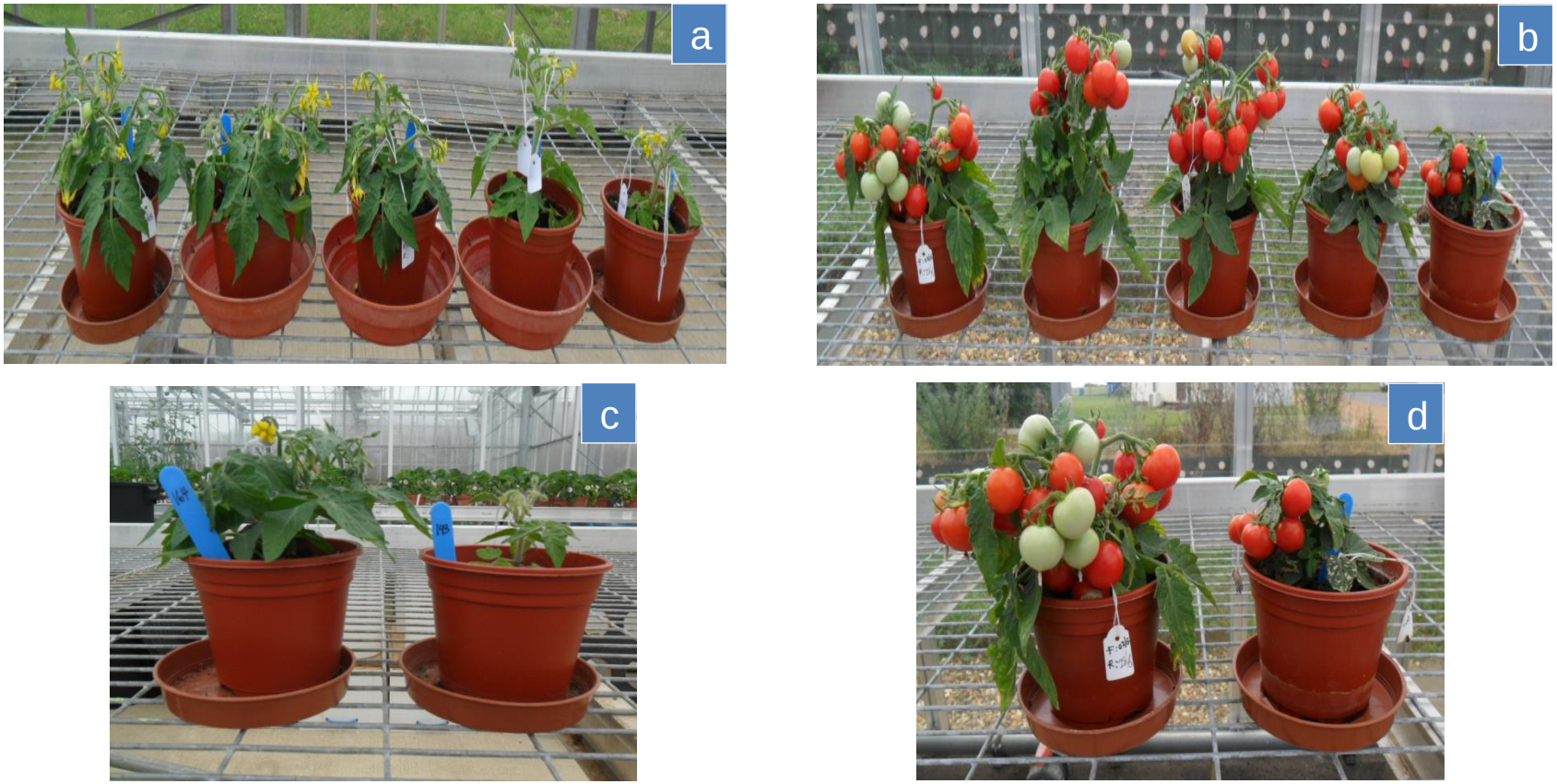

Figure 6-3: Growth of Micro-Tom tomatoes at different CRIV: (a) Before fruiting, (b) During ripening, (c) Before fruiting: 0 mg/kg TPH Control – Left, 5,000 mg/kg TPH - Right, and (d) During ripening: 0 mg/kg TPH Control – Left, 5,000 mg/kg TPH - Right

6.3.3 Samples preparation and analysis

At the end of the experiment, the fruits and roots samples obtained after harvesting were transferred to the laboratory for appropriate processing and storage prior to analysis (see Chapter 3, section 3.2.6.2 for details).

6.3.4 Statistical analysis

Microsoft Excel package, 2010 Version (Microsoft corporation) and IBM SPSS Statistics (IBM Corp. Released 2013. IBM SPSS Statistics for Windows, Version 22.0. Armonk, NY: IBM Corp.) were used in analysing the changes in the tomatoes' growth, postharvest measurements and chemical composition due to crude oil contamination. Shapiro-Wilk's and Levene's tests were carried out to assess the normality of the data and the homogeneity of variances respectively while One-way ANOVA was used to compare the means involving Tukey HSD *post-hoc* analysis when the difference is statistically significant ($p < 0.05$). SigmaPlot for Windows Version 11.0 (Systat Software, Inc., 2008) was also used where applicable.

6.4 Results and discussion

6.4.1 Physiochemical properties results

Although the pH of the Sinclair growing medium (5.69) was below the most favourable range (6.5 – 8.0) for oil degradation reported by Vidali (2001), the obtained value is between the pH range of 4 - 8 of typical (ThermoFisher Scientific, 2014) and most agricultural soils (Hesse, 1971) as well as that stipulated by Naika et al. (2005) (pH 5.5 – 6.8) to be required for tomatoes agrifood production. The moisture content (prior to water addition for the experiment) was also found to be suitable for optimum oil degradation (Table 6.2) while its electrical conductivity of 0.138 S/m is below the range in which the yields of very sensitive crops could be restricted (Richards, 1954).

The C/N and C/P ratios of this growing medium were also above the acceptable theoretical values for a mixed microbial population in soil of 10:1 (Waksman, 1924; Dibble and Bartha, 1979) and 100:1 (Thompson et al., 1954,

cited in Dibble and Bartha, 1979, p. 737) respectively. According to Dibble and Bartha (1979), optimal values of these ratios for petroleum biodegradation are expected to be higher than the theoretical values.

Table 6-2: Physicochemical properties of the Sinclair growing medium

Property	Value
pH	5.69 ± 0.02
Moisture Content (%)	51.89 ± 0.49
Electrical Conductivity (S/m)	0.138 ± 0.00
Total Carbon (%)	42.21 ± 0.33
Total Organic Carbon (%)	39.14 ± 0.72
Total Nitrogen (%)	0.95 ± 0.01
Total Phosphorus (mg/kg)	1795.94 ± 43.83
C/N Ratio	44.29 : 1.00
C/P Ratio	235.03 : 1.00

Values are the means of three replicates ± SE.

6.4.2 Soil hydrocarbons results

Figure 6.4 showed that the gradual increase in the amount of crude oil added to the Sinclair growing medium to achieve CRIV between 750 – 5,000 mg/kg TPH led to the progressive increase in the distribution of alkanes in the crude oil-containing treatments. Meanwhile, alkane concentration in the control treatment was not reported as a result of the recorded irregular pattern of alkanes' distribution in this treatment. Also, based on technical limitations, analysis of PAHs distribution in all the treatments could not be completed.

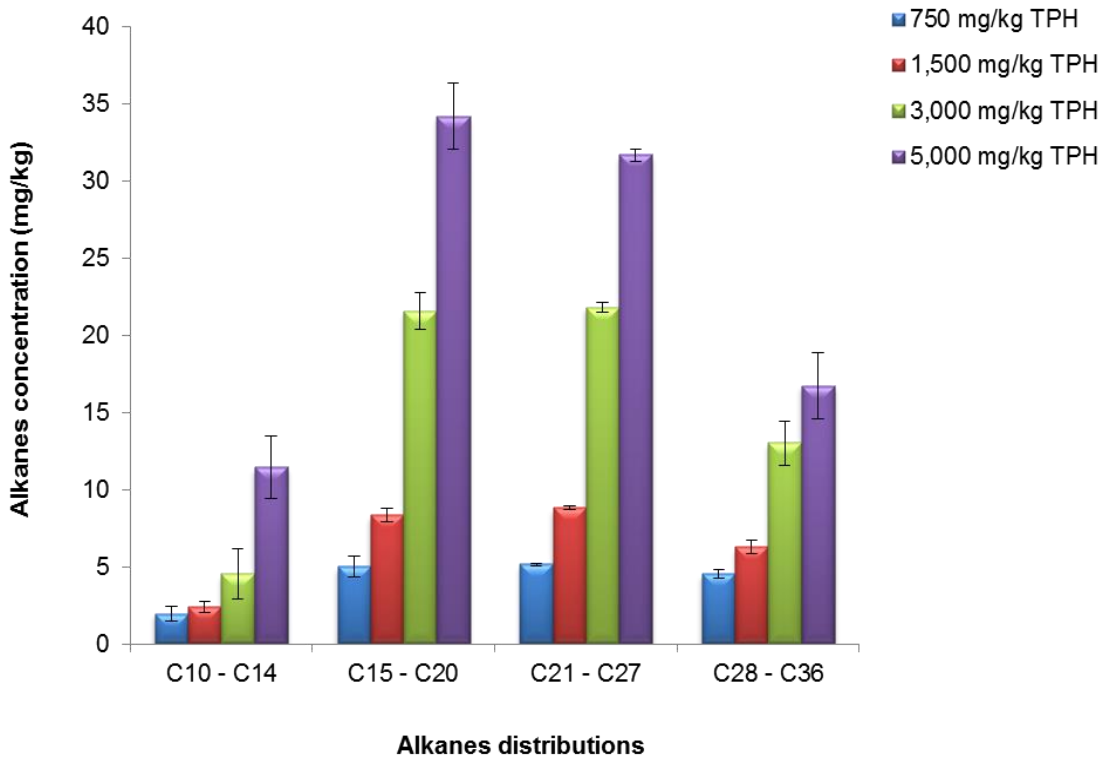

Figure 6-4: Initial alkanes' distribution in the Sinclair crude oil-containing treatments at the start of the experiment in the glasshouse (Day 0). Values are the means of three replicates ± SE.

6.4.3. Results and discussions from the glasshouse experiment and analysis of agricultural produce

6.4.3.1 Growth parameters and postharvest measurements

6.4.3.1.1 Variation in plant growth and fruit production

The experimental results as shown in Figure 6.5 revealed that CRIV of 5,000 mg/kg TPH had the most reducing effect on the Micro-Tom tomato growth, fruit production and fruit ripening. As pointed out in Chapter 5, this decrease in growth can be attributed to the: (1) toxic compounds involved in petroleum hydrocarbons contamination (Bossert and Bartha, 1985, cited in Merkl et al., 2004, p. 182), (2) limitation caused by the oil on transpiration and photosynthesis (Amakiri and Onofeghara, 1983; Akapo et al., 2011; Baruah et al., 2014), (3) effect of petroleum hydrocarbons on soil physicochemistry leading to poor wetting and aeration of the soil (Nwazue,

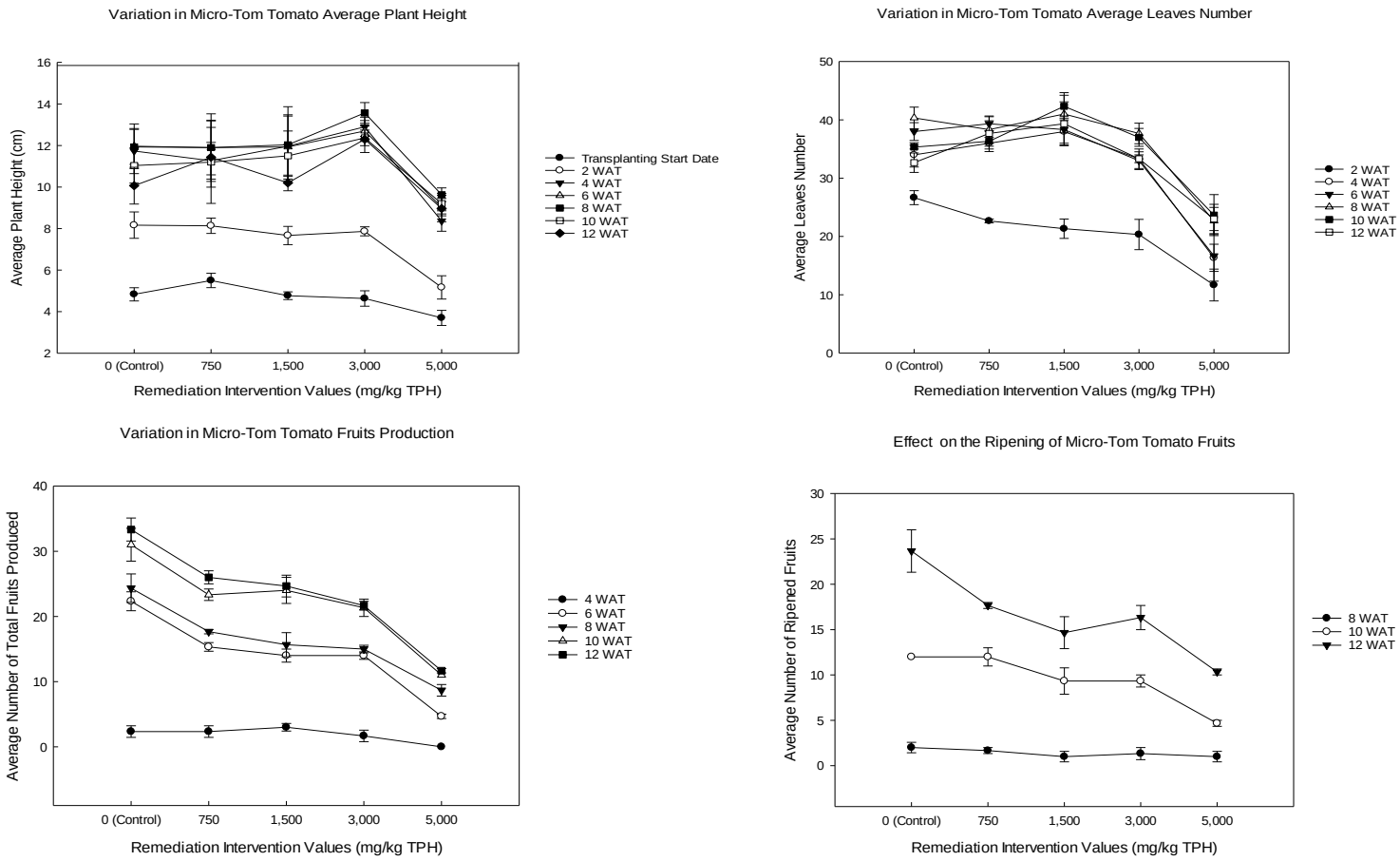

Figure 6-5: Effect of different CRIV on Micro-Tom tomato growth parameters and fruits production. Values are the means of three replicates ± SE. WAT = Weeks after transplanting.

2011), as well as (4) the ability of hydrocarbons to coat plant roots which affect water and nutrient absorption (Xu and Johnson, 1995).

Meanwhile, assessment of the effect of the different CRIV (750 – 5,000 mg/kg TPH) on M82 tomato cultivar growth parameters was only conducted on the plant yield (total fruit mass per plant and root fresh weight) as discussed in Section 6.4.3.1.2.

6.4.3.1.2 Effect on tomatoes fruit and root yield

Only the crude oil contamination at 5,000 mg/kg TPH, compared with the control treatment, significantly reduced ($p < 0.05$) both the fruit and root yield of the Micro-Tom tomato cultivar (Table 6.3). Following the report of Inckot et al. (2011), the decrease in shoot development as recorded in the Micro-Tom tomato fruits arising from the use of low TPH concentration such as 5,000 mg/kg TPH in this experiment, is as a result of physicochemical alterations in the soil but not directly linked to petroleum effect. In line with these authors (i.e. Inckot et al., 2011) as well as Acosta Gallegos and Shibata (1989), this decrease in shoot development of the Micro-Tom tomato cultivar at this CRIV (i.e. 5,000 mg/kg TPH), suggests a survival strategy of the plant in reducing the rate of water use and delaying the onset of more severe stress.

Table 6-3: Effect of different CRIV on Micro-Tom tomato yield

Treatments	Yield	
	Total fruit mass per plant (g/plant)	Root fresh weight (g/plant)
Treatment 1 (0 mg/kg TPH, Control)	159.63 ± 3.71^{a}	5.20 ± 0.59^{a}
Treatment 2 (750 mg/kg TPH)	154.50 ± 1.83^{a}	$5.01 \pm 0.49^{a,b}$
Treatment 3 (1,500 mg/kg TPH)	142.63 ± 9.98^{a}	$4.94 \pm 0.54^{a,b}$
Treatment 4 (3,000 mg/kg TPH)	133.60 ± 6.72^{a}	$4.59 \pm 0.36^{a,b}$
Treatment 5 (5,000 mg/kg TPH)	59.70 ± 3.97^{b}	3.04 ± 0.19^{b}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Although, Merkl et al. (2005a) pointed out that some authors (e.g. Xu and Johnson, 1995; Merkl et al., 2005b) indicated that petroleum hydrocarbons also affect root production, the yields (fruit and root) of the M82 tomato cultivar were unaffected ($p > 0.05$) by crude oil contamination within the range of 750 – 5,000 mg/kg TPH (Table 6.4). This revealed that crude oil contamination at these remediation intervention values is not phytotoxic to the shoot/root development of the M82 tomato cultivar, and that this tomato cultivar could survive in crude oil-contaminated soils with relatively higher concentration of petroleum hydrocarbons.

Generally, the experimental results support the earlier report by Baker (1970), Baek et al. (2004) and Inckot et al. (2011) that the response of plants to petroleum hydrocarbons contamination varies as the Micro-Tom tomato cultivar was more sensitive to crude oil contamination than the M82 tomato cultivar within the experimental range.

Table 6-4: Effect of different CRIV on M82 tomato yield

Treatments	Yield	
	Total fruit mass per plant (g/plant)	Root fresh weight (g/plant)
Treatment 1 (0 mg/kg TPH, Control)	2076.20 ± 99.37[a]	64.60 ± 6.03[a]
Treatment 2 (750 mg/kg TPH)	2003.05 ± 97.85[a]	60.75 ± 6.85[a]
Treatment 3 (1,500 mg/kg TPH)	1953.90 ± 55.05[a]	59.35 ± 4.85[a]
Treatment 4 (3,000 mg/kg TPH)	1943.68 ± 13.33[a]	52.25 ± 1.85[a]
Treatment 5 (5,000 mg/kg TPH)	1938.45 ± 87.43[a]	47.85 ± 0.35[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

6.4.3.1.3 Effect on tomatoes firmness

With the exception of the Micro-Tom tomato fruit samples from the 3,000 mg/kg TPH treatment, crude oil contamination in the range of 750 - 5,000 mg/kg TPH had no significant effect on the firmness of the harvested (Micro-Tom and

M82) tomatoes. The significant difference ($p < 0.05$) in the firmness of Micro-Tom tomato fruits from the 3,000 mg/kg TPH relative to the control treatment (no crude oil contamination) samples is as a result of delayed fruit production of samples from the crude oil-containing treatments (Figure 6.5) as tomatoes lose their firmness as they ripen (Wann, 1996; Nunes, 2008).

Table 6-5: Effect of different CRIV on tomatoes firmness (N/mm)

Treatments	Firmness (N/mm)	
	Micro-Tom tomatoes	M82 tomatoes
Treatment 1 (0 mg/kg TPH, Control)	2.87 ± 0.11^{b}	4.22 ± 0.39^{a}
Treatment 2 (750 mg/kg TPH)	$3.12 \pm 0.04^{a,b}$	4.42 ± 0.31^{a}
Treatment 3 (1,500 mg/kg TPH)	$3.32 \pm 0.10^{a,b}$	5.28 ± 0.44^{a}
Treatment 4 (3,000 mg/kg TPH)	3.55 ± 0.03^{a}	5.15 ± 0.01^{a}
Treatment 5 (5,000 mg/kg TPH)	$3.16 \pm 0.20^{a,b}$	5.47 ± 0.03^{a}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

On the other hand, with firmness (N/mm) defined as average slope of the force/deformation curve (Adegoroye et al., 1989, cited in Batu, 2004, p. 473), the firmness of the two tomato cultivars' samples being > 1.46 N/mm indicated that all the harvested tomato fruits are very firm (Batu, 2004).

6.4.3.1.4 Effect on tomatoes colour parameters

Although external colour measured in terms of hue angle is considered the most vital indicator of tomato quality level (Shewfelt and Prussia, 1993, cited in Radzevicius et al., 2009), assessment of the effect of crude oil contamination on the colour of the harvested tomato fruits revealed that CRIV in the range of 750 – 5,000 mg/kg TPH as used in this experiment, had no significant effect ($p > 0.05$) on the tomato fruits' colour (lightness, chroma and hue angle) of the two

tomato cultivars - Micro-Tom and M82 - as shown in Table 6.6 and 6.7 respectively.

Table 6-6: Variation in the Micro-Tom tomato fruits colour

Treatments	Variation in the Micro-Tom tomato fruits colour		
	Lightness	Chroma	Hue Angle
Treatment 1 (0 mg/kg TPH, Control)	41.14 ± 0.27[a]	42.26 ± 0.79[a]	41.88 ± 0.23[a]
Treatment 2 (750 mg/kg TPH)	40.33 ± 0.41[a]	39.59 ± 0.35[a]	42.29 ± 1.26[a]
Treatment 3 (1,500 mg/kg TPH)	40.13 ± 0.65[a]	38.84 ± 2.17[a]	41.65 ± 0.76[a]
Treatment 4 (3,000 mg/kg TPH)	39.87 ± 0.59[a]	38.78 ± 1.36[a]	41.83 ± 0.65[a]
Treatment 5 (5,000 mg/kg TPH)	39.36 ± 0.04[a]	38.79 ± 0.57[a]	40.83 ± 0.59[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post- hoc* test.

Broadly, the results of the tomato fruits colour parameters agree with the outcome of the levels of lycopene, the carotenoid pigment responsible for the red colour of tomatoes (Ronen et al., 1999), in the two selected tomato cultivars (see section 6.4.3.3.3, Figure 6.14 and Figure 6.15) which although could be altered by environmental growing conditions (Hart and Scott, 1995; Abushita et al., 2000; Sahlin et al., 2004) were statistically unaffected ($p > 0.05$) by the presence of petroleum hydrocarbons (between 750 - 5,000 mg/kg TPH) in the growing medium.

Table 6-7: Variation in the M82 tomato fruits colour

Treatments	Variation in the M82 tomato fruits colour		
	Lightness	Chroma	Hue Angle
Treatment 1 (0 mg/kg TPH, Control)	38.63 ± 0.96[a]	40.25 ± 2.32[a]	39.05 ± 0.69[a]
Treatment 2 (750 mg/kg TPH)	39.43 ± 0.17[a]	43.18 ± 1.07[a]	39.24 ± 0.79[a]
Treatment 3 (1,500 mg/kg TPH)	39.29 ± 0.32[a]	42.53 ± 0.01[a]	38.41 ± 0.49[a]
Treatment 4 (3,000 mg/kg TPH)	38.55 ± 0.34[a]	42.04 ± 0.39[a]	38.74 ± 0.56[a]
Treatment 5 (5,000 mg/kg TPH)	38.27 ± 0.33[a]	40.58 ± 0.26[a]	38.99 ± 1.04[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

6.4.3.2 Nutritional composition analysis results

6.4.3.2.1 Total soluble solids (TSS) and non-structural carbohydrate (sugars) distribution

Brix test

The total soluble solids (TSS) determination which is a measure of the sugar content in tomatoes (Vinha et al., 2014) and their ability to deviate light was evaluated using a refractometer (Kader, 2008) [specifically the ATAGO Pallette digital refractometer (PR-32α, Brix 0 ~ 32%; see Chapter 3, Figure 3.14)]. The statistical analysis result as shown in Table 6.8 revealed that the total soluble solids (TSS) contents of the harvested (Micro-Tom and M82) tomato fruits were unaffected ($p > 0.05$) by crude oil contamination in the range of 750 – 5,000 mg/kg TPH used in this experiment. Nonetheless, the brix chart from the refractive index of crop juices (calibrated in % Sucrose or ^{o}Brix) as provided in Bionutrient Food Association (2015), classifies the TSS contents of the Micro-

Tom tomato fruits as average while those of the M82 tomato fruits were slightly below the average (6 ^{o}Brix) but above the poor (4 ^{o}Brix) level.

Table 6-8: Effect of different CRIV on tomatoes Total Soluble Solids (TSS)

Treatments	Total Soluble Solids (^{o}Brix)	
	Micro-Tom tomatoes	M82 tomatoes
Treatment 1 (0 mg/kg TPH, Control)	6.18 ± 0.40^{a}	5.30 ± 0.02^{a}
Treatment 2 (750 mg/kg TPH)	6.20 ± 0.08^{a}	5.33 ± 0.01^{a}
Treatment 3 (1,500 mg/kg TPH)	6.22 ± 0.45^{a}	5.36 ± 0.09^{a}
Treatment 4 (3,000 mg/kg TPH)	6.23 ± 0.01^{a}	5.77 ± 0.05^{a}
Treatment 5 (5,000 mg/kg TPH)	6.24 ± 0.09^{a}	5.81 ± 0.03^{a}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

In addition, the total soluble solids (TSS) evaluation, which in the view of Kader (2008) and Beckles (2012), is a measure of the sugars (sucrose and hexoses; 65%), acids (citrate and malate; 13%) and other minor components (phenols, amino acids, soluble pectins, ascorbic acid and minerals) present in the tomato fruit pulp, indicated that the Micro-Tom tomatoes have higher amount of dissolved solids in solution (Beckles, 2012) (Table 6.8). The outcome of the HPLC analysis also agrees with the TSS results as the sucrose (Figure 6.6), malic, ascorbic and citric acid contents (Figure 6.12) of the Micro-Tom tomato fruits were found to be higher than those of the M82 tomatoes.

HPLC Analysis

Crude oil contamination in the range used in this experiment (750 – 5,000 mg/kg TPH) had no significant effect on the sugars' (total sugar, fructose, glucose and sucrose) concentration in the Micro-Tom tomato fruits when compared with the amount found in the control treatment samples (Figure 6.6).

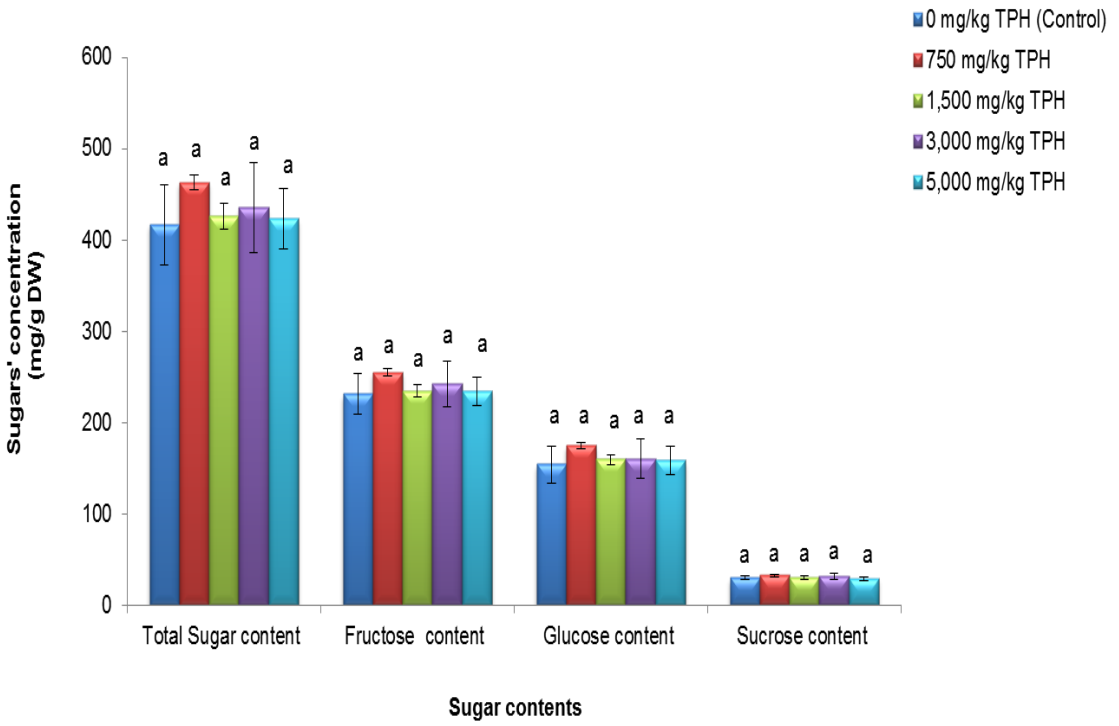

Figure 6-6: Effect of different CRIV on the sugars' distribution in Micro-Tom tomatoes. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

A similar effect was also observed in the M82 tomato fruits' sugars content (Figure 6.7). Meanwhile, despite the non-significant effect ($p > 0.05$) of crude oil contamination (at CRIV between 750 – 5,000 mg/kg TPH) on the yield of the M82 tomato cultivar as shown in Table 6.4, sucrose was only detected in the M82 tomato fruits from the crude oil-containing treatments.

As mentioned earlier in Chapter 5 (Section 5.4.3.2.1) and following the account of Wang and Frei (2011), the recorded sucrose content (> LOD) in the M82 tomato fruits from only the crude oil-contaminated treatments indicated that the application of crude oil in these treatments affected the physiology of the plants. In line with the report of Kameli (1990, cited in Akinci and Losel, 2012, p. 21) that the accumulation of sugars is part of the main effects of water stress, the experimental results suggest that the M82 tomato plants in the crude oil-

containing treatments were under induced water stress arising from the crude oil contamination.

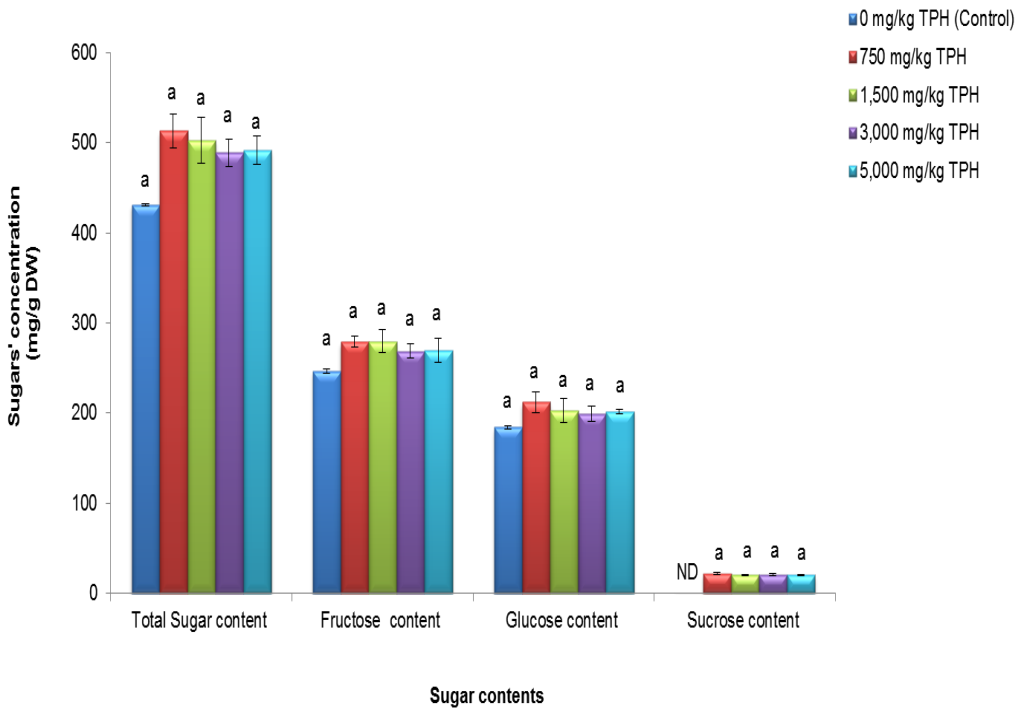

Figure 6-7: Effect of different CRIV on the sugars' distribution in M82 tomatoes. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test. ND = Not Detected.

Practically, results of the sugars distribution in the two tomato (Micro-Tom and M82) cultivars agree with the view of Davies and Kempton (1975, cited in Velterop and Vos, 2001, p. 299) that most of the sugars in tomato fruits are in the form of fructose and glucose while sucrose may be present in low quantities as observed in Figure 6.6 and 6.7 as well as some of the HPLC sugars chromatogram in Figure 6.8.

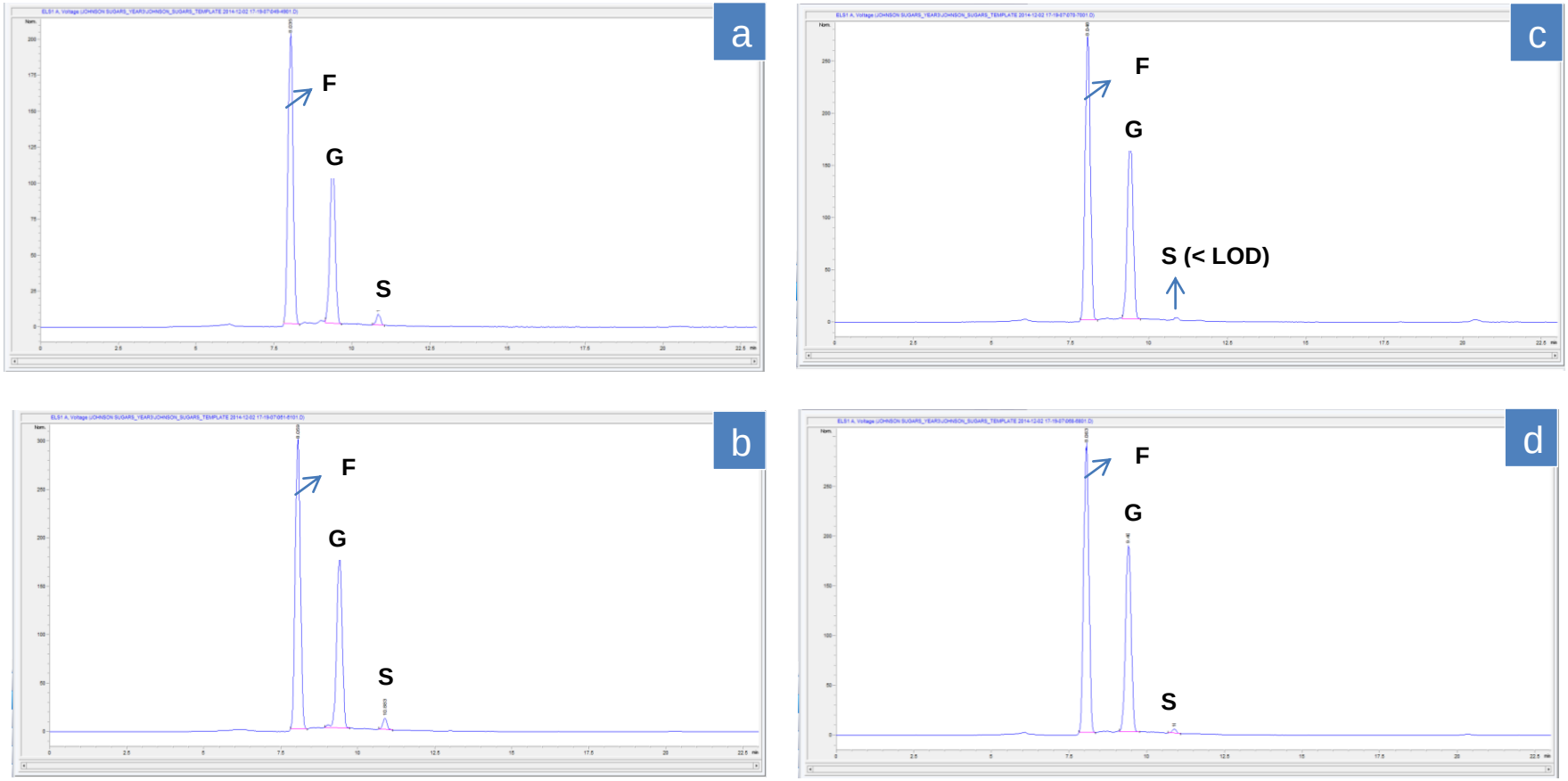

Figure 6-8: Sugars chromatograms in the: Micro-Tom tomatoes - (a) and (b); M82 tomatoes – (c) and (d) at 0 mg/kg TPH (Control) and 5,000 mg/kg TPH respectively [Peak F, G and S = Fructose, Glucose and Sucrose (Retention times = 8.064, 9.418 and 10.882 mins correspondingly); LOD = Limit of Detection].

Sugar ratios

As shown in Table 6.9, the F/G ratios of the two tomato cultivars (Micro-Tom and M82) were not affected ($p > 0.05$) by crude oil contamination between the CRIV of 750 – 5,000 mg/kg TPH. However, following from the explanation provided in Chapter 5 (Section 5.4.3.2.1), the harvested tomato samples with F/G ratio > 1, would present a low tolerance level of fructose.

Table 6-9: Sugar ratios in the fruits of the selected tomato cultivars

Tomato cultivars	Treatments	F/G ratio
Micro-Tom	0 mg/kg TPH (Control)	1.50 ± 0.06^{a}
	750 mg/kg TPH	1.46 ± 0.01^{a}
	1,500 mg/kg TPH	1.47 ± 0.01^{a}
	3,000 mg/kg TPH	1.51 ± 0.04^{a}
	5,000 mg/kg TPH	1.48 ± 0.05^{a}
M82	0 mg/kg TPH (Control)	1.34 ± 0.03^{a}
	750 mg/kg TPH	1.32 ± 0.04^{a}
	1,500 mg/kg TPH	1.38 ± 0.03^{a}
	3,000 mg/kg TPH	1.35 ± 0.02^{a}
	5,000 mg/kg TPH	1.34 ± 0.05^{a}

F/G ratio = Fructose/Glucose ratio.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

6.4.3.2.2 Crude protein contents

The statistical analysis result showed that crude oil contamination at CRIV between 750 - 5,000 mg/kg TPH had no significant effect ($p = 0.228$) on the crude protein content of all the Micro-Tom harvested tomato fruit samples (Figure 6.9). Similarly, the crude protein distribution in the M82 tomato fruits was also unaffected ($p = 0.358$) by the different concentrations of crude oil used in this experiment.

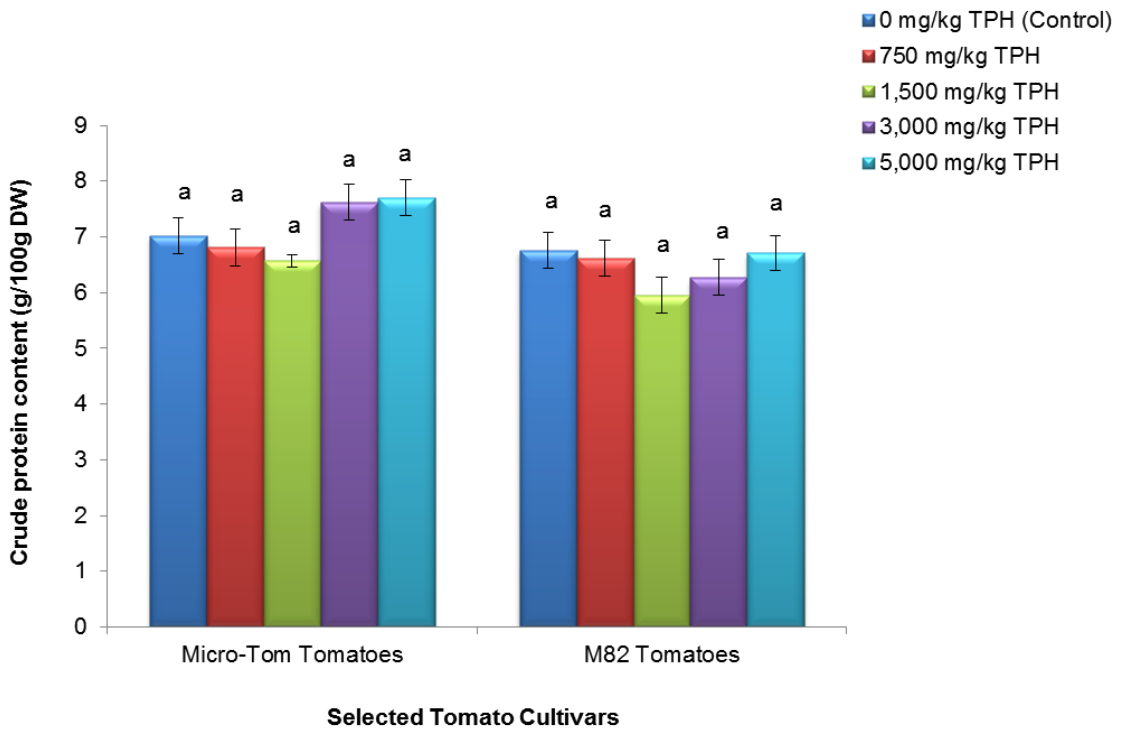

Figure 6-9: Crude protein distribution in the selected tomato cultivars. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Although this outcome of the crude protein content of the two tomato cultivars' samples involving environmental stress with no significant effect is uncommon with respect to published literature (Wang and Frei, 2011), the experimental results suggest that the intensity of the crude oil contamination (at 750 - 5,000 mg/kg TPH)/induced abiotic stresses were not up to the level that could affect the crude protein content of the harvested tomato fruits.

6.4.3.2.3 Minerals analysis results

Surprisingly, although crude oil contamination at 5,000 mg/kg TPH had a significant reducing effect ($p < 0.05$) on the yield of Micro-Tom tomato fruits (see Table 6.3), the concentrations of most of the minerals tested in the Micro-Tom tomato fruits (with the exception of Mo and Sr) at this CRIV, compared with the control treatment samples, were unaffected by the crude oil application. This indicated that there was no direct interaction between crude oil contamination in

the range of 750 – 5,000 mg/kg TPH on the concentration of most of the minerals in the Micro-Tom tomato fruits, including the major dietary minerals as shown in Figure 6.10. Crude oil contamination in this experimental range also had no significant effect ($p > 0.05$) on the yield of the M82 tomato fruits (see Table 6.4) and most of the minerals tested [i.e. response involved no change in parameter (Jarrell and Beverly, 1981)] in the harvested fruits of this tomato cultivar.

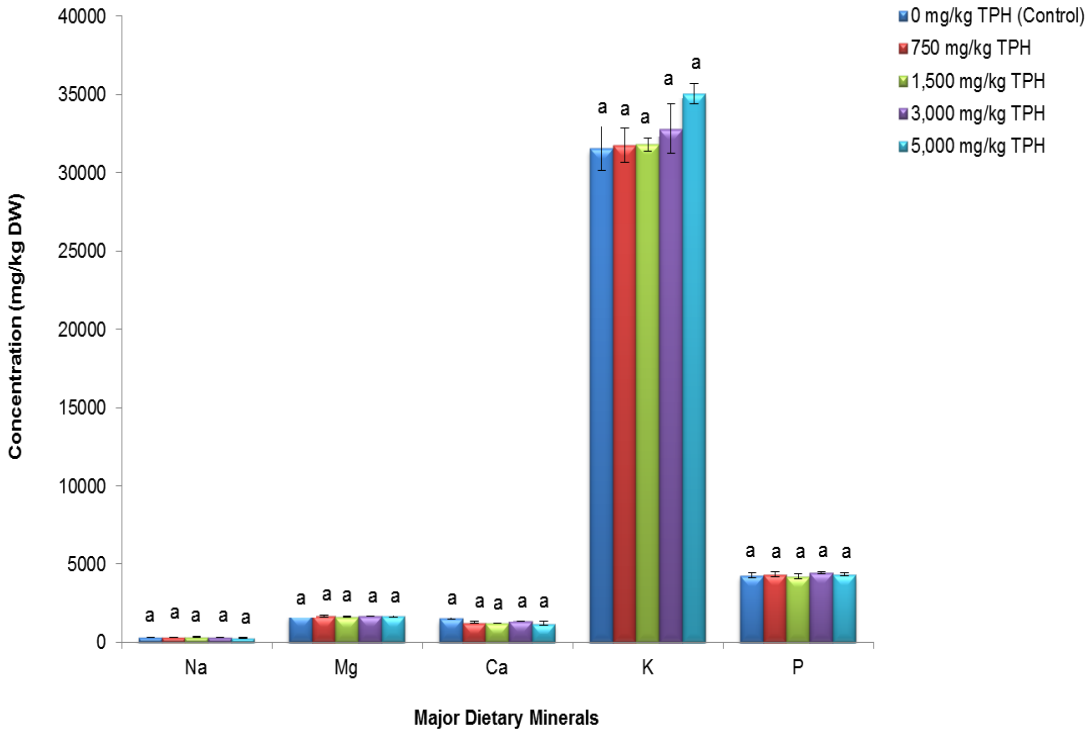

Figure 6-10: The distribution of the major dietary minerals in Micro-Tom tomato fruits. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The results of the major dietary minerals distribution in the two tomato cultivars (Micro-Tom and M82) support the findings in literature that fruits and vegetables are poor sources of sodium (U.S.A National Research Council, 1954, cited in Halevy et al., 1957, p. 412) while the high K content in these cultivars (Figure 6.10 and 6.11) indicate that they may be used with advantage in potassium-repletion diets (U.S.A National Research Council, 1954, cited in Halevy et al., 1957, p. 412).

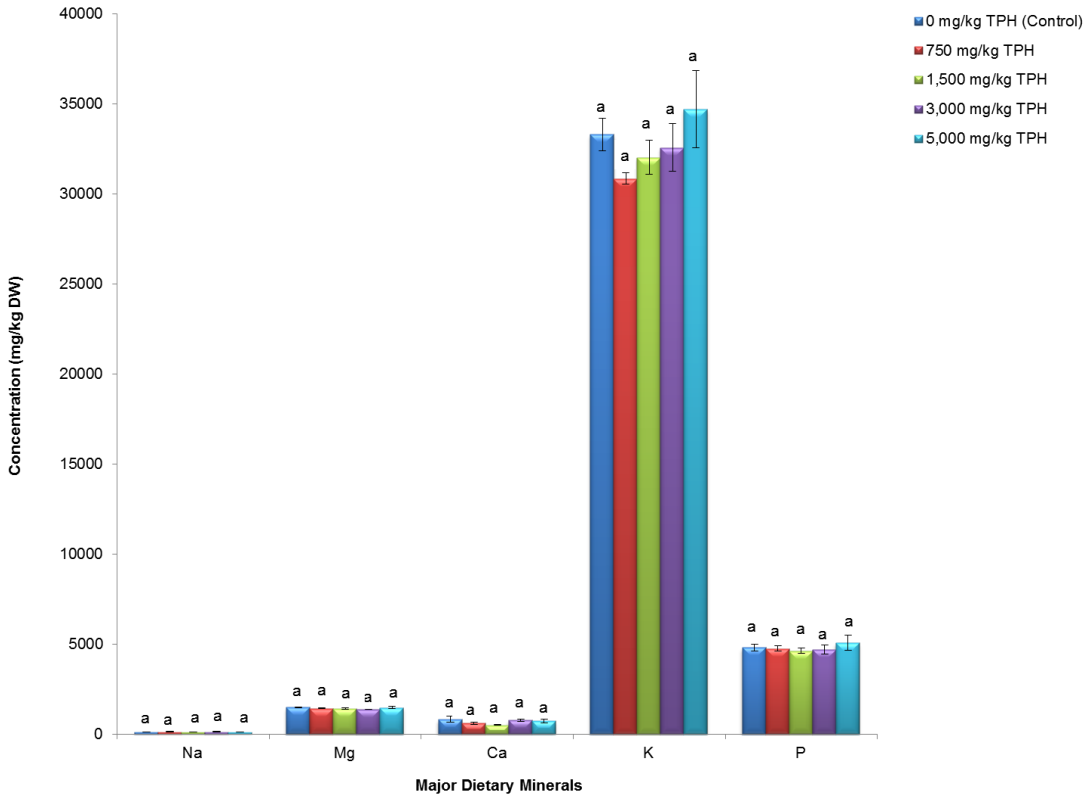

Figure 6-11: The distribution of the major dietary minerals in M82 tomato fruits. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Furthermore, the mineral analysis results revealed that there was an antagonistic effect (Jarrell and Beverly, 1981) of crude oil contamination in the range of 1,500 – 5,000 mg/kg TPH on the concentration of Mo in the harvested Micro-Tom tomato fruits (Table 6.10) from these treatment pots. The antagonistic effect of crude oil application at 5,000 mg/kg TPH and 750 – 1,500 mg/kg TPH also led to reduced Sr and Ba contents respectively in the Micro-Tom tomato fruits. In this latter case, however, the reduction in the Sr and Ba concentrations in the Micro-Tom tomato fruits from these treatments is beneficial as the two minerals are regarded as contaminants which have harmful effect on human health when in excess (Nieman et al., 1992). Meanwhile, the Se content of the M82 tomato fruits (Table 6.11) from the 5,000 mg/kg TPH treatment was only significantly different ($p < 0.05$) from that obtained in samples from the 3,000 mg/kg TPH treatment as a result of the different level of crude oil contamination.

Table 6-10: Distribution of minor dietary minerals (mg/kg) in Micro-Tom tomato fruits

Treatments	Distribution of Minor Dietary Minerals (mg/kg)									
	Cr	Mn	Co	Cu	Zn	Mo	Fe	Se	V	Ni
Treatment 1 (0 mg/kg TPH, Control)	1.22 ± 0.09^{a}	17.87 ± 0.63^{a}	0.043 ± 0.01^{a}	4.42 ± 0.35^{a}	12.08 ± 0.22^{a}	6.01 ± 0.95^{a}	25.60 ± 0.50^{a}	0.375 ± 0.06^{a}	0.063 ± 0.01^{a}	0.31 ± 0.08^{a}
Treatment 2 (750 mg/kg TPH)	1.03 ± 0.11^{a}	15.83 ± 0.76^{a}	0.044 ± 0.00^{a}	3.90 ± 0.12^{a}	10.33 ± 0.30^{a}	3.44 ± 0.85^{a,b}	24.47 ± 0.93^{a}	0.367 ± 0.04^{a}	0.042 ± 0.01^{a}	0.24 ± 0.09^{a}
Treatment 3 (1,500 mg/kg TPH)	0.97 ± 0.12^{a}	16.65 ± 0.97^{a}	0.043 ± 0.01^{a}	3.98 ± 0.16^{a}	11.45 ± 0.82^{a}	2.30 ± 0.29^{b}	25.87 ± 0.35^{a}	0.397 ± 0.04^{a}	0.048 ± 0.00^{a}	0.40 ± 0.06^{a}
Treatment 4 (3,000 mg/kg TPH)	0.96 ± 0.03^{a}	17.34 ± 1.00^{a}	0.038 ± 0.01^{a}	4.59 ± 0.07^{a}	13.41 ± 0.27^{a}	2.08 ± 0.28^{b}	26.60 ± 1.78^{a}	0.464 ± 0.05^{a}	0.049 ± 0.01^{a}	0.46 ± 0.19^{a}
Treatment 5 (5,000 mg/kg TPH)	0.95 ± 0.05^{a}	17.69 ± 0.53^{a}	0.036 ± 0.00^{a}	4.71 ± 0.04^{a}	13.49 ± 1.21^{a}	1.87 ± 0.12^{b}	27.13 ± 0.79^{a}	0.474 ± 0.03^{a}	0.065 ± 0.01^{a}	0.61 ± 0.14^{a}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Table 6-11: Distribution of minor dietary minerals (mg/kg) in M82 tomato fruits

Treatments	Distribution of Minor Dietary Minerals (mg/kg)									
	Cr	Mn	Co	Cu	Zn	Mo	Fe	Se	V	Ni
Treatment 1 (0 mg/kg TPH, Control)	1.19 ± 0.50^{a}	10.48 ± 2.23^{a}	0.034 ± 0.01^{a}	4.89 ± 0.90^{a}	19.29 ± 1.96^{a}	5.99 ± 1.71^{a}	27.90 ± 1.70^{a}	0.34 ± 0.02^{a,b}	0.021 ± 0.00^{a}	2.06 ± 0.85^{a}
Treatment 2 (750 mg/kg TPH)	0.81 ± 0.05^{a}	7.49 ± 0.04^{a}	0.017 ± 0.00^{a}	4.39 ± 0.47^{a}	18.31 ± 1.15^{a}	4.38 ± 0.37^{a}	24.50 ± 3.50^{a}	0.29 ± 0.01^{a,b}	0.023 ± 0.00^{a}	0.57 ± 0.00^{a}
Treatment 3 (1,500 mg/kg TPH)	0.88 ± 0.04^{a}	8.95 ± 1.38^{a}	0.019 ± 0.00^{a}	4.63 ± 0.17^{a}	19.27 ± 0.99^{a}	4.72 ± 0.43^{a}	25.25 ± 3.75^{a}	0.32 ± 0.00^{a,b}	0.026 ± 0.00^{a}	0.65 ± 0.25^{a}
Treatment 4 (3,000 mg/kg TPH)	0.92 ± 0.04^{a}	10.35 ± 0.10^{a}	0.019 ± 0.00^{a}	4.71 ± 0.15^{a}	19.97 ± 2.49^{a}	5.22 ± 0.51^{a}	26.00 ± 4.00^{a}	0.33 ± 0.01^{b}	0.028 ± 0.01^{a}	0.77 ± 0.19^{a}
Treatment 5 (5,000 mg/kg TPH)	0.96 ± 0.05^{a}	10.95 ± 0.48^{a}	0.029 ± 0.01^{a}	4.79 ± 0.25^{a}	20.66 ± 0.20^{a}	6.25 ± 2.48^{a}	26.40 ± 1.80^{a}	0.37 ± 0.01^{a}	0.031 ± 0.01^{a}	1.13 ± 0.38^{a}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The outcome of the minerals analysis also revealed that among all the harvested Micro-Tom and M82 tomato fruits, only the Micro-Tom tomatoes from the control treatment (no crude oil contamination), had a Pb content (0.071 mg/kg) (Table 6.12) above the FAO/WHO Codex maximum level (ML) of 0.05 mg/kg for fruiting vegetables (Codex Alimentarius, 2015). However, this Pb concentration in the Micro-Tom tomato control samples, as identified by Reilly (1980, cited in Samara, 1992, p. 64), is below the maximum permissible concentration of the element (2.0 – 2.5 mg/kg DW) in vegetables for human consumption. Meanwhile, Al was not detected in all the harvested M82 tomato fruits as shown in Table 6.13.

Table 6-12: Distribution of mineral contaminants (mg/kg) in Micro-Tom tomato fruits

Treatments	Distribution of Mineral Contaminants (mg/kg)					
	Al	As	Sr	Ba	Cd	Pb
Treatment 1 (0 mg/kg TPH, Control)	1.61 ± 0.37[a]	0.049 ± 0.01[a]	1.52 ± 0.09[b]	0.59 ± 0.07[b]	0.049 ± 0.00[a]	0.071 ± 0.01[a]
Treatment 2 (750 mg/kg TPH)	1.72 ± 0.52[a]	0.049 ± 0.00[a]	1.27 ± 0.07[a,b]	0.33 ± 0.01[a]	0.052 ± 0.00[a]	0.044 ± 0.01[a]
Treatment 3 (1,500 mg/kg TPH)	1.70 ± 0.16[a]	0.046 ± 0.00[a]	1.09 ± 0.06[a,b]	0.33 ± 0.09[a]	0.046 ± 0.00[a]	0.041 ± 0.01[a]
Treatment 4 (3,000 mg/kg TPH)	1.55 ± 0.39[a]	0.046 ± 0.00[a]	1.07 ± 0.02[a,b]	0.35 ± 0.01[a,b]	0.046 ± 0.00[a]	0.039 ± 0.00[a]
Treatment 5 (5,000 mg/kg TPH)	0.22 ± 0.05[a]	0.042 ± 0.01[a]	1.01 ± 0.18[a]	0.48 ± 0.03[a,b]	0.039 ± 0.00[a]	0.038 ± 0.01[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Table 6-13: Distribution of mineral contaminants (mg/kg) in M82 tomato fruits

Treatments	Distribution of Mineral Contaminants (mg/kg)					
	Al	As	Sr	Ba	Cd	Pb
Treatment 1 (0 mg/kg TPH, Control)	ND	0.033 ± 0.00[a]	0.75 ± 0.31[a]	0.32 ± 0.13[a]	0.048 ± 0.01[a]	0.027 ± 0.01[a]
Treatment 2 (750 mg/kg TPH)	ND	0.030 ± 0.00[a]	0.24 ± 0.01[a]	0.03 ± 0.00[a]	0.045 ± 0.00[a]	0.037 ± 0.01[a]
Treatment 3 (1,500 mg/kg TPH)	ND	0.030 ± 0.00[a]	0.37 ± 0.14[a]	0.21 ± 0.06[a]	0.049 ± 0.01[a]	0.026 ± 0.01[a]
Treatment 4 (3,000 mg/kg TPH)	ND	0.032 ± 0.00[a]	0.56 ± 0.04[a]	0.25 ± 0.00[a]	0.051 ± 0.00[a]	0.021 ± 0.01[a]
Treatment 5 (5,000 mg/kg TPH)	ND	0.035 ± 0.01[a]	0.71 ± 0.17[a]	0.34 ± 0.07[a]	0.051 ± 0.01[a]	0.019 ± 0.01[a]

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.
ND = Not Detected.

Mineral ratios of the selected tomatoes

As shown in Table 6.14, crude oil contamination in the range used for this experiment (750 – 5,000 mg/kg TPH), had no significant effect ($p > 0.05$) on the mineral ratios of the harvested tomato fruits while the consumption of the two tomato (Micro-Tom and M82) cultivars' fruits would not contribute to high blood pressure as a result of their very low Na/K ratios (< 0.6). However, the Ca/P ratios (< 0.5) of these samples suggest that they would promote the loss of more than normal amount of calcium in the urine which reduces the calcium level in bones (Nieman et al., 1992).

Table 6-14: Mineral ratios of the selected tomatoes

Treatments	Ca/P[1] ratio		Na/K[2] ratio	
	Micro-Tom	M82	Micro-Tom	M82
Treatment 1 (0 mg/kg TPH, Control)	0.36 ± 0.02[a]	0.17 ± 0.04[a]	0.01 ± 0.00[a]	0.004 ± 0.00[a]
Treatment 2 (750 mg/kg TPH)	0.29 ± 0.02[a]	0.13 ± 0.02[a]	0.01 ± 0.00[a]	0.004 ± 0.00[a]
Treatment 3 (1,500 mg/kg TPH)	0.29 ± 0.01[a]	0.11 ± 0.01[a]	0.01 ± 0.00[a]	0.004 ± 0.00[a]
Treatment 4 (3,000 mg/kg TPH)	0.31 ± 0.01[a]	0.17 ± 0.01[a]	0.01 ± 0.00[a]	0.004 ± 0.00[a]
Treatment 5 (5,000 mg/kg TPH)	0.28 ± 0.03[a]	0.15 ± 0.01[a]	0.01 ± 0.00[a]	0.003 ± 0.00[a]

[1]Ca/P ratio = Calcium: Phosphorus ratio
[2]Na/K ratio = Sodium: Potassium ratio
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

6.4.3.3 Phytochemicals composition analysis results

6.4.3.3.1 Total phenolics and total flavonoids contents

Crude oil contamination at CRIV between 750 - 5,000 mg/kg TPH had no significant effect on the total phenolics content (TPC) ($p = 0.132$) as well as the total flavonoids distribution ($p = 0.705$) in all the harvested Micro-Tom tomato fruits as shown in Table 6.15. Similar results with no significant effect ($p > 0.05$) of crude oil contamination on the phenolics distribution in M82 tomato cultivar fruits were also recorded (Table 6.16).

Table 6-15: Phenolics distribution in Micro-Tom tomato fruits

Treatments	Phenolics distribution	
	Total phenolics content (mg GAE/g DW)*[1]	Total Flavonoids content (mg QE/g DW)*[2]
Treatment 1 (0 mg/kg TPH, Control)	8.93 ± 0.35[a]	10.97 ± 0.56[a]
Treatment 2 (750 mg/kg TPH)	8.69 ± 0.01[a]	11.14 ± 0.63[a]
Treatment 3 (1,500 mg/kg TPH)	9.09 ± 0.14[a]	11.09 ± 0.75[a]
Treatment 4 (3,000 mg/kg TPH)	8.58 ± 0.18[a]	10.63 ± 0.31[a]
Treatment 5 (5,000 mg/kg TPH)	8.19 ± 0.29[a]	10.05 ± 0.70[a]

*[1]Total phenolics content (TPC) values were expressed as mg of gallic acid equivalents (GAE) per g of dry weight extract.
*[2]Total flavonoids content values were expressed as mg of quercetin equivalents (QE) per g of dry weight extract.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The analytical results revealed that crude oil contamination in the range used in this experiment had no significant effect on the metabolic activity of these plants.

Table 6-16: Phenolics distribution in M82 tomato fruits

Treatments	Phenolics distribution	
	Total phenolics content (mg GAE/g DW)*[1]	Total Flavonoids content (mg QE/g DW)*[2]
Treatment 1 (0 mg/kg TPH, Control)	5.14 ± 0.03[a]	4.97 ± 0.09[a]
Treatment 2 (750 mg/kg TPH)	5.97 ± 0.40[a]	5.80 ± 0.21[a]
Treatment 3 (1,500 mg/kg TPH)	6.04 ± 0.10[a]	6.88 ± 0.32[a]
Treatment 4 (3,000 mg/kg TPH)	6.57 ± 0.49[a]	6.91 ± 0.77[a]
Treatment 5 (5,000 mg/kg TPH)	6.71 ± 0.37[a]	7.02 ± 0.76[a]

*[1]Total phenolics content (TPC) values were expressed as mg of gallic acid equivalents (GAE) per g of dry weight extract.
*[2]Total flavonoids content values were expressed as mg of quercetin equivalents (QE) per g of dry weight extract.
Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

6.4.3.3.2 Non-volatile organic acids distribution

Statistical analysis of the organic acids distribution in the two tomato cultivars (Micro-Tom and M82) revealed that crude oil contamination in the range used in this experiment (750 – 5,000 mg/kg TPH) had no significant effect ($p > 0.05$) on the tartaric and citric acid concentrations as well as the malic, ascorbic and citric acid concentrations of the Micro-Tom and M82 tomatoes respectively. In the Micro-Tom tomatoes, crude oil contamination at 1,500 and 3,000 mg/kg TPH led to a reduction in the malic acid content. Meanwhile, contrary to the view of Noctor and Foyer (1998, cited in Gaur and Sharma, 2014, p. 133) that vitamin C loss is the most sensitive indicator of stress exposure, there was a significant increase ($p = 0.018$) in the concentration of ascorbic acid found in the Micro-Tom tomato samples from the 3,000 mg/kg TPH treatment (Figure 6.12).

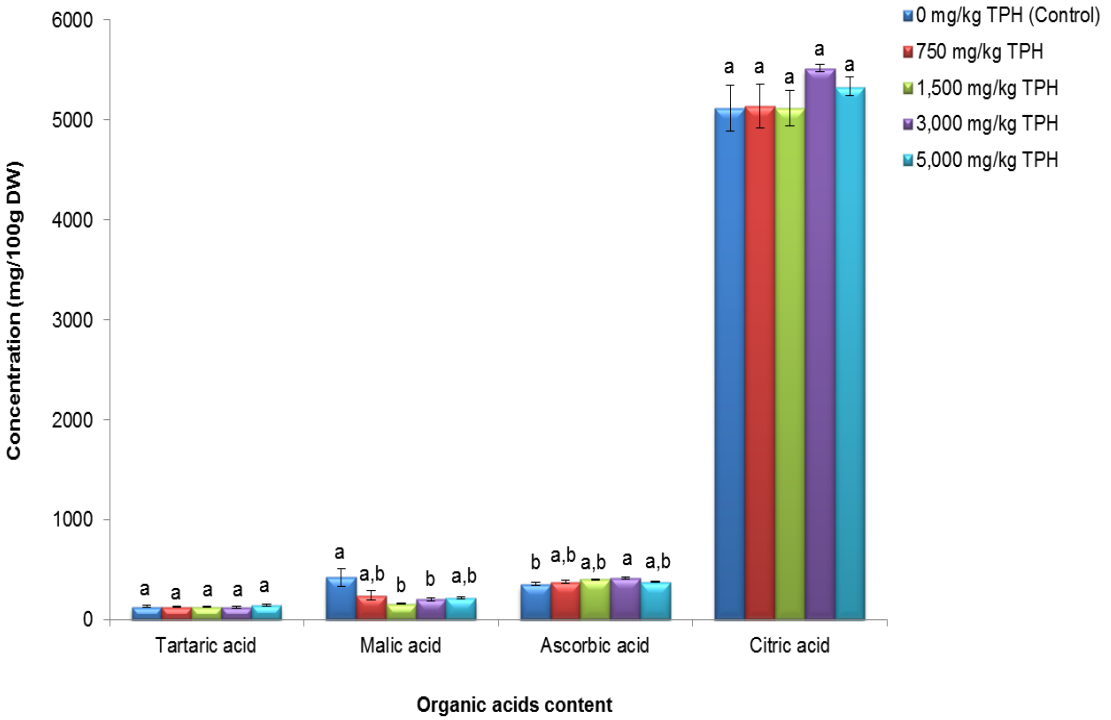

Figure 6-12: Organic acids distribution in Micro-Tom tomatoes. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

The significant increase in the L-ascorbic acid content of the Micro-Tom tomato fruits from the 3,000 mg/kg TPH treatment (419.54 mg/100g DW) is as a result of the induced water stress on the plants via the presence of crude oil in the growing medium. In line with the report of Akinci and Losel (2012), this led to the accumulation of this organic acid which contributes to osmotic adjustment. To some extent, results from previous experiments on the effect of environmental stress on ascorbate (vitamin C) content in harvested products have, however, been controversial (Wang and Frei, 2011).

On the other hand and similar to the observation in the leafy vegetables, tartaric acid was only detected in the M82 tomato samples from the treatment with the highest level of crude oil contamination (5,000 mg/kg TPH) used in this experiment (Figure 6.13). Largely, the result of the organic acids distribution in the M82 tomatoes, with the exception of tartaric acid content, revealed that the

concentrations of organic acids in the tomato cultivar's fruits were not affected ($p > 0.05$) by crude oil contamination in the range of 750 – 5,000 mg/kg TPH.

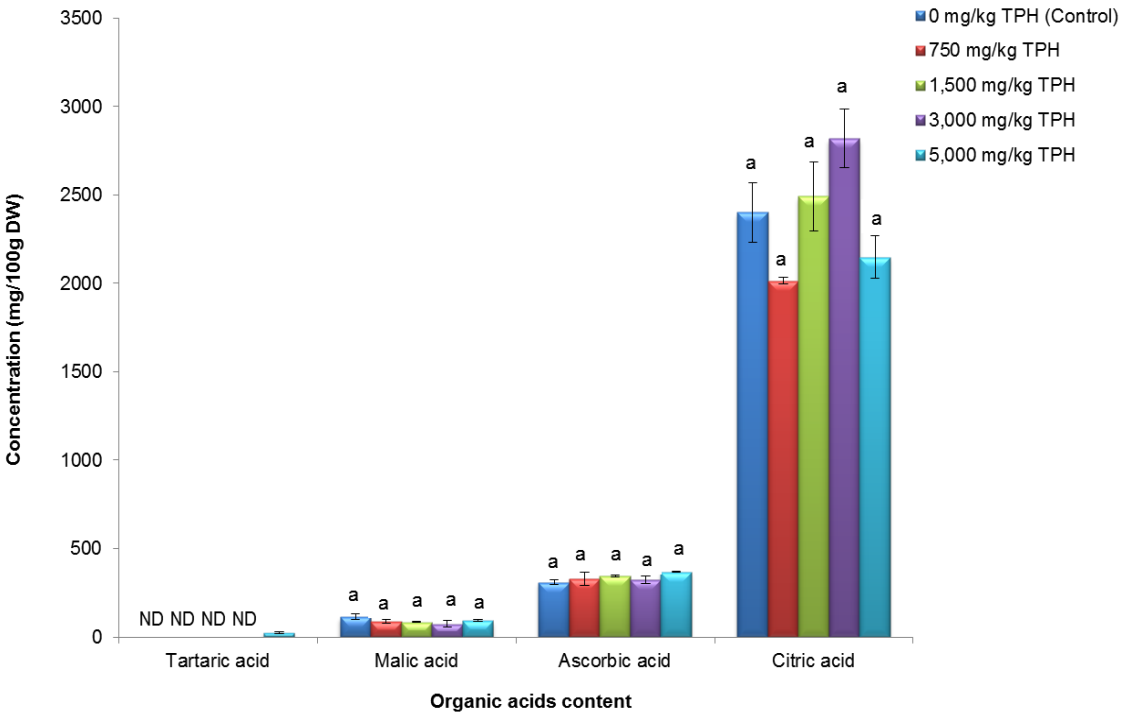

Figure 6-13: Organic acids distribution in M82 tomatoes. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test. ND = Not Detected.

Nonetheless, crude oil contamination in the experimental range altered the usual trend of tomatoes' organic acids distribution in the harvested Micro-Tom tomato fruits. In line with Emin (2001), citric and malic acids are the main organic acids in tomatoes but this was only true for Micro-Tom tomato fruit samples from the control treatment (i.e. with citric acid > malic acid > ascorbic acid > tartaric acid). The organic acids contents of the M82 tomato fruit samples from both the control and crude oil-containing treatments contradict this view of Emin (2001).

Malic acid/Citric acid ratio

Following the report of Meredith et al. (1989), the significantly higher malic acid/citric acid ratio ($p < 0.05$) in the Micro-Tom tomato fruits from the control treatment compared with those obtained in this cultivar's fruits from the crude

oil-contaminated treatment (Table 6.17), indicate that the Micro-Tom tomato fruits from the control treatment have increased maturity. This agrees with the data presented in Figure 6.5 in which Micro-Tom tomato fruits from the control treatment had the highest average number of ripened fruits. Meanwhile, the malic acid/citric acid ratio of the M82 tomato cultivar, which could be used as an index of maturity (Meredith et al., 1989; Esti et al., 1997) of the plant, was unaffected ($p > 0.05$) by crude oil contamination in the experimental range.

Table 6-17: Malic acid/Citric acid ratios in the tomato cultivars' fruits

Treatments	Micro-Tom	M82
0 mg/kg TPH (Control)	0.08 ± 0.01^{a}	0.05 ± 0.01^{a}
750 mg/kg TPH	0.05 ± 0.01^{b}	0.04 ± 0.00^{a}
1,500 mg/kg TPH	0.03 ± 0.00^{b}	0.03 ± 0.01^{a}
3,000 mg/kg TPH	0.04 ± 0.00^{b}	0.03 ± 0.01^{a}
5,000 mg/kg TPH	0.04 ± 0.00^{b}	0.04 ± 0.01^{a}

Values are the means of three replicates ± SE.
Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

Generally, the results of the malic acid/citric acid ratio of the two tomato cultivars (Table 6.17) support the view of Emin (2001) that the malic acid/citric acid ratio in red ripe tomato is ≤ 0.5.

6.4.3.3.3 Lycopene and β-carotene contents

Although some studies involving the effect of environmental stresses on lycopene content in tomato fruits reported by Wang and Frei (2011) indicated an increase in lycopene concentration, such increment according to Krauss et al. (2006), Navarro et al. (2006) as well as Wang and Frei (2011) could partly be as a result of concentration effect arising from reduced water content in the stressed crops. This increase in carotenoid content of crops has also been linked with enhanced biosynthesis (Krauss et al., 2006; Wang and Frei, 2011).

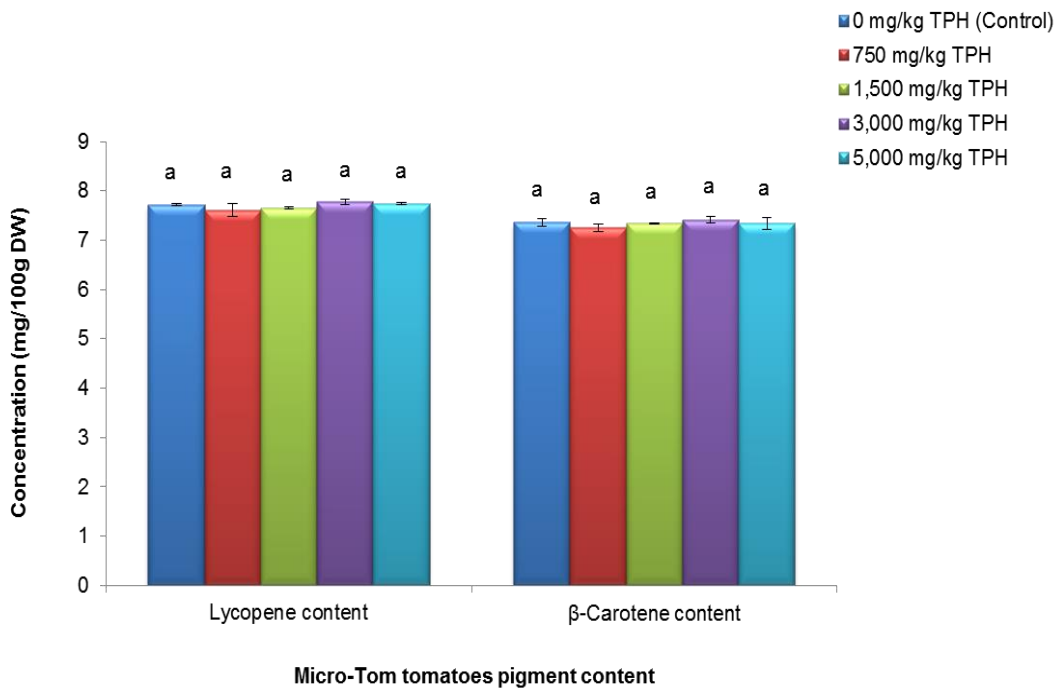

Figure 6-14: Pigments distribution in Micro-Tom tomato ripe fruits. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

In this experiment, the results of the carotenoids (lycopene and β-carotene) distribution in the two tomato cultivars (Figure 6.14 and 6.15), determined on a dry weight basis, showed that crude oil contamination between 750 - 5,000 mg/kg TPH had no significant effect ($p > 0.05$) on the lycopene and β-carotene contents of the harvested tomato fruits as well as on their biosynthesis.

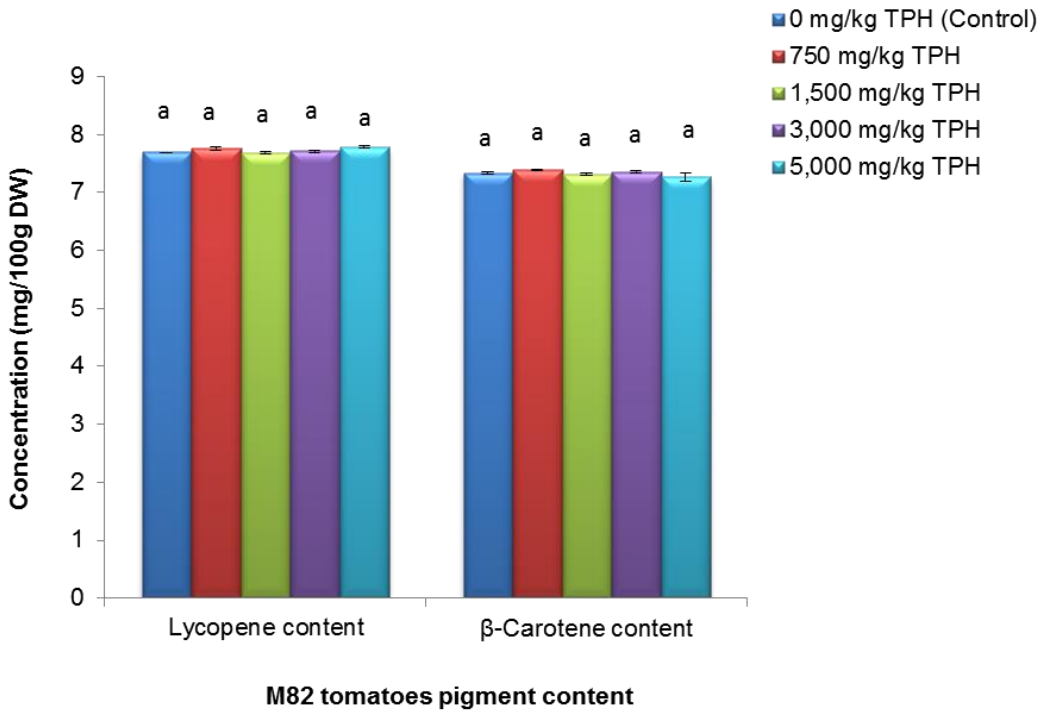

Figure 6-15: Pigments distribution in M82 tomato ripe fruits. Values are the means of three replicates ± SE. Means followed by different letters are significantly different ($p < 0.05$) according to Tukey *post-hoc* test.

6.4.3.4 Bioaccumulated PAHs

As mentioned in Chapter 5 (Section 5.4.3.4), evaluation of the bio-accumulated PAHs content of the harvested tomato fruit samples could not be completed as a result of technical limitations.

6.5 Conclusion

Only the yield of Micro-Tom tomato cultivar was affected by crude oil contamination at 5,000 mg/kg TPH. Aside from the organic acids and some minerals distribution in the Micro-Tom tomatoes as well as the sucrose level (> LOD) in the M82 tomato samples from the crude oil-contaminated treatments with Se content variation, most of the nutritional and phytochemical contents of the selected tomato cultivars were unaffected (on dry weight basis) by the different remediation intervention values considered in this experiment. The

elimination of ‘concentration effects’ and observed changes in some of the chemical compositions (e.g. minerals and organic acids) tested further re-affirm that apart from genetics, environmental factors (such as crude oil contamination in the range of 750 – 5,000 mg/kg TPH) may affect tomato fruit quality as well as yield (Baxter et al., 2005).

Although all the harvested (Micro-Tom and M82) tomatoes have beneficial Na/K ratios (< 0.6), crude oil contamination in the experimental range had no significant effect on the F/G, Ca/P and Na/K ratios of the two tomato cultivars.

Generally and to the best of the author’s knowledge, the investigations in this Chapter provide the first evidence on the effect of remediation intervention values (between 750 - 5,000 mg/kg TPH) on tomato yield and quality. The experimental results were in line with the literature (e.g. Baker, 1970; Baek et al., 2004; Inckot et al., 2011) that the response of plants to petroleum hydrocarbons contamination varies as the Micro-Tom tomato cultivar was more sensitive to the different CRIV than the M82 tomato cultivar, and also consistent with the finding of Alexander and Webb (1987) in which Libyan crude oil of up to 5 mg/g did not affect the growth of *S. alterniflora*. Nonetheless, more studies are required to assess the impact of other classes of crude oil (involving different density and sulphur content) vis-à-vis their effect on the yield and quality of crops as well as possible contribution to PAHs bioaccumulation.

CHAPTER 7
GENERAL CONCLUSIONS AND RECOMMENDATIONS

7.1 Project conclusions

The project conclusions arising from all the experiments carried out to achieve the aim and objectives of the research are summarized in Figure 7.1:

RESEARCH AIM (Chapter 1, Section 1.3)

↓

OBJECTIVE 1: Literature review - Research gaps (Chapter 2, Section 2.6)

↓

KEY RESEARCH FINDINGS

↓

OBJECTIVE 2:

- The experimental crude oil (Pr/Ph = 0.98) with higher concentrations of: C_{10} – C_{14}, C_{15} – C_{20} and C_{21} – C_{27} alkanes than the C_{28} – C_{36} alkanes, as well as phenanthrene and anthracene permits easier degradation.

↓

OBJECTIVE 3:

- RENA application enhanced the degradation of C_{15} - C_{20} and C_{21} - C_{27} alkanes more than the bioaugmentation treatment;
- Of the two bioremediation strategies, RENA treatment showed better support for agrifood production.

↓

OBJECTIVE 4:

- *B. juncea* had the least tolerance to crude oil stress among the leafy vegetables;
- The yield of all the selected vegetables, except M82 tomato cultivar, were in most cases impaired at CRIV > 3,000 mg/kg TPH;
- *p*-Xylene presented the greatest toxic potential among the VOCs emitted at CRIV of 5,000 mg/kg TPH.

↓

OBJECTIVE 5:

- Crude oil stress at 10,000 mg/kg TPH:
  - Enhanced some minerals and crude protein contents of *B. oleracea* and *L. sativa*;
  - Enhanced sucrose, total sugars, total phenolics and total flavonoids content of the *L. sativa* vegetable samples;
  - Resulted to accelerated leaf senescence of the *L. sativa* vegetable (Table 5.13).
- Most of the quality parameters of the two tomato cultivars were unaffected by crude oil contamination in the range of 750 – 5,000 mg/kg TPH.

Figure 7-1: Summary of key research findings

7.2 New insights from the research outcome

As established in Chapter 2 that crude oil spills affect soil and plants in the environment, the research findings as summarised in Figure 7.1, add to the body of knowledge on the effect of crude oil contamination on food crops and plant tissues as recommended by Agbogidi et al. (2007). The study of the impact of fresh oil, with higher toxic potential than weathered oil (Mendelssohn et al., 2012), on agrifood production at remediation intervention values, aid the assessment of the effect of slight increase in the concentration of petroleum hydrocarbons in the environment on crop growth and quality.

The experimental results in this study agree with the view of Ibemesim (2010) as well as Meudec et al. (2007) that the direct impacts of oil on plants result from both physical effects and chemical toxicity. These can be attributed to: (1) the chemical action of crude oil compounds (like the PAHs) which may affect plant physiological processes (Ibemesim, 2010) at some concentrations, and (2) the negative effect of crude oil-induced abiotic stresses (see Figure 2.5) on plants.

As some CRIV had no significant effect on the yield and quality of the selected vegetables, the research findings re-affirm the need for the consideration of bioavailabilities of PAHs at crude oil remediated sites in the setting of cleanup levels by federal and state regulatory agencies as the conventional bulk measurements of TPH in a sample do not provide a sufficient basis for a human health risk assessment (McMillen et al., 2001). This also becomes necessary in order to avoid overestimation of the exposure to toxic organic compounds at the remediation intervention or target values, and associated risk by the currently used chemical methods. To some extents, the experimental results in Chapter 6 points to the acceptance of the view of Alexander (2000, p.4261) that “if the total concentration at a polluted site is greater than the regulatory level but the bioavailable concentration is below that value, a site that might be slated for expensive cleanup might, instead, be deemed to present an acceptable risk”.

Furthermore and following the account of Jarrell and Beverly (1981), this study underscores the significance of adequate understanding of the

phenomena/mechanisms of ‘dilution effect’ and ‘concentration effect’ as well as ‘non-interactive effect’ and ‘interactive effect’ in the evaluation of the impact of crude oil contamination on plant nutrition and soil fertility. Also, and in line with these authors (i.e. Jarrell and Beverly, 1981), the experimental results (e.g. in Chapter 4 mineral analysis) emphasize the importance of not regarding concentration value alone as an overall measure of plant nutrient status without considering data on total nutrient uptake and yield wherever possible.

In view of the report of Lillo et al. (2008) in which the susceptibility of flavonoid synthesis to environment factors was indicated, the outcome of this research showed that manipulation of crude oil contamination below the suggested critical TPH concentration of 1.5% for both animal toxicity and phytotoxicity by Tang et al. (2011), such as that used in the leafy vegetables’ experiment in Chapter 5 (i.e. between CRIV of 1,500 - 10,000 mg/kg TPH) provide, to some extent, an alternative to genetic engineering in carrying out special modifications on the level of plant components in situations where PAHs bioaccumulation is not a source of concern.

Finally, as fresh crude oil spills lead to the release of VOCs into the environment, quantitative analysis of VOCs associated with the various soil remediation intervention values used in different national environmental regulations is required for effective comparison with health-based screening concentrations (also referred to as “screening levels”) at contaminated sites in which measurements below the health-based screening level generally point to a low potential for health concerns for exposures up to a year (US EPA, 2016).

7.3 Contributions to knowledge and research limitations

Sequel to the literature and experimental findings, the following contributions to knowledge in the field of environmental food safety are presented:

1. Provision of a mechanistic link between crude oil contamination and the identified crude oil-induced abiotic stresses as it affects agrifood production;

2. The use of RENA as a suitable bioremediation candidate for treating petroleum-derived spray oils (PDSOs) contaminated sites with alkanes between C_{21} - C_{25};
3. The effectiveness of RENA for the degradation of low-molecular-weight PAHs (such as phenanthrene and anthracene) and to subsequently support agrifood production;
4. The ability of CRIV ≤ 3,000 mg/kg TPH to support the cultivation of commonly consumed vegetables;
5. The potential of crude oil stress at 10,000 mg/kg TPH to enhance the concentrations of some essential nutrients and health-promoting phytochemicals in certain crops; and
6. The possibility of human contact with *p*-xylene during agricultural activities in crude oil contaminated farmlands.

Principally, the research contributions are very much applicable to environmental situations in regions of the world with CRIV in the range of 750 – 10,000 mg/kg TPH such as the Nigerian Niger Delta.

Research limitations

- The insufficiency of the experimental crude oil affected the experimental design, particularly in the VOCs and Chapter 6 experiments;
- Unavailability of adequate space influenced the experimental set-up of the M82 tomato cultivar's experiment;
- The major focus of this study was on the chemistry involved in the link between the two main parts of the research (bioremediation and food science);
- The limited studies on the impact of crude oil stress on crop quality constrained the extent of the results discussion;
- Owing to the low sensitivity of the MS used, the actual level of the bio-accumulated PAHs in the harvested agricultural products could not be ascertained.

7.4 Recommendations for future work

From all the analyses carried out in this research, the following are recommended for further investigations:

- ✓ As only vegetables were considered in this study, there is a need to examine the effect of remediation intervention values on other food plant families;
- ✓ A field study is required to look at the impact of the different CRIV on crops' mineral distribution which depend on climatic conditions and soil properties, among other factors (Szefer and Nriagu, 2007; Martínez-Ballesta et al., 2010; Ismail et al., 2011);
- ✓ There is a need to assess the effect of bioremediation and crude oil stress on the cancer preventive properties of glucosinolates found in *Brassica* crops (van Doorn et al., 1998; Cartea et al., 2008);
- ✓ At CRIV of 10,000 mg/kg TPH, the crude protein contents of two of the selected green leafy vegetables were enhanced. Future studies are required to investigate the effect of crude oil stress on crops' amino acids distribution, particularly the essential amino acids and those utilised as compatible solutes (e.g. proline) under stress conditions;
- ✓ As only the total phenolics and total flavonoids contents were considered in this study, future work is needed to determine the impact of crude oil contamination and RENA application on the individual members of this group, especially the distribution of flavonols and flavones in *L. sativa*;
- ✓ The method used for the sugar analysis in this study was very effective for *L. sativa* as it indicated the presence of more polar compounds (i.e. heavier sugars or sugar alcohols) (see Section 5.4.3.2.1, Figure 5.14) present in the plant. It may be necessary to further improve/optimize this method to make it a universal method solely for sugar analysis in lettuce. The other eluted compounds also need to be identified;
- ✓ Further work is required to assess: the levels of the bio-accumulated PAHs using more sensitive equipment, the effect of other classes of crude oil on agrifood production and the levels of bioavailable PAHs at the different CRIV;

- ✓ A field study is required to ascertain and quantify the VOCs emitted from fresh crude oil-contaminated farmlands in order to investigate their adverse effect on human health;
- ✓ There is a need for more study in the area of plant science and system biology involving the series of -omics (such as: phenomics, transcriptomics, proteomics and metabolomics) (Cramer et al., 1994, cited in Kumar et al., 2014, p. 140), to provide additional information regarding the effect of crude oil stress on plants' constituents.

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APPENDICES

Table A: PAHs LOD and LOQ

Polyaromatic hydrocarbons	Limit of Detection (LOD) mg/kg	Limit of Quantification (LOQ) mg/kg	Limit of Quantification (LOQ) mg/kg used by Commercial Laboratories for PAHs in Nigerian Soils
Acenaphthylene	0.018	0.060	0.020
Fluorene	0.009	0.030	0.020
Phenanthrene	0.019	0.063	0.020
Anthracene	0.024	0.081	0.020
Pyrene	0.019	0.063	0.020
Benz[a]anthracene	0.019	0.064	0.020
Chrysene	0.011	0.038	0.020
Benzo[b]fluoranthene	0.014	0.048	0.020
Benzo[k]fluoranthene	0.014	0.046	0.020
Benzo[a]Pyrene	0.033	0.111	0.020
Indeno[1,2,3-cd]Pyrene	0.014	0.045	0.020
Dibenz[a,h]anthracene	0.004	0.013	0.020
Benzo[g,h,i]perylene	0.011	0.037	0.020

Table B: Alkanes LOD and LOQ

Alkanes	Limit of Detection (LOD) mg/kg	Limit of Quantitation (LOQ) mg/kg
C10	0.197	0.658
C11	0.150	0.500
C12	0.111	0.369
C13	0.067	0.224
C14	0.118	0.394
C15	0.096	0.322
C16	0.077	0.258
C17	0.075	0.251
Pristane	0.133	0.442
C18	0.087	0.289
Phytane	0.074	0.245
C19	0.049	0.162
C20	0.052	0.173
C21	0.057	0.190
C22	0.061	0.204
C23	0.040	0.134
C24	0.042	0.141
C25	0.035	0.117
C26	0.054	0.179
C27	0.059	0.196
C28	0.053	0.176
C29	0.087	0.292
C30	0.077	0.256
C31	0.061	0.202
C32	0.075	0.249
C33	0.072	0.239
C34	0.108	0.361
C35	0.172	0.572
C36	0.106	0.353

Table C: Statistical result involving Independent Samples T-Test of decane concentrations at 5,000 mg/kg TPH (For homogeneity of crude oil and soil mixing assessment)

Table C-1: Case Processing Summary

	Treatments involving the homogeneous mixing of crude oil and soil at 5,000mg/kg	Cases					
		Valid		Missing		Total	
		N	Percent	N	Percent	N	Percent
Concentration of Decane in Treatments' replicate	Treatment B	5	100.0%	0	0.0%	5	100.0%
	Treatement C (For RENA)	5	100.0%	0	0.0%	5	100.0%

Table C-2: Tests of Normality

	Treatments involving the homogeneous mixing of crude oil and soil at 5,000mg/kg	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
		Statistic	df	Sig.	Statistic	df	Sig.
Concentration of Decane in Treatments' replicate	Treatment B	.205	5	.200*	.945	5	.703
	Treatement C (For RENA)	.298	5	.168	.843	5	.174

*. This is a lower bound of the true significance.

a. Lilliefors Significance Correction

Table C-3: Group Statistics

	Treatments involving the homogeneous mixing of crude oil and soil at 5,000mg/kg	N	Mean	Std. Deviation	Std. Error Mean
Concentration of Decane in Treatments' replicate	Treatment B	5	3.3641	1.33451	.59681
	Treatement C (For RENA)	5	5.1303	2.71957	1.21623

Table C-4: Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
Concentration of Decane in Treatments' replicate	Equal variances assumed	5.451	.048	-1.304	8	.229	-1.76620	1.35477	-4.89029	1.35790
	Equal variances not assumed			-1.304	5.821	.242	-1.76620	1.35477	-5.10609	1.57370