

**ISOLATION AND DEVELOPMENT OF A
BACTERIAL CONSORTIUM FOR THE
POTENTIAL BIOREMEDIATION OF SOIL
CONTAMINATED WITH
USED LUBRICATING OIL**

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UNIVERSITI SAINS MALAYSIA

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CONTAMINATED WITH
USED LUBRICATING OIL**

by

NURFARAH AINA BINTI MOHAMED RAZALLI

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LIST OF ABBREVIATIONS

°C	Celsius
μL	microlitre
μm	micrometer
alkB	alkane hydroxylase
API	Analytical Profile Index
AR	Analytical agent
AS	Activated sludge
AT	Aerated tank
BATH	Bacterial Adhesion To Hydrocarbon
BFT	Baffled flask test
BTEX	benzene, toluene, ethylbenzene and xylenes
cc/mL	cubic centimeter per milliliter
CDW	cell dry weight
CFU	colony forming unit
cm	centimeter
DCPIP	2,6 Dichlorophenolindophenol
DNA	Deoxyribonucleic acid
DO	dissolve oxygen
E ₂₄	Emulsification test
g	gram
g/cm ³	gram per cubic centimeter
g/L	gram per liter
GC-MS	gas chromatography mass spectrometry

h	hour
H ₂	hydrogen
HDB	hydrocarbon degrading bacteria
HDBC	hydrocarbon degrading bacteria consortium
HOCs	hydrophobic organic compounds
ICU	intensive care unit
JSTP	Jelutong Sewage Treatment Plant
KOH	potassium hydroxide
L	litre
M	Molar
mg	milligram
MIC	microbiologically influenced corrosion
mL	millilitre
mPa.s	millipascal-second
MSM	mineral salt medium
N:P:K	nitrogen: phosphorus: calcium
NA	nutrient agar
NaCl	sodium chloride
NB	nutrient broth
NBCT	North Butterworth Container Terminal
NCBI	National Centre for Biotechnology Information
O ₂	oxygen
OD ₆₀₀	optical density at 600 nanometer
OST	oil spreading test
PAH	polycyclic aromatic hydrocarbon

PAO	Polyalphaolefin
PBS	Phosphate buffer solution
pH	power of Hydrogen
PW	Port Weld
rDNA	recombinant Deoxyribonucleic acid
rpm	rotation per minute
SP	Sungai Pinang
TPH	total petroleum hydrocarbon
TTC	Triphenyl Tetrazolium Chloride
ULO	used lubricating oil
(v/v)	volume per volume
v/w	volume per weight
vvm	volume of air per volume of unit medium per minute
w/w	weight per weight
WMC	Waste Management Centre

**PEMENCILAN DAN PEMBANGUNAN KONSORTIUM BAKTERIA UNTUK
POTENSI BIOREMEDIASI TANAH TERCEMAR OLEH MINYAK
PELINCIR TERPAKAI**

ABSTRAK

Mutakhir ini, terdapat peningkatan isu berkaitan dengan salah urus bahan buangan minyak yang menyebabkan pencemaran alam sekitar. Antara contoh permasalahan yang dihadapi sekarang termasuklah laporan tentang pembuangan sisa minyak hidrokarbon secara haram ke dalam sungai, laut dan kawasan kebun tanaman. Oleh itu, penyelidikan ini ingin membangunkan konsortium bakteria pengurai hidrokarbon yang dipencil dari kawasan tercemar untuk remediasi sebatian hidrokarbon dengan menggunakan minyak pelincir terpakai (ULO) sebagai model bahan cemar. Kajian bermula pemencilan konsortia mikrob dari empat lokasi berbeza yang dikenalpasti sebagai Kawasan tercemar di sekitar Pulau Pinang, Malaysia. Antaranya ialah Sungai Pinang (SP), Pelabuhan Weld (PW), Pengkalan Kontena Butterworth Utara (NBCT) dan Loji Rawatan Kumbahan Jelutong (JSTP). Enam konsortia diperkaya dalam medium garam minimum (MSM) yang ditambah dengan 3% (v/v) ULO. Setiap konsortium ditakrifkan untuk penilaian bagi penyaringan kemampuan dalam menguraikan ULO. Berdasarkan hasil analisis, kedua-dua konsortium dari JSTP (konsortium AT dan AS) telah dipilih berdasarkan keupayaan yang menggalakkan dalam penyaringan awal dan kemudian digabungkan menjadi satu konsortium yang dikodkan sebagai konsortium ATAS. Pembangunan konsortium ATAS sebagai pengurai hidrokarbon diteruskan dengan penilaian beberapa faktor pengkulturan termasuk sumber nitrogen berbeza yang diuji pada kepekatan yang berbeza dan kepekatan ULO berbeza menggunakan kaedah uji kaji faktorial penuh.

Penghasilan biojisim dipilih sebagai pemboleh ubah yang bertindak balas sebagai petunjuk bagi penguraian hidrokarbon. Penghasilan biojisim tertinggi (4.62 ± 2.02 g/L) direkodkan dalam medium yang ditambah dengan 5 g/L ammonium klorida sebagai sumber nitrogen dan 3% (v/v) ULO. Fermentasi berkelompok diteruskan dalam sebuah reaktor tangki teraduk 3.6 L dengan mengintegrasikan keadaan kultur yang dioptimumkan daripada kajian dalam kelalang goncang sebelumnya. Sepanjang 7 hari fermentasi, biojisim tertinggi direkodkan sebanyak 2.5 OD₆₀₀ pada jam yang ke-120 dengan minyak berbaki sebanyak 9.74 g/L pada akhir eksperimen. Matlamat untuk mengurai sebatian hidrokarbon telah dibuktikan melalui analisis GC-MS. Ekstrak sisa minyak menunjukkan penurunan ketara bagi bilangan puncak hidrokarbon dalam kromatogram apabila dibandingkan sebelum dan selepas proses fermentasi. Sel-sel mikrob yang dikumpul daripada proses penapaian dalam bioreaktor kemudiannya diterapkan pada tanah yang dicemari secara buatan untuk percubaan bioremediasi. Tanah yang ditapis dicampurkan dengan 10,000 mg ULO/kg tanah. Terdapat 4 set persediaan mikrokosmos; 1) tanah tanpa bahan pencemar minyak, 2) ditambah dengan kaldu MSM yang baru disediakan, 3) tambahan oleh kaldu terpakai dan 4) tanpa konsortium ATAS. Selepas 30 hari, analisis GC-MS telah dijalankan untuk mengesan penguraian hidrokarbon. Keputusan dianalisis secara kualitatif dan mendapati Set 2 dan 3 menunjukkan bilangan puncak hidrokarbon mengalami penurunan yang ketara apabila dibandingkan dengan puncak kromatogram asal. Interaksi antagonistik dan sinergistik terhadap ahli bakteria dalam konsortium ATAS telah dinilai dengan kaedah silang calit. Keputusan menunjukkan pertembungan pertumbuhan calitan bagi semua pencilan bakteria yang diuji terhadap satu sama lain menunjukkan interaksi sinergistik antara ahli konsortium bakteria ATAS.

**ISOLATION AND DEVELOPMENT OF A BACTERIAL CONSORTIUM
FOR THE POTENTIAL BIOREMEDIATION OF SOIL CONTAMINATED
WITH USED LUBRICATING OIL**

ABSTRACT

Recently, there has been an uprising issue regarding the mismanagement of waste oil material which has been contaminating the environment. Reports on illegal discharge of waste hydrocarbon oil into rivers, oceans and crop soils are among the examples for this current predicament. Hence, this research endeavoured to develop a hydrocarbon degrading bacterial consortium isolated from a heavily polluted environment to remediate hydrocarbon compounds using used lubricating oil (ULO) as a model of contaminant. The work was initiated with isolation of microbial consortia from four different locations that are known to be contaminated area around Penang, Malaysia. The locations were Sungai Pinang (SP), Port Weld (PW), North Butterworth Container Terminal (NBCT) and Jelutong Sewage Treatment Plant (JSTP). Six consortia were enriched in a minimal salt medium (MSM) supplemented with 3% (v/v) of ULO. Each consortium was subjected for evaluation on screening their capacity in degrading the ULO. Based on the result of the analyses, both consortia from JSTP (consortium AT and AS) were selected due to their excellent performance in the preliminary screening and hence were combined as one consortium coded as ATAS consortium. Development of ATAS consortium as hydrocarbon degrader was proceeded by assessing several cultivation factors including different types of nitrogen source tested at different ULO concentrations using full factorial design in shake flask. Biomass production was selected as the responding variable as

an indicator for hydrocarbon degradation. The highest biomass production (4.62 ± 2.02 g/L) was recorded in a medium supplemented with 5 g/L of nitrogen concentration using ammonium chloride as nitrogen source and 3% (v/v) of ULO. Batch fermentation was continued in a 3.6 L stirred tank bioreactor by integrating the optimized cultivation condition from previous shake flask study. Throughout the 7 days fermentation, the highest biomass was recorded at 2.5 OD₆₀₀ at 120th hour with 9.74 g/L of residual oil at the end of experiment. The aim to degrade hydrocarbon compounds was verified by GC-MS analysis. The extracted residual oil displayed a significant reduction of hydrocarbon peaks in the chromatogram when comparing the sample before and after fermentation process. Microbial cells harvested from fermentation in bioreactor were introduced to an artificially contaminated soil for bioremediation attempt. Prior sieved loam soil was spiked with 10,000 mg ULO/kg soil. There were 4 sets of microcosm setup; 1) soil without oil contaminants, 2) supplemented with freshly prepared MSM broth, 3) additional of spent broth and 4) without ATAS consortium (control). After 30 days, GC-MS analysis was done to detect the hydrocarbon degradation. The results was analysed qualitatively and demonstrated that Set 2 and 3 showed significant reduction in the amount of hydrocarbon peaks when compared to the peaks in control chromatogram. Antagonistic and synergistic interactions of the bacterial members in ATAS consortium was evaluated by a cross-streak method. The results displayed an emerging growth streak of all bacterial isolates tested towards each other indicating synergistic interaction among the bacterial members of ATAS consortium.

CHAPTER 1

INTRODUCTION

1.1 General introduction

Since the 19th century, active exploitation of crude oil has become the ultimate focal point for developers in the industrial sector (Soetaert & Vandamme, 2006). However, vigorous consumption and exponential trend of petrochemical production to fulfil human needs have led to unexpected misfortunes, mainly as oil spillage and illegal oil discharge issues (Gdara *et al.*, 2018; Obida *et al.*, 2018; Rosell-Melé *et al.*, 2018). Researchers have been reporting on the toxicity and recalcitrant properties of polycyclic aromatic hydrocarbon (PAH). This compound has been identified as one of the most carcinogenic elements, capable of causing severe detrimental effects towards living organism (Eisler, 1987) and soil fertility (Grant & Briggs, 2002).

Nowadays, there are countless emerging issues circulating on a series of deleterious contamination of hydrocarbon which mostly affects soil crops, rivers and marine ecosystem (Hassan *et al.*, 2018; Pérez-Fernández *et al.*, 2018). Hence, in addressing this problematic tribulation, several strategies to curb and remediate the problem are introduced, which also raise common awareness in the community. This includes implementing bioremediation treatments towards hydrocarbon contamination sites by applying physicochemical and biological methods while at the same time taking action in managing the hydrocarbon waste products (Cheng *et al.*, 2017; Guo *et al.*, 2017).

The basic composition of crude oil is an association of different groups of hydrocarbons. Predominantly, the monomers that make up hydrocarbon comprises

carbon and hydrogen molecules, hence it is categorized as an organic compound (Haritash & Kaushik 2009; Abbasian *et al.*, 2015). These molecules assemble in various conformational structures of short and long carbon chains to form a stable and complex hydrocarbon mixture (Ghosal *et al.*, 2016). Overall, there are four fundamental groups of hydrocarbon, namely aliphatic, aromatic, resins and asphaltenes (Varjani, 2017b). Hydrocarbon is classified based on their chemical structures, the number of double bonds and the presence of aromatic rings. Undoubtedly, benzene and polycyclic aromatics are the most minacious contaminants due to their high toxicity (Haritash & Kaushik, 2009). They can remain deposited deep down in the soil and potentially seep through the pipelines and eventually contaminating the underground water table (Cozzarelli *et al.*, 1990; Solyanikova & Golovleva, 2011).

Petroleum pollution is notorious for its detrimental impact on the environment (Korte & Boedefeld, 1978; Duan *et al.*, 2017). The reason behind this is that petroleum constitutes several complex chains of hydrocarbon compounds, the majority of which are pernicious towards nearby inhabitants (Eisler, 1987; Beyer *et al.*, 2016). Most contaminated sites were initially caused by the discharge and leakage of oil in excessive amounts into the body of water such as rivers and oceans (Rogowska & Namiesnik, 2010). This eventually gives rise to the formation of thickened oil-in-water emulsion film on the upper aqueous surface (Vila *et al.*, 2015). The formation of an impenetrable layer will then block sunlight from penetrating the surface, causing inhibition of photosynthesis process. This prevents oxygen from being replenished, and eventually disrupts the oxygen cycle, endangering life within the aquatic ecosystem (El-Fadel & Khoury, 2001). In addition, hydrocarbon contamination can cause adverse health effects in human. This is supported by experimental studies,

providing scientific evidence linking these compounds to liver cancer and leukaemia in tested laboratory animals (López *et al.*, 2008; Pinedo *et al.*, 2014). There is also evidence that confirms hydrocarbon contamination affects the environmental ecosystem in terms of causing changes in microalgae and microbial activity (Zhang *et al.*, 2012), low soil fertility (Ojinnaka *et al.*, 2012) and provoke deathly illness towards wildlife (Eisler, 1987; Duan *et al.*, 2017).

Various treatments and amendments on managing the pollutant had been carried out. These include bioremediation since it is potentially potent, environmental-friendly and cost-effective (Varjani, 2017b). The premise of this is that microorganisms are equipped with the ability to degrade hydrocarbon via various metabolic pathways such as phototrophic anoxygenic, chemotrophic aerobic and chemotrophic anaerobic (Widdel & Rabus, 2001). Since pure culture isolates can often only degrade a portion or specific group of hydrocarbon compound, a combination of several microbial species in a mixed culture, also known as a bacterial consortium, offers high chances of acquiring different enzymatic capabilities. Consequently, this will result in a boost of performance in hydrocarbon biodegradation (Salam, 2016). The literature is replete with studies focussed on assessing the ability of a diverse species of bacteria, either as a pure isolate or in a mixed culture, to degrade different types of hydrocarbon compounds (Leahy & Colwell, 1990; Kanaly & Harayama, 2000). Experiments on microbial communities, such as developing bacterial consortia, capable of degrading mixed hydrocarbon, are actively being studied and has been rewarded with positive and encouraging outcomes, providing momentum to the idea of applying mixed cultures in bioremediation, rather than pure cultures (Dua *et al.*, 2002; Rizzo *et al.*, 2018). It is anticipated that, mixed culture with diverse catabolic pathways would provides enhanced degrading abilities and shows potential in

bioremediation applications in hydrocarbon-contaminated sites (Field *et al.*, 1995; Cerqueira *et al.*, 2011).

The main aim of this research is to develop a bacterial consortium isolated from a heavily hydrocarbon-contaminated environment. In addition, it is also aimed to promote a multifaceted hydrocarbon degrading ability by the consortium to degrade toxic waste hydrocarbon contaminants, simultaneously. Each consortium sample acquired were subjected to preliminary assessment to screen for the best hydrocarbon degrading ability. Unlike most studies, which isolated pure bacterial isolates from a mixed culture to be screened individually for their hydrocarbon degrading ability (Barathi & Vasudevan, 2001; Bhattacharya *et al.*, 2015; Bibi *et al.*, 2018; Dorr de Quadros *et al.*, 2018), this work focusses on developing a bacterial consortium culture obtained from the original collected sample. The reason of introducing the mixed culture into a hydrocarbon contaminated environment is to enhance and stimulate the biodegradation ability of the bacterial members to work in synergy in breaking down different types of toxic contaminants present in the complex hydrocarbon oil (Varjani, 2017b).

1.2 Problem statement

Used lubricating oil from automobile engine was adopted as the model of contaminant in this research to represent the hydrocarbon contaminant in the environment. This waste oil was chosen due to the lack of reports focusing in treating the real waste oil that remains prevalent in the environment (Luther, 2017). Preferentially, most researchers focused on degrading crude oil and single-target-contaminants such as pyrene, phenanthrene, benzene, BTEX compounds and many

more (Jindrova *et al.*, 2002; Field & Sierra-Alvarez, 2008; Sekar & DiChristina, 2017; Jiang *et al.*, 2018), presumably out of convenience and simplicity. However, in reality, there has been mounting evidence regarding illegal dumping and discharging of waste oil into the environment, none of which was closely related to the bulk of reports to date (Kang, 2014; Luther, 2017).

In addition, the application of a single-species culture has been proven to be limited in their specific action, attacking only certain fractions of the hydrocarbon group (Cerqueira *et al.*, 2011; Patel *et al.*, 2019). Indeed, the degradation by a single culture may often be incomplete and require a longer time to breakdown the contaminants (Yang *et al.*, 2019). For instance, Verma *et al.* (2006) reported that single-culture isolates, such as *Bacillus* sp. SV9, *Acinetobacter* sp. SV4 and *Pseudomonas* sp. SV17 showed a higher performance in degrading only the simpler forms of hydrocarbon, namely the n-alkane fragments compared to the aromatic fragments when they are grown as pure cultures.

This provides a strong research motivation to unfold discovery and strategy in addressing this dire situation. This work develops a hydrocarbon-degrading consortium comprising different species of bacterial members for the degradation of used automotive lubricating oil. Consequently, the relevance of this research is in its attempt to simulate the conditions in an environment of a microbial community of a mixed bacterial population, degrading hydrocarbon waste oil.

1.3 Aim and objective of the research

This research focusses to develop a hydrocarbon-degrading bacterial consortium from the environment that is capable of utilising and degrading used lubricating oil as a model of waste hydrocarbon contaminants. The specific objectives for this study are:

1. To isolate and screen bacterial consortia for hydrocarbon-degrading ability.
2. To scout for optimal hydrocarbon-degradation cultivating condition for the selected bacterial consortium.
3. To assess hydrocarbon degradation by the bacterial consortium using Gas Chromatography Mass Spectrometry (GC-MS) analysis.
4. To determine the synergistic and antagonistic interactions among the members of the selected hydrocarbon-degrading bacterial consortium.

CHAPTER 2

LITERATURE REVIEW

2.1 Petroleum

Petroleum is an organic material that exists in a form of sticky, yellowish-black in colour liquid which can be found in selected locations beneath the earth's crust (Morse & Turgeon, 2018). It is very essential as it has become one of the primary sources of energy and fuel in powering the transportation system, machinery and electricity generation (Wauquier, 1995; Ahn *et al.*, 1999; Krutof & Hawboldt, 2016). Almost every household item used in our daily life has some portions that can be traced back to petroleum feedstocks known as petrochemicals. The majority of these petrochemicals are very versatile, allowing their incorporation into a wide range of industrial products, namely plastics, chemical solvents, detergents, preservatives, medicines, fertilizers, pesticides and cosmetic products (Helmenstine, 2018).

Petroleum recovery begins with crude oil extraction from the oil reservoir. From there, the crude oil is transported to an oil refinery via ocean-going oil tankers or a network of pipelines for processing (Meyers, 2004). The crude oil undergoes a series of refinery processes, including a separation process known as fractional distillation. This separation process utilises different boiling points of the components in the crude oil, producing different petrochemical products such as gasoline, kerosene, diesel, lubricants and asphalts (Cozzarelli & Baerh, 2003; Morse & Turgeon, 2018). During the process, crude oil is heated and transformed into an extremely hot vapour and boiling liquid. These heated mixtures are then channelled to a distillation tower which is incorporated with a tier of collection trays. Each tray collects different categories of purified oil through a condensation process. The vapours of compounds

with low boiling points surges rapidly upward in the distillation tower, whereas high boiling point compounds deposit as liquids at the bottom (Speight, 2001; Meyers, 2004).

Recovery products from the distillation process are called distillates or fractions. Each distillate from a different level of trays is fed off from the tower to be collected and further refined to remove any impurities (Stauffer *et al.*, 2008). The overall process of petroleum distillation is illustrated by a schematic diagram in Figure 2.1. The movement of these volatile compounds can be visualised in the red coloured arrows, showing how crude oil is fractionated into a different category of petroleum distillates.

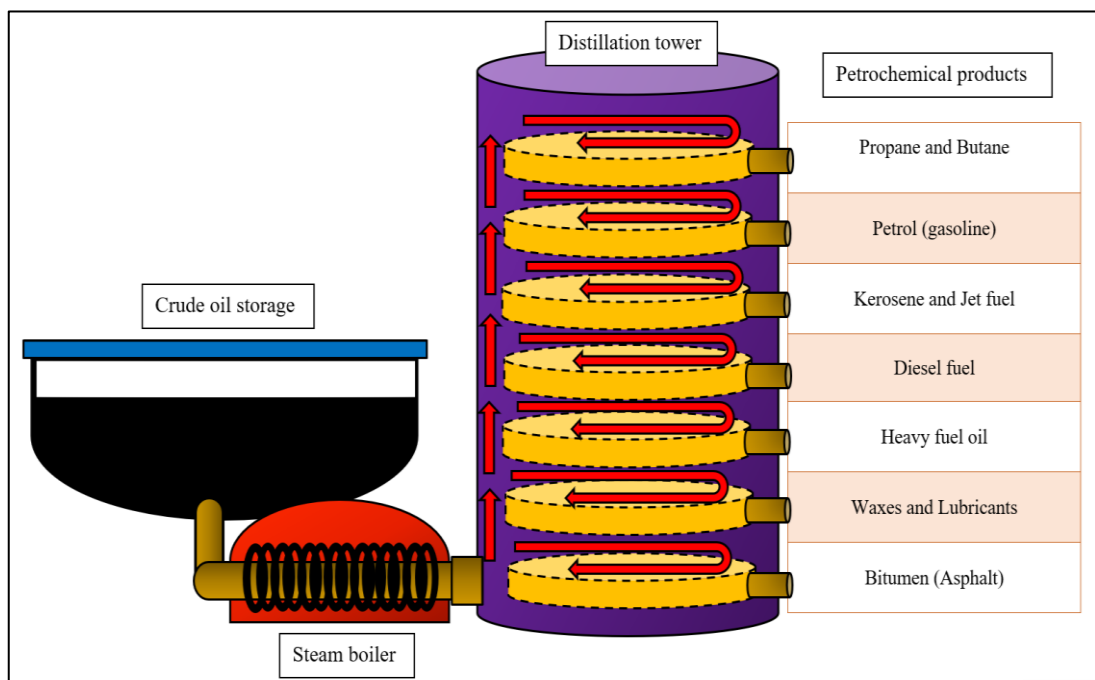


Figure 2.1: Schematic diagram of crude oil distillation process (Speight, 2001)

2.1.1 Composition of petroleum

Petroleum is an organic mixture which has a complex chemical composition. Predominantly, it comprises of hundreds of different hydrocarbon chains, which are made up by carbon and hydrogen atoms. Additional non-hydrocarbon elements such as oxygen, sulphur and nitrogen-bearing compounds, are also incorporated in petroleum with varying concentrations (Cozzarelli & Baehr, 2003). On the other hand, residual of trace elements and metals such as aluminium, iron and silicon are present at very low concentration (Doble & Kumar, 2005). The pie chart in Figure 2.2 below summarizes the average percentage estimated for each elemental components comprised in a petroleum mixture (Huth & Heilos, 2013).

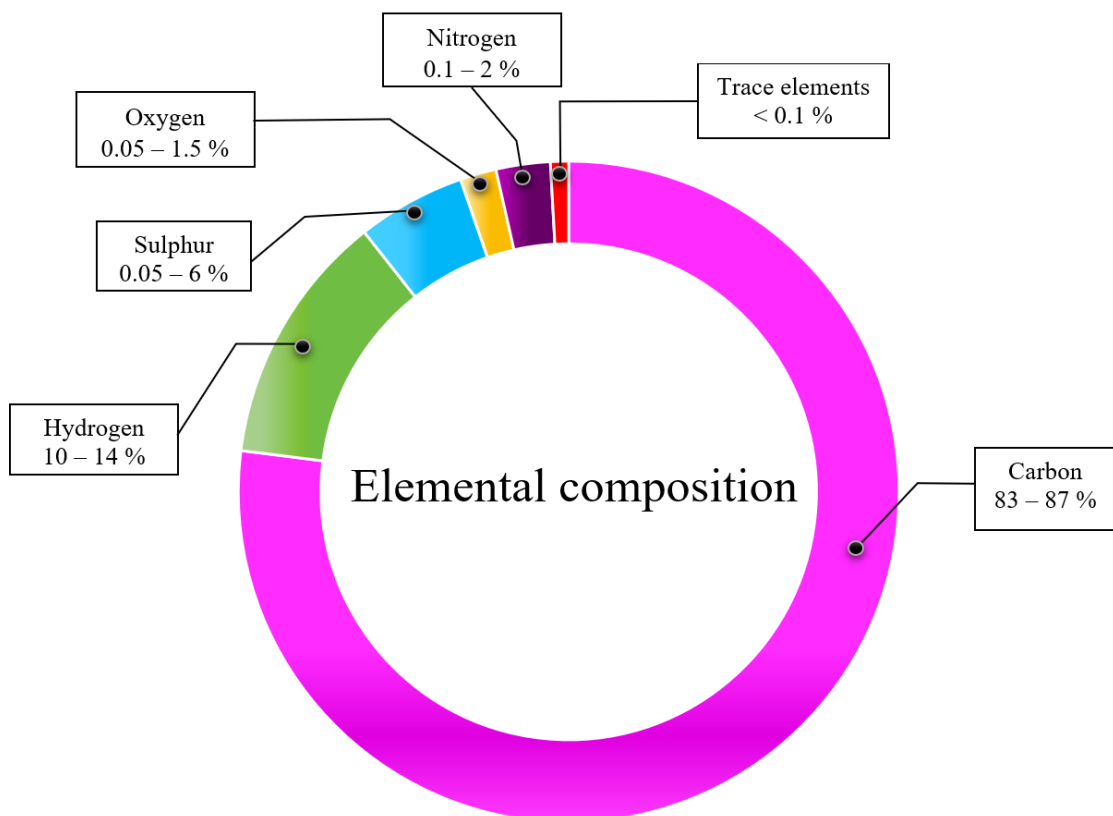


Figure 2.2: Primary elemental components of petroleum (Huth & Heilos, 2013)

The chemical composition of petroleum differs from each source of oil reservoir, largely due to the environmental and geophysical variations (Stauffer *et al.*, 2008). The elemental composition of petroleum differs from each source of oil reservoir. The abundance of hydrocarbon constituents further influences the chemical properties of petroleum oil. In addition, different molecular weight and the composition of different types of hydrocarbon groups would also determine the physicochemical property of the crude oil (Fuentes *et al.*, 2014; Abbasian *et al.*, 2015).

2.2 Hydrocarbon

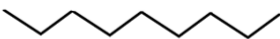
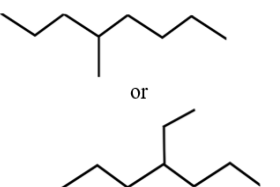
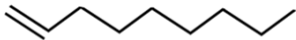
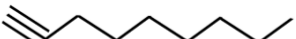

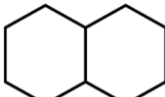
The core building unit in crude oil is an amalgamation of simple and complex hydrocarbon mixture. These carbon and hydrogen molecules bind together in various forms of complex branches and lengthy molecular conformations of short and long carbon chains (Fuentes *et al.*, 2014). Different molecular structures determine the amount each carbon chain holds, thus dictating the chemical and physical properties. A hydrocarbon mixture can be classified based on chemical structures of its constituents, the amount of double to triple bonds and the existence of aromatic ring structures. Based on this, they can be divided into four fundamental categories, namely aliphatic, aromatic, resins and asphaltenes (Varjani, 2017b). Each group possesses a distinct hydrocarbon structure to generate an intricate yet stable compound.

2.2.1 Aliphatic hydrocarbon

Aliphatic hydrocarbons typically exist abundantly in the hydrocarbon mixture. It is estimated that 66 % of the total hydrocarbon weight in a crude oil comprise a mixture of paraffin compounds (Cozzarelli & Baehr, 2003; Tissot & Welte, 2013).

Alkanes, alkenes, alkynes, iso-alkanes and cycloalkanes are among the paraffins that fall in this category (Table 2.1) (Abbasian *et al.*, 2015). Aliphatic hydrocarbon can be represented by saturated, unsaturated and cyclic carbon chain. These compounds are categorized according to the existence of single, double and triple bonds between the carbon atoms (Varjani, 2017b). Apart from having hydrogen molecules binding to the carbon chain, other elements such as sulphur, nitrogen and oxygen could also be incorporated within the aliphatic structure. Typically, paraffin products such as methane and butane are highly combustible with most of them serving as a source of fuel (Mango, 1997; Zhao *et al.*, 2010).

Table 2.1: Example of the organic compound in an aliphatic hydrocarbon group (Abbasian *et al.*, 2015)

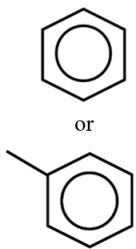
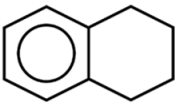
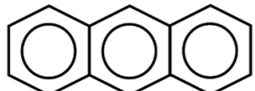
Chemical classification	Structure	Name and formula
Alkane		Nonane C ₉ H ₂₀
Iso-alkane		4-Methyloctane C ₉ H ₂₀ or 4-Ethylheptane C ₉ H ₂₀
Alkene		Nonene C ₉ H ₁₈
Alkyne		Nonyne C ₉ H ₁₆
Cycloalkane (Monocycloalkane)		Cyclooctane C ₈ H ₁₆
Cycloalkane (Dicycloalkane)		Decahydronaphthalene C ₁₀ H ₁₈

2.2.2 Aromatic hydrocarbon

Aromatic hydrocarbon compounds primarily display an additional feature of a stable-ringed structure known as benzene. These compounds can be further divided into two groups, namely the monocyclic aromatic and polycyclic aromatic hydrocarbons (PAHs) (Ahn *et al.*, 1999; Haritash & Kaushik, 2009). Generally, monocyclic aromatic hydrocarbon contains only one structure of benzene ring (Table 2.2). Among the monocyclic aromatic examples are benzene, toluene, ethylbenzene and xylenes (BTEX) (Bamforth & Singleton, 2005).

By the same token, compounds with multiple structures of benzene ring that are attached together are classified as PAHs (Coates *et al.*, 1997; Ghosal *et al.*, 2016). PAH compounds with four benzene rings or more are classified as high molecular weight compounds. In contrast, naphthalene (two-ringed), anthracene and phenanthrene (three-ringed) are considered as low molecular weight PAHs (Varjani, 2017b).

Table 2.2: General chemical structure of aromatic hydrocarbon (Bamforth & Singleton, 2005)

Chemical classification	Structure	Name and formula
Aromatic (Monocyclic aromatic)		Benzene C_6H_6 or Methylbenzene C_7H_8
Aromatic (Cycloaromatics)		Tetrahydronaphthalene $C_{10}H_{12}$
Aromatic (Polycyclic aromatic)		Anthracene $C_{14}H_{10}$

2.2.3 Asphaltenes and resins

Asphaltenes and resins represent the non-hydrocarbon group are classified as high molecular weight molecule because they hold a combination of aromatic and polycyclic compounds with long alkyl branch (Speight, 2004), thus, making them to be more recalcitrant in nature (Balba *et al.*, 1998; Alegbeleye *et al.*, 2017). Trace elements (nitrogen, sulphur, oxygen and trace metals) that are associated within these non-hydrocarbon compounds create polar functional groups to the compound (Varjani, 2017b). Asphaltenes and resins play a crucial role as peptizing agents to stimulate consistency and act as stabilizers in crude oil (Ancheyta & Speight, 2007).

2.3 Used lubricating oil (ULO)

Lubricant is a viscous-oily substance that assists as a grease film between two adjacent surfaces of a mechanical part that is in contact. Lubricating oil is applied in an engine of a machine such as in automobiles, steam-turbine generator, sewing machines and many more (Seifert & Westcott, 1972; Prabhakaran & Jagga, 1999; Zhu *et al.*, 2017). It serves to minimize the effect of friction, heat generation and wear due to long term and repeated use of moving surfaces by creating a protective layer on the surfaces (Okpokwasili & Okorie, 1988; Nagendramma & Kaul, 2012).

There are three types of automotive lubricating oils sold worldwide; mineral, synthetic and semi-synthetic oils (Rizvi, 2009). Mineral oil is commonly derived from one fraction of crude oil distillate (Prince, 1997; Rizvi, 2009). Fractionation of mineral oil requires boiling point ranging from 287.8 to 565.6°C and prior refined to remove waxes and other impurities (Lilly, 1984; Rudnick, 2005). In contrast, synthetic oils are synthesized from a synthetic base oil known as Polyalphaolefin (PAO) compounds

(Rosenbaum *et al.*, 2006). It is specially formulated to incorporate several similarities in terms of chemical structures of mineral oils. Specific groups of hydrocarbon compounds, for instance alkylated aromatics, polyalkylene glycols, diesters, polyol esters and phosphate esters, are being selected and incorporated into the synthetic oils (Dawson & Vanoosthuyze, 2004; Nagendramma & Kaul, 2012). Several chemical additives, such as detergents and dispersants are also augmented into the synthetic oils (Traise *et al.*, 1969; Brown *et al.*, 2010). These additives produce formulated oils with chemically stable properties. Synthetic oil formulations, by design, possess the least amount of impurities in comparison with mineral and semi-synthetic oils. This results in better rheological properties, thereby provide better maintenance of the overall engine performance in an automobile (Murphy *et al.*, 2002; Wu *et al.*, 2007).

The downside of this is the relative high cost of industrial synthetic oils, due to the overall cost in producing customized synthetic oils that meet the desired specification for specific engine systems (Rudnick & Shubkin, 1999; Brown *et al.*, 2010). Alternatively, semi-synthetic oils are mixtures of controlled portions of mineral and synthetic oils (Rudnick, 2005) to maximize the advantages offered by synthetic oils while still offering competitive pricing (Atadashi *et al.*, 2010).

It is advisable to replace the engine lubricants regularly after a certain mileage usage or duration. This is to avoid any possibility of the lubricating oil accumulating contaminating particulate matter or oil weathering. The former is a direct result of repeated moving parts and combustion, producing metal particles and combustion by-products while the latter is a physicochemical change that may affect rheological properties of the oil. This is rectified by replacing the used lubricating oils with new lubricating oils. Consequently, the old and used oils are discharged as a by-product waste to the environment, causing an abundant and continuous production of waste oil

(Vazquez-Duhalt, 1989; Luther, 2017). This petroleum by-product is termed as used lubricating oil (ULO).

Discharged ULO consists of several notorious types of pollutants (Hamad *et al.* (2005)). The contaminants that are constantly present are heavy metal, water, salts, dirt, and the occasional residual products from the oxidation process (Bhattacharya *et al.*, 2015). Therefore, it is only prudent to treat the toxic ULO. As is always the case with environmental protection, the cost of treatment is finite and is seen as a burden which sometimes results in unreliable and irresponsible handling the ULO wastes (Luther, 2017).

Illegal dumping and discharging of ULO into rivers, drain and crop soil has caused serious damage to the environment (Mohammed *et al.*, 2013). This irresponsible act has consequentially contributed to an increasing number of unfortunate oil-contaminated sites. Hence, implementation and approach on the remediation treatments to curb and solve this serious predicament is very crucial among the responsible parties and researchers.

2.4 Petroleum contamination

The majority of incidents associated with hydrocarbon oil pollution caused by vigorous anthropogenic activities. More precisely, the oil and gas industry is one of the contributors to most oil spillage incidents, particularly in offshore drilling activities. In many cases, the entire process of extracting refining and transporting petroleum and natural gas have caused spillage and leakage of the fuel (Ellis Jr & Adams Jr, 1961; Muangchinda *et al.*, 2018; Borah *et al.*, 2019; Lee *et al.*, 2019).

Unfortunately, the amount of frequent spillage is massive and manifold. As oil has a lower density than water, these typically cause oil to spread on the surface of the ocean, covering vast areas (Clark *et al.*, 2010). Eventually, the oil spill reaches the coastline, damaging the natural seascape along with its flora and fauna. The latest massive oil spillage recorded in the last decade was the Deepwater Horizon oil drilling platform spillage in April 2010. The Deepwater Horizon spillage was instigated by an explosion in the oil drilling rig, causing enormous amounts of crude oil to be discharged into the ocean (Eckle *et al.*, 2012). Table 2.3 summarizes a series of massive oil spillage events recorded for the past century.

**Table 2.3: Summary of oil spillage incidents in the past century
(Lim *et al.*, 2016)**

Name of oil spill	Date of the incident occurred	Venue of oil spilled occurrence	Type of contaminants	Quantity of oil spilled (million gallons)	Estimated clean-up cost in 2010 (US dollars)
Deepwater Horizon	20 th April 2010	Mexican Gulf, Mexican	Crude oil	210	\$ 10 billion
ABT Summer	28 th May 1991	Off coast of Angola, Africa	Iranian crude oil	80	\$ 163.2 million
M/T Haven Tanker oil spill	11 th April 1991	Mediterranean Sea; Genoa, Italy	Crude oil	45	\$ 85 million
Gulf War	23 rd January 1991	Persian Gulf, Kuwait	Crude oil	240 – 336	\$ 540 million
Odyssey oil spill	10 th November 1988	North Atlantic, off the coast of Nova Scotia	North Sea crude oil	43	\$ 86.7 million
Castillo de Bellver oil spill	6 th August 1983	Table Bay; Saldanha Bay, South Africa	Light crude oil	78.5	\$ 153 million
Nowruz oil field	10 th February 1983	Persian Gulf, Nowruz Field Platform	Oil	80	\$ 161.5 million
Atlantic Empress oil spill	19 th July 1979	Caribbean Sea, off the coast of Trinidad and Tobago	Light crude oil	9	\$ 187 million
Ixtoc 1 oil well	3 rd June 1979	Mexican Gulf, Bay of Campeche	Crude oil	140	\$ 283.9 million
Amoco Cadiz	16 th March 1978	Brittany coast, up to Channel Islands; Portsall, France	Light Iranian and Arabian crude oil & bunker fuel	68.7	\$ 136 million

Environmental oil contamination can also be caused by leakage of petroleum-product-transporting pipelines. Leakage from these pipelines can be due to the corrosion and deterioration of the pipeline structure. One of the reasons that could cause the corrosion is the microbiologically influenced corrosion (MIC) (Rajasekar *et al.*, 2010). This natural phenomenon is initiated by microorganisms that carry out electrochemical activity in the presence of carbon dioxide and chloride. This process stimulates a vigorous decomposition reaction of steel surface (Jana *et al.*, 1998). This uncontrollable biochemical reaction has caused massive maintenance cost to reconstruct and maintain the deterioration of metal-based structures. Oil pipelines and storage tanks are among the many affected structures that face this microfouling issues (Benka-Coker *et al.*, 1995).

The mismanagement of waste oil materials is also one of the main contributors to oil pollution. This irresponsible disposal has caused ill-fated consequences to the environment (Khelifi *et al.*, 2006). To make matters worse, these wastes are often not treated prior to disposal of out misplaced greed and utter disregard for the safety of others. Waste oils are simply discharged into the river and crop soils, ignoring the standard operating procedure in handling hazardous wastes. Toxic contaminants, namely hydrophobic organic compounds (HOCs), are distributed over a large area and thus affecting the residential area (Dawson & Mercer, 1986). Ultimately, this process would reach a saturation point where the widespread contaminants can no longer be constrained and mitigated, thus, making removal and treatment more involved and complicated. In Malaysia, the proper waste management service for treating, recovering and recycling these hazardous wastes is the 'Kualiti Alam' Waste Management Centre (WMC).

2.4.1 Local reports on the contamination of toxic wastes

In March 2019, Malaysia encountered a massive water pollution incident . The affected area was reported to be at Sungai Kim Kim, Pasir Gudang, Johor where an illegal discharge of chemical wastes, composed of marine oil was deliberately dumped into the river by a lorry tanker. An estimated 2.43 tonnes of hazardous chemical wastes were collected during the clean-up operation. In addition, the Johor Department of Environment also found a number of barrels containing toxic wastes by the riverbank of Sungai Masai and Sungai Sembilang, Johor. These barrels had been disposed illegally by the irresponsible parties, with some of the barrels leaking out toxic content, contaminating the affected land and river area (Yap *et al.*, 2019).

Several locations including Sungai Kim Kim, Sungai Masai and Sungai Sembilang, Johor were identified as areas with evidence of illegal waste disposal activity. Figure 2.3 illustrates the clean-up activity and sample collection of the contaminated water for content analysis. Further investigation unveiled that the chemical discharged was a mixed of waste marine oils that typically contain toluene, xylene, benzene and other hydrocarbon compounds, suggesting that the waste was from marine engine compressors (Irwan Shafrizan & Nurul Amanina, 2019). This waste oily mixture released very toxic and lethal fumes of methane and benzene gas, eventually affecting up to 111 buildings of educational institute and 92 premises of kindergartens and nurseries, which had to be shut down in Pasir Gudang, Tebrau and Tenggara (Ibrahim *et al.*, 2019).



Waste chemical pollution along Sungai Kim Kim



Figure 2.3: Clean-up activity, collection of samples for analysis and evidence of illegal disposal of several barrels of waste chemical along Sungai Kim Kim

(<https://www.bharian.com.my/kolumnis/2019/04/547870/isu-sungai-kim-kim-tuntut-tindakan-berkesan> (April 2019))

(<https://www.hmetro.com.my/mutakhir/2019/03/435063/sekolah-tutup-sampai-cuti-sekolah-metrotv> (March 2019))

Shortly after that, another pollution incident occurred in Selangor. This time, the chemical waste oil was contaminating the Sungai Klang. Figure 2.4 shows the unprecedented images recorded as the oil-mixture started spreading on the surface of the water. According to a report, the waste oil was originally discarded into a reservoir pond and drain located near Taman Eng Ann (Chan, 2019). Eventually, the waste oil

slowly finds its way towards Sungai Klang, spreading to areas downstream of the entry point. The director of Lembaga Urus Air Selangor (LUAS) issued a statement, ensuring the public that the waste pollution would not affect the water supplies in Selangor as it located downstream and would not cause harmful effect towards nearby community. Nevertheless, the aquatic creatures living in the vicinity of the river would possibly be affected by the discharged waste (Muhammad, 2019).



Figure 2.4: Discharge of waste oil chemical into the Sungai Klang and the drain nearby Taman Eng Ann

(<https://www.channelnewsasia.com/news/asia/pollution-detected-in-klang-river-believed-to-be-chemical-waste-11345464> (March 2019))

In mid-July 2019, yet another illegal discharge of waste chemical incident was reported in Sungai Selangor (Musalib, 2019). This time, the entire water supply, including four water treatment plants (Sungai Selangor Phase 1, Sungai Selangor Phase 2, Sungai Selangor Phase 3 and Rantau Panjang) in Selangor area were forced to halt operations for several days due to contamination of diesel oil. Earlier, prior to the shutting down of water supplies, there were complaints from nearby residents of an odour pollution in the water supplies. Following the complaint, authorities

involving Selangor Water Quality Unit, Lembaga Urus Air Selangor (LUAS) and Hulu Selangor District Council (MDHS) began the search for tracing the source of the toxic odour. The investigation revealed that two oil drums without the cover were placed on a non-operational pontoon (Hasnan, 2019). This caused an estimated 100 litre of chemical waste oil to leak and contaminate Sungai Selangor (Figure 2.5).



Figure 2.5: Contamination of diesel oil in Sungai Selangor triggered by the spillage of oil drums placed on the non-operational pontoon

(<http://www.astroawani.com/berita-malaysia/eksklusif-pencemaran-air-selangor-nampak-tumpahan-diesel-ada-individu-tawar-duit-kopi-213374> (July 2019))

The incident was allegedly caused by either an act of sabotage, carelessness or deliberate disposal of chemical waste by an irresponsible party. Eventually, a witness confessed that the waste oil dumping was by the operators of the Selangor's sand mining project nearby. Selangor Water Management Sdn. Bhd. initiated the clean-up process which required six tonnes of activated carbon released into the river to fully absorb the organic contaminants (Nur & Lakshamanan, 2019).

2.5 Toxicity effect of petroleum hydrocarbon contaminants

Spillage, leakage and illegal dumping of hydrocarbon contaminants are typically originated from oil and gas industries plus other industrial sectors which involved the usage of hydrocarbon oil such as factories and workshops . This issue poses grave effects towards the environment, including any living beings in the affected areas. In fact, prolong exposure from hydrocarbon contaminants may be fatal (Varjani, 2017b). In another aspect, the viscous nature of the waste hydrocarbon oils may eventually cause detrimental consequences to life. Apart from that, the hydrocarbon contaminants tend to persist in an environment for a long period, increasing the risk over an extended period of time. (Vazquez-Duhalt, 1989). Eventually, this would further increase the risks of contamination problem.

2.5.1 Impact on animals and marine life

Massive oil spillage in the oceans had endangered large numbers of birds and marine lives. Figure 2.6 shows the grim reality following an oil spill. Thick layers of oil completely overwhelm animals and birds. It blocks the airways of the animals and suffocates them. Oil residues get taken up by both marine flora and fauna, poisoning them. Eventually, hydrocarbon oil ingested by birds would potentially be carcinogenic, giving rise to cancerous cells (Leighton, 1993; Troisi *et al.*, 2016; Horak *et al.*, 2017). The immediate obvious effect on the majority of ‘oil-covered-birds’ is the failure of flight due to the thick oil layer adhering to their feathers. In addition, this eventually alters the physical insulating role of the feathers since it has become stickier and heavier (Troisi *et al.*, 2016). Disruption in the function of feathers greatly affects the ability of birds to regulate their body temperature (Perez-Umphrey *et al.*,

2018). This condition would later cause hypothermia and death to these birds (Jenssen, 1994).



Figure 2.6: Marine animal and birds fully coated with oil from oil spillage
(<https://blogs.ntu.edu.sg/hp331-2015-15/pollution/> (March 2019))

Oil contaminant residues in seawater contain largely low and high molecular weight hydrocarbon molecules (Varjani, 2017b). However, BTEX and PAHs are categorized as the most detrimental toxicants towards marine lives (Leighton, 1993). Phytoplankton, zooplankton and the young of marine organisms are reported to be the most vulnerable to the exposure of these harmful contaminants. This is due to the lipophilic properties of PAHs, making them accumulate within the body with the eventual toxic aftermath (Jiang *et al.*, 2010). Moreover, aquatic organisms such as fish, shellfish and turtles, may inadvertently ingest the spilt oil residues, causing blockage to their respiratory tract and digestive system (Duan *et al.*, 2017; Duan *et al.*, 2018).

The extent of damage does not stop there. Life of the coral reef ecosystem can also be affected by oil spills (DeLeo *et al.*, 2016). The coral reef ecosystem is known to be sensitive, particularly to toxic hydrocarbon compounds. These pollutants

generally compromise the optimal environment conditions for coral to live and reproduce (Negri *et al.*, 2016). This would consequentially upset the food pyramid in the reef ecosystem. Affected coral will perish, thereby reducing the diversity of coral species. If left unabated, coral species would soon become extinct in the near future (Beyer *et al.*, 2016).

2.5.2 Impact on soil ecosystem

Petroleum contaminated soil impairs the quality of the soil, microbiota and macro-community surrounding it. The presence of these contaminants tend to alter the role of each indigenous microbial species, and in some cases, changing the microbial population and diversity in the community (Nie *et al.*, 2009). This pernicious pollutant, classified as hydrocarbon organic carbons (HOCs), are mainly composed by PAHs and other groups of hydrocarbons. Most of them are capable to persist within the soil horizon for a long time. Plants that grow within the area would eventually die and those nearby are more to be susceptible to illness. The creeping roots that absorb the nutrients and water from the soil would co-adsorb HOCs into the plant tissues. This may trigger a consequential genetic mutation to the chromosome, thereby affecting the fertility of the plant (Hoshina & Marin-Morales, 2009). In addition, vapours originating from volatile organic compounds (VOCs) also have an impact on the quality of air, used in plants for respiration. Ultimately, these hydrocarbon compounds would eventually find their way into the metabolic and respiration systems of plants through leaves and roots.