The use of microcosms to assess the potential impact of underground bunker crude oil on freshwater aquatic organisms

by

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DECLARATION

I, **NGOAKWANA SONIA CHIPU** (Student No: 16023640), hereby declare this dissertation for the **MASTER OF ENVIRONMENTAL SCIENCE** (School of Geography & Environmental Science) Degree at the University of Venda, hereby submitted by me, has not been submitted previously for a degree at this or any other university, that it is my own work in design and in execution, and that all reference material contained therein has been duly acknowledged.

Signature  **Date:** 30.09.2022

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ABSTRACT

Historically, crude oil was stored in old underground coal mines collectively known as the Ogies Terminal. A study was conducted to assess the potential environmental impacts of underground bunker crude oil on freshwater ecosystems. The study assessed the impact of sediment contaminated with crude oil (from the bunker area) on freshwater resources, particularly wetlands and pans. Controlled laboratory conditions, indoor microcosms were being used for this purpose. Artificial sediments of the experimental microcosms were weighed (450 g per chamber) and washed three times with Milli-Q® deionized water before the sediment was spiked with four concentrations of crude oil. Sediments were spiked with 50 mg/kg, 25 mg/kg, 12.5 mg/kg, and 6.25 mg/kg crude oil per dry weight sediment and mixed well before overlaying it carefully with filtered, dechlorinated tap water. Each exposure, including the control microcosm, was conducted in triplicate. The key findings of the study revealed that the impacts on aquatic ecosystems in a crude oil spill will be devastating. From different trophic levels, organisms (*Daphnia magna*, *Physa spp*., *Lemna giba* and *Neocaridina spp*) had different responses, but overall, were severely affected by the crude oil contamination. Crude oil spill was lethal to *D. magna* and *Neocaridina spp*. in all experimental concentrations, 100% mortality was observed within 24 hours of the experiment. While *L. giba* and *Physa spp*. were slightly less sensitive to the oil concentrations of 6,25mg/kg and 12,5mg/kg. Therefore, when an oil spill should occur, it will have repercussions for the structure and functioning of an aquatic ecosystem and, subsequently, the goods and services that people rely on for their well-being.

Keywords: crude oil, mining, microcosms, ecotoxicity testing

GLOSSARY

Algae	Algae is an informal term for a large, diverse group of photosynthetic organisms which are not necessarily closely related.
Anthropogenic	Environmental pollution and pollutants originate in human activity.
Biomonitoring	Use of biological attributes of a water body to assess its environmental health or condition.
Crude Oil	A naturally occurring, unrefined petroleum product composed of hydrocarbon deposits and other organic materials.
Daphnia	A genus of small freshwater planktonic crustaceans (commonly known as waterflea) that is 0.2–5 millimetres (0.01–0.20 in) in length.
Duckweed	A tiny aquatic flowering plant that floats in large quantities on stagnant water, often forming a continuous green mat on the surface.
Ecotoxicology	A scientific discipline combining ecology and toxicology methods to study the effects of toxic substances, especially pollutants, on the environment.
Hazard	A substance, state, or event can potentially threaten the surrounding natural environment and/or adversely affect people's health.
Macroinvertebrate	Organisms without backbone, which are visible without the aid of a microscope.
Microcosm	Small-scale artificial simplified ecosystems that are used to simulate and predict the behaviour of natural ecosystems under controlled, laboratory conditions.
Multi-trophic	They involve species of different trophic levels of the same food chain.
Risk assessment	Process for analysing and evaluating the possibility of adverse ecological effects caused by environmental pollutants.
Teratogenic	Any agent that can disturb the development of an embryo or foetus.
Vascular plants	A plant that is characterised by the presence of conducting tissue.

Table of Contents

LIST OF TABLES

LIST OF FIGURES

ACCRONYMS AND ABBREVIATIONS

AMAP	Arctic Monitoring and Assessment Programme
ANOVA	One-Way Analysis of Variance
APHA	American Public Health Association
API	American Petroleum Institute
BTEXN	Benzene, Toluene, Ethylbenzene, Xylenes, and Naphthalene
CAF	Central Analytical Facilities
CSIR	Centre for Scientific & Industrial Research
DMR	Department of Mineral Resources
DWA	Department of Water Affairs
DWAF	Department of Water Affairs & Forestry
EC	Electrical Conductivity
ERA	Ecological Risk Assessments
FAO	Food & Agriculture Organisation
FEPA	Federal Environmental Protection Agency
KZN	Kwazulu-Natal
GC-MS	Gas Chromatography-Mass Spectrometry
HMWHs	High Molecular Weight Hydrocarbons
ITOPF	International Tankers Owners Pollution Federation
LMWHs	Low-Molecular-Weight Hydrocarbons
LSC	Louisiana Sweet Crude (Oil)
MP	Microplastic
NRE	Natural Resource and Environment
OECD	Organisation Of Economic Cooperation and Development
OMA	Oil Mineral Aggregation
PAH	Polyaromatic Hydrocarbons
PS I	Photosystem I
PS II	Photosystem II
ROSE	Recycling Oil Saves Environment
SAPIA	South African Petroleum Industry Association
SFF	Strategic Fuel Fund
TDS	Total Dissolved Solids

TPH	Total Petroleum Hydrocarbons
UCM	Unresolved Complex Mixture
US EPA	US Environmental Protection Agency
WAF	Water Accommodated Fraction
WCC	Witbank Consolidated Collieries
WFS	Wildlife & Fish Services
WHO	World Health Organisation
WMA	Water Management Areas
WSF	Water soluble fraction

CHAPTER ONE

1.1 INTRODUCTION

This study seeks to address the ecotoxicological hazard that crude oil may pose to freshwater ecosystems, especially once crude oil has settled in sediments. The study assesses the impact of sediment contaminated with crude oil (from the bunker area) on freshwater resources, particularly wetlands and pans. This chapter will set the tone for the study by providing the context within which the study was undertaken. By way of a brief background, the study's problem statement is espoused. The research aims and objectives are also identified and the specific research questions that this study's has undertaken to address

Historically, crude oil was stored in old underground coal mines collectively known as the Ogies Terminal. These old bunkers are located approximately 100 km east of Johannesburg in the Witbank coal mining belt, close to Ogies, Mpumalanga Province (Oberholster et al., 2016).

Crude oil and other liquids produced from fossil fuels are refined into petroleum products used for many different purposes. South Africa has no crude oil reserves, and about 60% of its crude oil requirements are met by imports from the Middle East and in other African countries such as Nigeria, Ghana, and Saudi Arabia (SAPIA, 2017). The crude oil is refined at South Africa's four crude oil refineries from where it is moved via pipelines, rail, sea, and road to approximately 200 depots, 4 600 service stations, and 100 000 direct consumers who are primarily farmers (SAPIA, 2017).

1.2 BACKGROUND OF THE STUDY AREA

Due to sanctions, South Africa experienced a shortage of crude oil during the 1960s, and old underground coal mines were subsequently used to store crude oil (Gaiya, 2016). For this reason, Klippoortjie became one of four mines in the Ogies area that was bought by the apartheid government from 1967 onwards to be converted into the world's most massive oil storage operation. Millions of barrels of crude oil were stored in the disused coal mine covering an area roughly the size of the Knysna lagoon. The oil was kept within the mine

walls by underground water, which means the only requirement for an oil-bearing mine is an adequate water Table (Mail & Guardian, 1996).

The Ogies Terminal comprises four old underground mines: Klippoortjie, Witbank Consolidated Collieries (WCC), Ogies Navigation Colliery, and Alpha Consolidated Collieries. Since the late 70s, crude oil has been stored and managed by the Strategic Fuel Fund (SFF) on behalf of the South African government. Although most of the crude oil has been removed from the bunkers and sent to a refinery in Durban, an unknown volume remains in spaces and crevices of the bunker, and should it seep into the surrounding groundwater or surface water resources, it may pose an ecological and human health risk. This is an important issue as the area's expansion of current mining activities is proposed in sections of the catchment below or above the bunkers (Oberholster et al., 2016).

The bunkers are situated in the Olifants Water Management Area (WMA) in a sub-catchment known as the Upper Olifants River catchment. The Olifants WMA is regarded as one of the most economically significant WMA's in South Africa. Economic activity in the WMA is highly diverse and is characterised by mining, metallurgic activities, commercial agriculture, dry land, subsistence agriculture, and eco-tourism (DWA, 2011). The rich mineral deposits present in the catchment are critical economic drivers in the area. Mining within the Upper Olifants sub-catchment consists almost entirely of coal mining supplying coal to the various power stations in the WMA. Due to the rich coal reserves, Eskom developed the Kendal and Wilge power stations during the 1970s and 1980s to provide for future electricity needs. Their newest coal-fired power station currently under construction and located in the upper Olifants is Kusile, which will be completed in 2018, and will further fuel the demand for coal supply in the catchment (DWA, 2011).

Although most of the crude oil has been removed from the bunker area, an unknown volume remains in the crevices of the bunker and may pose devastating consequences to both surface water and groundwater ecosystems, should a spill or seepage occur. Studies on oil spills in freshwater ecosystems, especially in South Africa, are less documented than the marine environment; however, that does not imply that freshwater oil spills are less significant.

In the current study area, approximately six mining permit applications have been lodged and accepted by the Department of Mineral Resources (DMR) for coal mining in the vicinity of the

Klippoortjie bunker (Gudani, 2015). This may increase the threat to freshwater ecosystems in the area. Given the current expansion of mining activities in the Klippoortjie region and the potentially devastating consequences to water resources in the area, it is necessary to increase our understanding of the potential impact that crude oil may have on freshwater ecosystems to minimise potential impacts.

Surface water resources in the upper catchment are mostly stressed from water quality and quantity points of view due to the numerous and extensive land-use practices in the catchment (Grobler et al., 1994; Oberholster et al., 2013). The expansion of old underground mine contaminated with crude oil will exacerbate the existing conditions of the water quality and water resources in the area. Therefore, it is crucial to assess the impacts that the crude oil will have on the receiving environment which is already stressed by other activities.

1.3 PROBLEM STATEMENT AND RATIONALE FOR THE STUDY

As the proposed mining operations will be taking place above and below the storage bunker, there is a risk that the crude oil that is still present in the bunker will, seep into the nearby aquatic ecosystems (surface water and groundwater). A crude oil spill or seepage can have devastating consequences for aquatic environment and ultimately the goods and services provided by these ecosystems, but it can pose a risk to water users in the catchment with the potential impact on human health a significant concern.

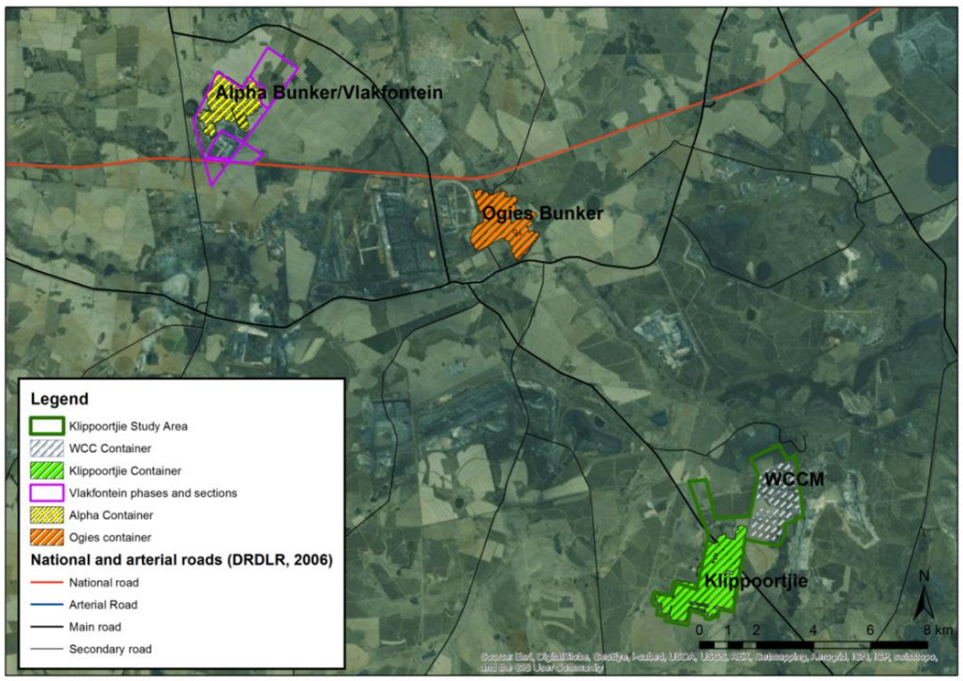

Figure 9-1: Ogies crude oil storage scheme: Location of the four-storage bunker in Mpumalanga Province, South Africa.

1.4 RESEARCH AIM AND OBJECTIVES

1.4.1 Overall aim

The overall aim of the current study is to assess the fate and ecotoxicological hazard/risk that sediment contaminated with crude oil (from the bunker area) may pose to freshwater resources, particularly wetlands and pans. Indoor microcosms, under controlled laboratory conditions, were used for this purpose.

1.4.2 Study objectives

The objectives of the study are to:

- Characterise the crude oil present in the bunker.
- Visually assess the behaviour and natural breakdown of the crude oil over the experimental period.
- To assess the effects that crude oil will have on the selected organisms and aquatic environments over a specific period of time.

- Determine management measures for the ecological hazard/risks to be implemented based on the study's outcome.

1.5 RESEARCH QUESTIONS

The following research questions will be addressed as part of this study:

- What are the characteristics of crude oil present in the bunker?
- How does crude oil break down and behave over the experimental period?
- What are the effects of crude oil on the selected organisms and aquatic environment over a selected period?
- What management measures should be implemented in the event of ecological hazard /risks in the freshwater contamination from an oil spill or seepage?

1.6 JUSTIFICATION OF THE STUDY

To date, very little research has been done on freshwater oil spills compared to marine oil spills globally. Because marine environments have very different characteristics and responses than freshwater ecosystems, it cannot be assumed that freshwater ecosystems will respond similarly.

In order to understand the ecotoxicity potential of freshwater oil seepage/spill situations, it is essential to consider and assess the effect that crude oil in different components of the ecosystem had on biota, i.e., the water column and sediments.

To improve our understanding of the effects of crude oil on freshwater resources, the potential risks posed to aquatic organisms, the behaviour of crude oil inputs into aquatic resources, biological responses, and what appropriate management actions needs to be taken in the event of a spill/seepage and after that, must be assessed and considered (Selala et al., 2013).

The current research gap is the impact of crude oil contaminated bottom sediment on freshwater aquatic organisms. Most of the oil spillage-related studies on freshwater systems have focused on the impact of the contaminated water column on organisms. This includes research in the proposed study area, where the impact on single species has been assessed (Oberholster et al., 2013; Oberholster et al., 2016). The use of aquatic microcosms is an

approach where experimental water enclosures provide a powerful research tool to simulate and mimic natural conditions to assess the impact of pollutants on organism's endemic to the study area. These microcosms are artificial, simplified ecosystems that are used to simulate and predict the behaviour of natural ecosystems under controlled conditions (Caquet et al., 1996; (Oberholster et al., 2012). They are a valuable tool that can be used to assess the freshwater ecological risk posed by crude oil as they allow assessing contaminant effects on community structure and functioning as opposed to the effect on individual organisms only.

1.8 DEFINITION OF FIVE KEY TERMS

i) **Biological monitoring:** Biomonitoring, or biological monitoring, is generally defined as "the systematic use of living organisms or their responses to determine the condition or changes of the environment. Aquatic organisms, such as diatoms and benthic macroinvertebrates, can serve as bioindicators to integrate their total environment and their responses to complex sets of environmental conditions. Biomonitoring has been proven to be a necessary supplement to those traditional monitoring techniques such as chemical monitoring.

ii) **Ecological risk assessment:** A powerful analytical tool that allows objective comparison of the relative risk contributed by a specific 'threat' to ecological structures being managed. This permits risks from multiple stressors to be evaluated and communicated logically, robust, and transparently. The process, therefore, facilitates optimum decision making for the management of natural resources through the full use of available information on potential environmental stressors.

iii) **Ecotoxicology**: A science that looks at the impacts of contaminants, including crude oil, on individuals, populations, natural communities, and ecosystems. The ultimate goal of this approach is to predict the effects of pollution so that the most efficient and effective action to prevent or remediate any detrimental effect can be identified. Ecotoxicological studies can inform the best course of action to restore ecosystem services and functions efficiently and effectively in those ecosystems already impacted by pollution.

iv) **Microcosm:** Artificial, simplified ecosystems that are used to simulate and predict the behaviour of natural ecosystems under controlled conditions. Open or closed

microcosms provide an experimental area for ecologists to study natural ecological processes. Microcosm studies can be beneficial for studying the effects of disturbance or determining the ecological role of critical species.

v) **Oil spill:** The accidental release of a liquid petroleum hydrocarbon into the environment due to human activity and can affect freshwater resources, marine areas and terrestrial bodies. An oil spill in a water resource is a form of pollution that can have severe consequences for the biota and plants in the affected aquatic ecosystems and pose serious human health risks. Clean-up and recovery from an oil spill are challenging and may take several weeks, months and even years, depending on the size of the spill and the type of environment.

CHAPTER TWO: LITERATURE REVIEW

2.1 INTRODUCTION

This chapter will advance pertinent issues that influence aquatic ecosystems and the impact of pollutants in the use of microcosms to assess the potential impact of underground bunker crude oil on freshwater aquatic organisms. The chapter presents a systematic interrogation of the key influencing factors for consideration in understanding the impact of underground bunker crude oil on freshwater aquatic organisms from a national and international perspective.

2.2 CRUDE OIL OVERVIEW

2.2.1 What is crude oil

Crude oil is a complex mixture of many compounds such as alkanes, aromatics, resins, and asphaltenes (Plaza et al., 2008). It is composed principally of hydrocarbons extracted from the earth in the liquid state and primarily associated with natural gases (Edema, 2012; Madu & Ugwu, 2017). Crude oils vary widely in molecular weight, physicochemical properties, environmental fate, behaviour, and toxicity (Grimwood, 2002).

2.2.2 Composition of crude oil

The primary classes of compounds found in crude oil are alkanes (hydrocarbon chains), cycloalkanes (hydrocarbons containing saturated carbon rings), and aromatics (hydrocarbons with unsaturated carbon rings) (Udofia, 2010; Dupius & Ucan-Marin, 2015). While straight-chained alkanes are more easily degraded in the environment than branched alkanes, cycloalkanes are incredibly resistant to biodegradation. However, aromatics (i.e., benzene, toluene, ethylbenzene, and xylene compounds) pose the most significant potential for environmental concern (Edema, 2012).

Udofia (2010) reported that a typical composition of crude oil consists of carbon (84-87%), hydrogen (13%), nitrogen (1%), oxygen (2%), water (1%), and heavy metals (0,1%). Although metals are of lower value in crude oil, (Kennon & Bouldin, 2015) noted that metals do have toxic effects which vary between organisms such as *Daphnia magna* (Baron et al., 2004) and *Hyllela Azteca* (water column and aquatic sediment organisms).

2.2.3 Characteristics of crude oil: physical and chemical properties

The physical and chemical characteristics of crude oil significantly influence the transport and ultimate fate of the oil once released to the environment. Light crude oils will evaporate more quickly, whereas heavy oils will persist for extended periods in the environment. Crude oils are characterised by physical and chemical properties, which play an essential role in understanding their geologic history and environment of origin (Madu & Ugwu, 2017). The physical properties that distinguish different oils include, according to the American Petroleum Institute (API), gravity, density, sulphur content, water content, flash point, pour point, viscosity, surface and interfacial tension, adhesion, the equation for predicting evaporation, emulsion formation, and simulated boiling point distribution (Lee et al*.,* 2015; Madu and Ugwu, 2017).

2.2.4 Crude oil spills and seepage in freshwater environments

Freshwater ecosystems such as wetlands, rivers, aquifers, and lakes are indispensable for life on the planet and vital for directly ensuring a range of benefits and services fundamental to the environment, society, and the economy. These include water for drinking, agriculture, industry, and energy production; critical habitats for fish, birds, mammals, reptiles, amphibians, insects, and other invertebrates; and natural solutions for purposes such as water purification and mitigating the impacts of development and floods and droughts (Daily, 1997).

According to (Baron et al., 2004), these ecological services are costly and often impossible to replace when aquatic ecosystems are degraded. However, today, aquatic ecosystems are being severely altered or destroyed at a greater rate than at any other time in human history and far faster than they are being restored. Sources of petroleum hydrocarbons are both natural and anthropogenic. Oil seeping from geological formations is the primary natural source. Anthropogenic oil contamination of water resources occur from: (i) chronic discharges from oil and gas development and production activities, (ii) accidental events such as oil spills from the rupture of pipelines, (iii) discharges from tankers and other ships along major routes, and atmospheric deposition (Das et al., 2002). Anthropogenic spills are more severe and devastating and vary in importance geographically across the world. Among the many concerns associated with a spill, water quality is one of the most critical issues affecting aquatic living organisms (AMAP Assessment Report, 1998).

Research directly assessing the impact and consequences of crude oil on freshwater ecosystems is not as extensive as compared to marine ecosystems. Yavari et al., 2015, conducted a study on the fate of crude oil spills in aquatic and land ecosystems and the environmental effects. Bhattacharyya et al., 2003, investigated the toxicity and temporal changes in this toxicity of two oils (South Louisiana Crude or SLC, and diesel) and two chemical additive treatments (the dispersant Corexit 9500, and the cleaner Corexit 9580) in laboratory microcosms containing fresh marsh soils. DeBofsky et al., 2020 was conducted to characterize microbiomes of common large-bodied fishes in the river and assess if microbiomes differed along a gradient of exposure to spilled oil. Abdelwahab, 2014, conducted a study to examine the efficiency of luffa fibers for the removal of various oils from water. Saba & Spotila (2003) Conducted a study to address concerns of rehabilitating and releasing oiled wildlife by observing post-release survival and behavior of oil exposed/rehabilitated (OER), possibly exposed (PE), and non-exposed (NE) freshwater turtles. Giari et al., 2012 conducted a study to investigate histologically and ultra-structurally the impact of the Lambro oil discharge on the main organs of *A. brama* inhabiting the lower Po River and to get information about the threat imposed by this incident. Therefore research on the assessment of environmental impacts associated with crude oil spillages in the freshwater ecosystem are lagging.

2.2.5 Rivers and Streams

Owens (2003) reported that, once oil reaches, or is spilt directly into, a river or stream, the oil enters a dynamic environment and is immediately and often rapidly transported downstream so that the size of the affected area increases and the length of time that the oil stays in motion also increases dramatically. Oil spilt into most rivers often collects along the banks, where the oil clings to aquatic plants and grasses. The animals that ingest these contaminated plants may also be affected. Rocks found in and around flowing water serve as homes for mosses, an essential fundamental element in a freshwater habitat's food chain. Spilt oil can cover these rocks, killing the mosses and disrupting the local ecology (USEPA, 1999). According to Poulton et al. (1997), oil spills in rivers are associated with decreased biodiversity and increased intolerant aquatic macroinvertebrates (e.g., Chironomidae and Oligochaeta). As a result, the structure and functioning of these ecosystems are severely impacted.

An example of the severe consequences of a freshwater oil spill was reported in August 2019, when a 1.6million litres of vegetable oil and caustic soda were spilt from Wilowton Oils in Pietermaritzburg into Msunduzi River after holding tanks at the factory collapsed (**Figure 2-1**). The chemicals that spilt into the water affected the pH of the river water significantly, making it very basic (10.78). It also affected the chemical and biological oxygen demand, which had a significant impact on the fish in the river. The sticky liquid clogged the gills and breathing systems of fish and other water creatures, choking the life out of the aquatic organisms (Carnie, 2019).

Figure 10-1 Dead fish due to oil spillage in the Msudunzi River, Kwazulu Natal, South Africa (Carnie, 2019).

2.2.5 Wetlands

According to the Ramsar Convention (2013); “wetlands are defined as areas of marsh, fen, peatland or water, whether natural or artificial, permanent or temporary, with water that is

static or flowing, fresh, brackish or salt, including areas of marine water the depth of which at low tide does not exceed six meters."

Wetlands are highly significant because they provide various benefits to human beings and the environment. Wetlands are rich in flora and fauna and are considered one of the world's most productive ecosystem types. Their aesthetic, economic, cultural, scientific, and recreational values are why wetlands are essential (Ibemesim & Bamidele, 2008). However, not much is known about the effect of oil spills on freshwater wetlands (Selala et al., 2013). When the oil is spilt into these ecosystems, it can be toxic to the frogs, reptiles, fish, waterfowl, and other animals that live here. Oiling plants and grasses rooted or floating in the water can also occur, harming the plants and the animals that depend on them for food and shelter.

2.2.6 Groundwater

Groundwater contamination by crude oil, and other petroleum-based liquids, is a widespread problem in the United States, each spilling an average of about 50, 000 barrels of crude oil (Delin & Herkeltrath, 1998). Ugwoha & Omenogor, (2017) mentioned that groundwater is one of the most valuable natural resources which support human health, social and economic development, and ecological diversity. For example, groundwater is a significant water source to many communities in Nigeria. Prolonged consumption of oil-polluted water harms consumers' health (Nwachukwu & Osuagwu, 2014). When the oil spill has occurred, it goes through the subsurface and the porous acquirers and reaches the groundwater resources. Duffy et al. (1980) reported that some oil spills on land could pose long-term threats to groundwater quality. For a given oil spill site, the extent of long-term groundwater contamination can be predicted easily by simple laboratory measurements and transport models. It is a renewable natural resource that is vulnerable to natural and human impacts.

2.3 BEHAVIOR AND EFFECTS OF CRUDE OIL IN A FRESHWATER ECOSYSTEM

The behaviour of released crude oil and the effects on environmental resources would depend on several factors (Dupuis & Ucán-Marín, 2015; Musk, 2012), namely: (i) physiochemical characteristics of the released oil (e.g., temperature, density), (ii) volume, duration, and rate of the release event; (iii) location and nature of the release event; (iv) physical conditions in the release area (e.g., currents, topography, soil porosity, vegetative cover); (v) weather (winds, light exposure, air temperature); (vi) type of habitat (marine/estuarine, freshwater, terrestrial); (vii) presence of environmental resources and

human populations; (viii) timing of species breeding cycles and seasonal migrations; (ix) locations of critical biological habitats; and (x) effectiveness of response efforts to stop or slow the release of oil.

As soon as crude oil is spilt into the environment, the oil and associated products undergo weathering processes. Weathering is a general term encompassing the changes in petroleum properties brought about by physical, chemical, and biological processes when oil is exposed to environmental conditions such as aquatic systems (**Figure 2-2**) (Musk, 2012).

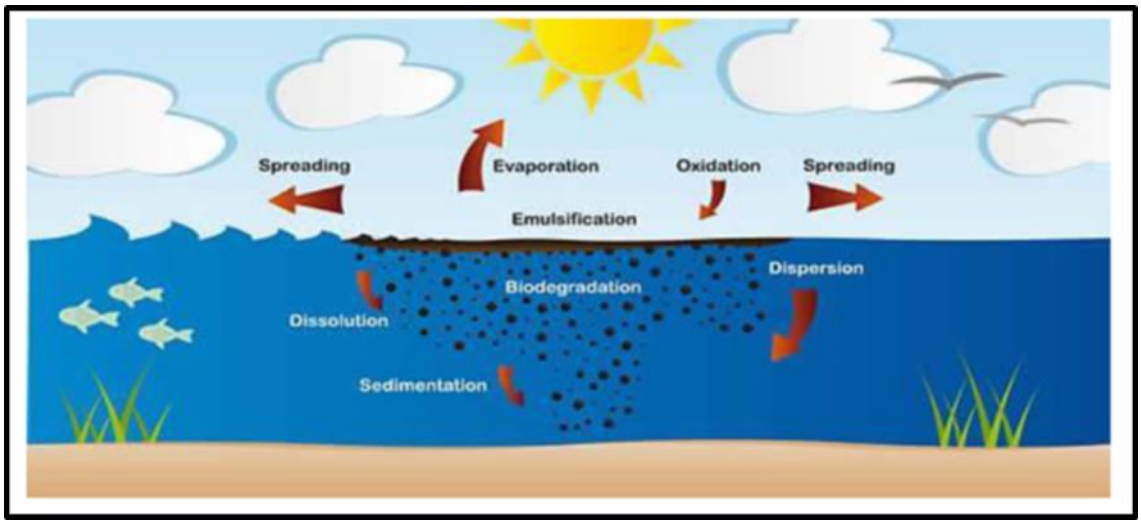

Figure 10-2: Weathering of oil spillage in aquatic environment (Musk, 2012)

Lee et al. (2015) highlighted that the rate of weathering occurs at different onset times resulting in progressive changes in oil composition and behaviour after the spill (**Figure 2-3**). Weathering processes, such as evaporation, begin immediately within hours or days; others, such as biodegradation, occur after a delay or more slowly (over months or years). Therefore, gross weathering rates are not constant and are generally highest immediately after the spill. Moreover, weathering processes are not constant in all areas of a spill site. These weathering processes are discussed in more detail below (**2.4.1 – 2.4.8**).

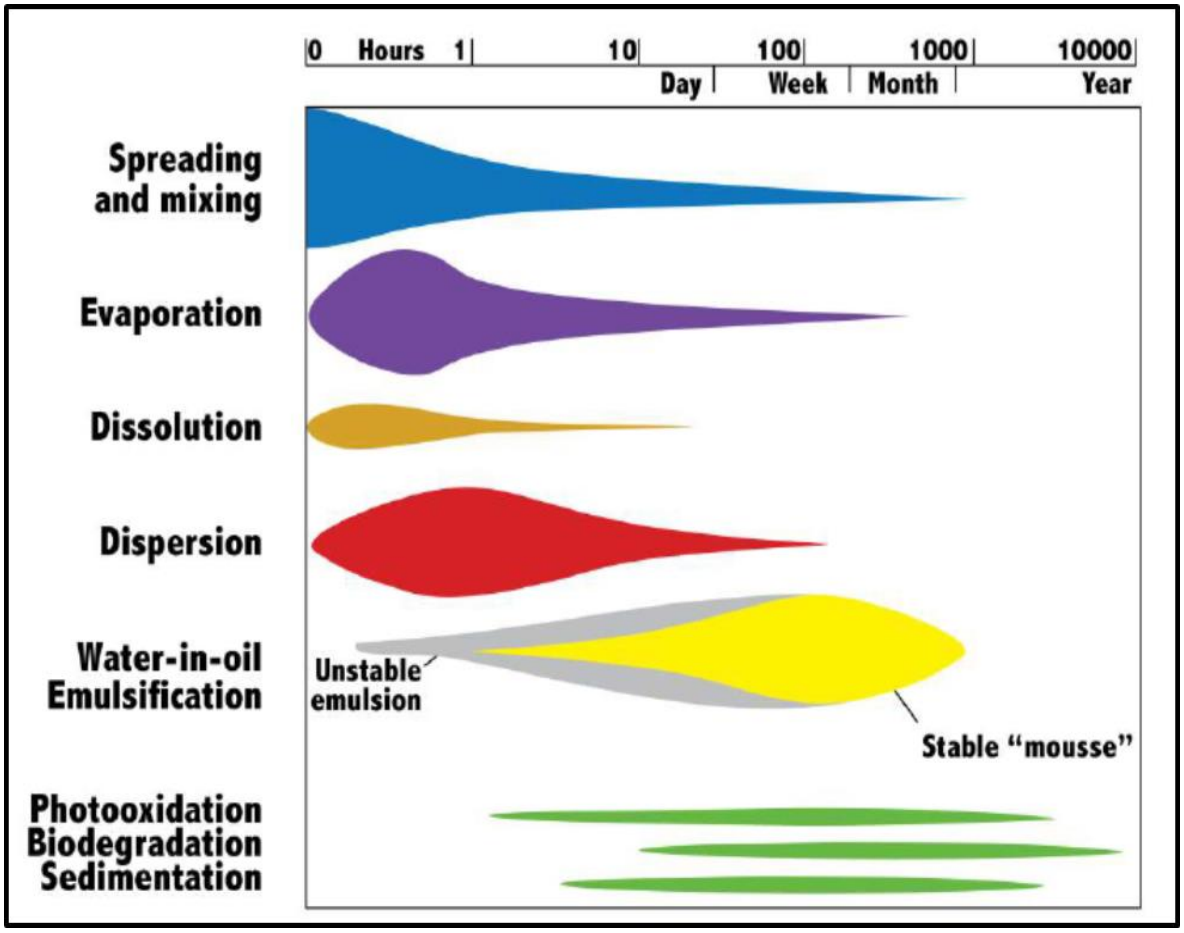

Figure 2-3: Weathering process overtime after an oil spill onto water (Lee et al., 2015)

2.3.1 Spreading

According to Esbaugh et al. (2016), when oil that is spilt onto the water is allowed to spread unhindered, it moves away from the source where the oil layer is thicker (a 'slick'), forming a thin 'sheen' at the edges, e.g., the familiar 'gasoline rainbow' of 5- µm. As it thins, the slick may form patches or 'ribbons'. Thus, spreading increases the spill area and decreases the average thickness of the oil layer. Spreading is influenced by the oil viscosity, water and air temperature, wind, wave and/or current action; in turn, spreading affects evaporative losses

2.3.2 Evaporation

Esbaugh et al. (2005) describe evaporation as the primary weathering force in removing oil from the sea surface. About 30 - 40% evaporates in the first 24 - 48 hours; these are the most poisonous (toxic) portions, as well as the portions that are the most soluble. The lighter oil fractions, such as benzene and toluene, are highly toxic but volatile and evaporate quickly. Heavier components of crude oil, such as polycyclic aromatic hydrocarbons (PAHs), appear

to cause the most damage; while they are less toxic than the lighter volatiles, they persist in the environment much longer (Ogeleka et al., 2017). The spreading process enhances evaporation as more oil is exposed to the air-oil interface. Warm air and water temperatures, high-level wind velocities, and solar heating increase the evaporative process (Xhelilaj & Sinanaj, 2010).

2.3.3 Dissolution

The rate and extent to which oil dissolves depend upon its composition, spreading, water temperature, turbulence, and degree of dispersion. Most of the dissolved hydrocarbons are more soluble and lightweight and classified as aromatic hydrocarbons that are usually considered toxic and become bioavailable to the organisms in the water column (ITOPF, 2002). However, the exposure area is usually localised and limited in duration due to the natural mixing and dilution in the water column (Scholz et al*.,* 2001).

2.3.4 Dispersion

Natural dispersion of oil in the water column occurs when the mechanical action of water or turbulence detaches oil droplets from the slick and forces them into the water column. Likewise, turbulent flow likely leads to oil dispersion in rivers having steep gradients, high velocity flows, and boulder/cobble substrates. Depending upon the droplet size, depth, and energy of the system, droplets may remain dispersed (i.e., suspended in the water column) or may resurface with or without coalescing with other droplets *(*Lee et al*.,* 2015*).* In a study by Lee et al. (2001), the findings were that the spreading of oil spilt into a freshwater ecosystem (Rio Desaguadero, Bolivia) could be attributed to significant oil dispersion and oil mineral aggregation (OMA).

2.3.5 Emulsification

Emulsification is the incorporation of water into the oil, forming a new product that is relatively resistant to other weathering processes (Scholz et al*.,* 1999). These emulsions significantly change the properties and characteristics of spilt oil. There are two emulsions: water-in-oil, or ‘chocolate mousse, and oil-in-water, a lighter mixture. The formation of the emulsions may occur during the first 8 hours after the spillage, but normally the rate and extent to which these processes take place depend upon the type of oil involved and the state of the water resource prevailing. The emulsion formation affects evaporation, degradation, and dissolution (Xhelilaj

& Sinanaj, 2010). For example, the viscosity of crude oil decreases when emulsified with water in the form of an oil-in-water type of emulsion. The stability of the oil-in-water emulsion increases as the surfactant concentration and speed of mixing of the emulsion increases (Ahmed et al*.*, 1998)

2.3.6 Photooxidation

Aromatic hydrocarbons (particularly PAHs and aromatic N-, S- and O-heterocycle) react with oxygen in the presence of sunlight, yielding oxygenated products that are more water-soluble and usually more resistant to biodegradability than the parent compounds. Although removing PAH from the oil is beneficial, as some PAHs contribute to its potential carcinogenicity and embryotoxicity, the increased mobility and persistence of the photo oxidised products in the water column may be detrimental to aquatic organisms (Scholz et al., 1999). In the study conducted by Robin et al*.* (1998), *Ceriodaphnia dubia* exposed to dissolve-phase diluent in environmentally realistic solar radiation exhibit 1.3 - 2.5 times greater sensitivity. Therefore, PAHs have the potential to cause toxicity effects to the aquatic organism under photooxidation conditions.

2.3.7 Sedimentation

Many contaminants produced by man's activities accumulate in sediment in aquatic ecosystems. The contaminants have low solubility and preferentially attach to sediment grains and particulate organic matter in the sediment. Because of this behaviour, the concentrations of many contaminants in sediment are usually far higher than concentrations in the water column (Oberholster et al*.,* 2013). Sediment characteristics are also an essential factor in determining the fate of crude oil in sediments.

Every aquatic environment is different in terms of climate, grain size distribution, and biological and chemical characteristics, and therefore this will affect the accumulation potential of oil in the sediments. Oil movement more in-depth into the sediment renders degradation difficult due to the limited availability of oxygen (Schiewer et al., 2015. Sedimentation occurs due to several processes that increase the specific gravity of the oil above that of the supportive water. Shallow waters are often laden with suspended solids providing favourable conditions for sedimentation (Xhelilaj & Sinanaj, 2010). For example, Katsimuti et al. (2013) found that freshwater wetland sediments (at a depth of 22 cm to 30 cm) contaminated by PAHs were toxic to organisms such as fish (*Hyphessobrycon reticulates*

and *Phalloceros caudimaculatus*) five years after the spill has occurred. In the study conducted by Oberholster et al. (2016), metals and hydrocarbons were found in the sediments, although the source could not be determined. Therefore, sediments can be a sink and a source of crude oil contaminants in aquatic ecosystems.

2.3.8 Biodegradation

The susceptibility of oil to biodegradation primarily depends on chemical composition (ratio of biodegradable alkanes and aromatics to resistant resins and asphaltenes), physical state (surface water available at the oil; water interface for microbial attachment or dissolution of light hydrocarbons and hence also dependence on spreading, dispersion and emulsification state of the oil); temperature (biochemical activity being slower at low temperatures); nutrients availability (particularly soluble nitrogen and sometimes phosphate) and available electron acceptors for redox reactions (aerobic or various anaerobic conditions). Oil degradation is generally faster and more efficient under aerobic conditions but may also occur slowly in buried sediments under anaerobic conditions (Lee et al., 2015).

2.4 EFFECTS AND IMPACTS OF OIL SPILL ON ORGANISMS

Organisms, including plankton, plants, invertebrates, fish, birds, and mammals that live in habitats that include the water column, sediments, beaches, wetlands, and forests, are susceptible to crude oil spills. The impact the oil has depends on many things, including the life stage of the organism (egg larvae, juvenile, adult), the time of year (wet or dry season), and other disturbances such as the presence of invasive species and the chronic effects of the spill (Lacerda et al., 2014).

Exposure of crude oil to aquatic organisms can be toxic, causing lethal or sub-lethal effects. Adverse effects resulting from exposure to crude oil can range from biochemical to organismal in scope. Oil is most toxic during the initial phases of a release before the lighter components have dissipated (WFS, 2010). The impact of the water accommodated fraction (WAF), as well as the water-soluble fraction (WSF) of crude oil on aquatic biota, have been studied previously (Oberholster et al., 2013). However, the impact of crude oil-contaminated sediments on the overlaying water has not been investigated in the study area in question.

In experimental tests conducted on different organisms, crude oil and its products showed various significant effects in studies conducted in Nigeria, United States, etc (Abdelwahab,

2020). For example, PAHs may also be associated with mutagenic, carcinogenic, and teratogenic effects. For example, terata were observed in fish exposed to naphthalene (239 µg/L for seven days), phenanthrene (85 µg/L for 27 days), and benzene (a)pyrene (0.2 µg for 36 days) (Dupuis & Ucan-Marin, 2015). An array of pollutants, including crude oil and its related products, are known to induce stress conditions, which impair the health of fish (FEPA, 1991). Daniel & Odioke (2017) revealed that toluene has adverse effects on growth performance and reproductive/hatchability success as their growths were retarded during the experiment. Yan et al. (2004) reported that besides direct chronic effects, oil pollution also postpones the pelagic succession of the ecosystem. Gautheir (2012) showed that biodiesel and crude oil had similar effects in altering the crayfish chemosensory ability to locate a food source during foraging trials compared to the control crayfish. Oil, with an aromatic content of 33%, reduces maise plants' growth to 31 percent (Edema, 2012). Romero-Lopez et al. (2012) tested strains of *Scenedesmus intermedius*, *Microcystis aeruginosa*, and *Dunaliella. tertiolecta* to increasing levels of crude oil and diesel. The exposure resulted in massive destruction of sensitive cells, though some cultures could grow due to the selection of toxin-resistant cells. On the contrary, diesel fuel did not affect the growth of *Scenedesmus* (Raybutt, 1972). Tudararo-aherobo et al. (2013) reported that sediment treated petroleum sludge is more toxic to freshwater shrimp (*Desmoscaris trispinosa*) than the brackish water shrimp (*Palaemonetes africanus*).

Ogeleka et al. (2016) found that the dispersants increased toxic hydrocarbon levels in fish by a factor of up to 100 and may kill fish and fish eggs. In a laboratory experiment conducted by the EPA (US EPA, 2010) using the Louisiana sweet (LSC), crude oil indicated that the mixture of LSC was classified as being toxic to moderately toxic depending on the test species and dispersants.

The known effects of oil spills on different organisms that live in or close to water ecosystems are discussed below.

2.4.1. Phytoplankton

These are unicellular primary producers, often called algae, which collectively form the base for the most spatially extensive food webs in nature. A floating mass of crude oil and its constituents, especially PAHs, can significantly impact phytoplankton. As concentrations increase, regardless of chemical profile, toxic impacts become apparent in the form of

increased cell diameter and reduced cell division, lower chlorophyll concentrations, and reduced photosynthetic activity resultant of electron chain transport interference in PSI and PSII. (Obaidy & Lami, 2014; Perhar & Arhonditsis, 2014). A study by Bordoloi & Burruah (2015) showed that there is a high infestation of two pollution tolerant centric diatom genera *Melosira* and *Cyclotella*, two species of *Cymbella* (*C*. *cymbiformis* and *C*. *gracilis*) and *Synedra* species (*S. tabulate* and *S. ulna*) in a highly oil-contaminated area. Therefore, phytoplankton can be used as a good bio-monitoring tool.

2.4.2. Zooplanktons

Zooplankton links primary producers to higher organisms and is a critical vector in marine and freshwater food webs. Initial exposure to oil constituents is followed by rapid dilution until an equilibrium state is established. The deleterious impacts of hydrocarbon spills, such as increased zooplankton mortality and reduced egg production/viability, can potentially alter food-web structure (Perhar & Arhonditsis, 2014). A study conducted by Lennuk et al. (2014) revealed that an increasing crude oil concentration above 100 mg/L sharply decreased the survival of *D. magna*, and survival varied among size classes. Being in contact with a concentration of 400 mg/L and above, all Cladoceran specimens died after 96 h. Hydrocarbons can inhibit zooplankton egg production by incorporating toxins into oocytes and altering the biosynthetic pathways involved in oogenesis (Perhar & Arhonditsis, 2014). According to Varela et al. (2006), some studies reported an increase in primary productivity, but it was not demonstrated whether this was caused by stimulation of photosynthesis or a decrease in zooplankton grazing caused by oil.

2.4.3. Invertebrates

Ogeleka et al. (2016) reported that aquatic invertebrate, which plays a vital role in water purification, habitat creation, and shoreline erosion control, could be affected by a spill in the aquatic environment. The negative impact of oil spills on aquatic invertebrates varies with the spill's location and magnitude and an invertebrate, life stage, habitat, sensitivity, feeding habits, and ability to avoid or process contaminants. The effect of oil on aquatic invertebrates, in general, include habitat degradation, smothering, fouling of gill structures, impaired reproduction, alterations of growth, development, feeding, immune response, and respiration, and disturbance of the food web. Also, benthic invertebrates can be adversely affected by oil trapped and buried in sediment, where it can persist essentially unchanged for years. For

example, Kennon & Bouldin (2015) reported toxicity tests using organisms such as *Ceriodaphnia dubia*, *Pimephales promelas*, and *Chironomus dilutus* in water and sediments, respectively, reduced reproduction and reduced growth. Sediments dwelling macroinvertebrates such as *Parantanytarss grimmi* adults have decreased in numbers due to sediments contaminated with synthetic motor oil at a concentration of more than 860 mg/kg (Pettigrove & Hoffmann, 2005).

2.4.4. Birds

When aquatic birds are covered in oil, they can lose their ability to fly, dive for food or float on the water, leading to drowning (Tseng, 1999). Inhalation and ingestion of toxic components can cause internal effects, which lead to pneumonia, gastrointestinal disturbances, hemolytic anaemia, immune suppression, and organopathy. **Figure 2-5** shows a bird covered with oil residue at the Con Joubert Sanctuary wetland (South Africa) after a vegetable oil spill in the year 2007 (Selala, 2013). A study conducted by Ramirez (2002) in Wyoming, USA, showed that the discharges of oil field produced water often create wetlands that provide habitat for wildlife. However, aquatic birds exposed to these oil field discharges observed impaired reproduction and embryo mortality.

Figure 2-4: Bird (Lemon Dove) affected by vegetable oil spillage at Con Joubert Bird Sanctuary, Gauteng, South Africa (Selala, 2013).

2.4.5. Fish

Fish can be affected by an oil spill through their gills, ingestion, or eating oiled prey. Several studies have been conducted on the effects of oil on shellfish, both bottom-dwelling (e.g., lobsters, crabs) and intertidal (e.g., clams, oysters) (FWS, 2010). Species living in bays, estuaries, and other shallow environments are at particular risk because the oil coming ashore may become concentrated (FWS, 2010). In addition to the toxic effects, heavy oils can smother and immobilise some invertebrate species. Sub-lethal effects are also seen, including growth, metabolism, reproduction, and behaviour changes. The toxic effects of hydrocarbons on fish include delayed growth, reduced survivorship, mid-development, and induced carcinogenic and mutagenic activity (Perhar & Arhonditsis, 2014). These responses could accentuate when exposure occurs at early life stages tightly linked with PAH

derivatives, metabolites, and chemically dispersed oil. Ubong et al. (2015) found that as little as 0.1 ppm of oil can seriously affect fish, amphibians, crustaceans, and plankton.

Udofia (2010) found that light crude oil blends caused 95% to 100% mortality of Nile tilapia (*Oreochromis niloticus)* fingerlings when exposed to different concentrations. The report was corroborated by Umar et al. (2018) which in their study, found that crude oil was very toxic to *O. niloticus* fingerlings.

2.4.6. Effects of oil spills on aquatic vegetation

According to FWS (2010), the impact of oil on wetland vegetation not only varies but is complex and can be both acute and chronic, ranging from short-term disruption of plant functioning to mortality. Once vegetation dies, the soil collapses. The soil then becomes flooded, and plants cannot re-grow. If plants cannot re-establish, soil erosion is accelerated, leading to even more flooding and further wetland loss (US EPA, 1999). If oil penetrates the sediments, roots are continuously exposed to oil, and chronic toxicity makes new shoots problematic (Michael and Fingus, 2016). Fouling of plant leaves can reduce photosynthesis and temperature regulation, while coating of roots can disrupt root architecture and water and nutrient uptake (Yavari et al., 2015). The anthracene exerts phytotoxic effects on *E. agilis* by disrupting growth, pigmentation, and photosynthesis. (Kottaparambil and Park, 2019).

A Water resource is a gift in nature and an essential for life for both humans, fauna, and flora in the ecosystem. Its contamination has adverse effects on life in general. Oil spillage can affect the aquatic animals in many ways including changing their reproduction and feeding rate and behaviour and causing tainting and loss of habitat. The environmental degradation caused by oil spillage has a socioeconomic impact in the communities and business (e.g., power stations require clean water to function). This pollution destroys the ecosystem services such as aesthetic values of water bodies and other qualities of water such as drinking, recreation, swimming, fishing, and domestic use. Mitigation measure to prevent, minimise and prevent the identified impacts will be required to improve the quality of the water resources.

2.5 APPROACHES TO ASSESSING IMPACTS OF CRUDE OIL ON FRESHWATER AQUATIC ECOSYSTEMS.

It has long been realized that the monitoring and assessment of biota can determine the extent of pollution or contamination in freshwater resources and provide vital information to determine what actions are required to protect both the resource and human health.

In their study on the evaluation of the endocrine disruptive potential of crude oil water accommodated fractions and crude oil contaminated surface water to freshwater organisms in Mozambique, Truter et.al. (2017) provide due justification on the tools adopted to solicit the data provided that evidence how crude oil pollution may lead to adverse health effects in freshwater fish and amphibians as a result of altered endocrine signaling.

This section looks at some of the more identifiable approaches to assessing the impact of crude oil on freshwater aquatic ecosystems relevant to this study. It sets the context of this study which seeks to examine how aquatic ecosystems respond when exposed to crude in the short and long term, what the effects of crude oil on the selected organisms and aquatic environment are over a selected period and what management measures should be implemented in the event of freshwater contamination from an oil spill or seepage.

2.5.1 Biological assessment of freshwater ecosystems

Physicochemical parameters, which provide snapshots of the condition of a water body, do not provide an integrative measure of the overall health of a stream and can, at times, inadequately identify impaired waters (Barbour et al. 1999). Because aquatic organisms are adapted to live within certain environmental conditions, changes in their environment may adversely affect a specific biological community's composition and abundance characteristics. For this reason, biological measures can provide an integrated and comprehensive assessment of the health of a water body over time (Karr, 2001).

2.5.2 Ecotoxicity testing

Ecotoxicology is an approach that aims to identify the effects that chemical pollutants, alone or in combination with other stressors, have on biota in the environment (Escher & Hermens, 2004). It is one of several approaches that can be used in freshwater assessments to

evaluate these ecosystems' state and monitor their rate of change over time (Li et al., 2010). A key advantage of toxicity testing is that it detects toxic compounds based on their biological activity without prior knowledge of the toxicant to identify its presence (Leusch & Chapman, 2011), therefore providing valuable information on the biological fraction of contaminants. Toxicity tests furthermore integrate the effects of all contaminants, including additive, synergistic and antagonistic effects, and those not considered or detected by chemical analyses (Schwarzenbach, 2006).

Application of toxicity tests with different selected organisms with different sensitivities to chemicals and pollutants can indicate general stress and provide information about the potential hazard to aquatic life (Lam & Gray, 2003). For this reason, toxicity tests include more than one species from different trophic levels to assess the toxicity potential or hazard posed by a water body or effluent (Persoone et al., 2003). However, one of the shortcomings of single organism toxicity tests is that it does not provide an integrated impression of how the functioning of the ecosystem is being affected. Microcosm tests, on the other hand, can address this gap to no small degree.

2.5.3 Microcosms

In situ aquatic communities form part of a complex system that consists of networks of multi-species interactions that are almost impossible to simulate. However, several tools and assessment approaches are available and continuously developed and refined to assist scientists and water resource managers in understanding how water resources respond when exposed to pollutants and contaminants. Microcosm tests are useful intermediates between bioassays and ecosystem experiments. The use of microcosms has been experimented within ecology and ecotoxicology since the 1970s. Microcosms were used as a requirement to register new chemicals, especially pesticides, before they could be used in the market to assess their potential ecological effects on the environment (Graney et al. 1989) and community level response in freshwaters (Caquet et al. 1996).

Microcosms provide controlled experimental conditions in the laboratory to study changes at any level (population or community, or ecosystem) caused by pollutants or other stressors. Microcosm studies are generally small, contain a few species, and are conducted indoors. Experimental tests to assess ecological effects can also be conducted in outdoor mesocosm studies (Strauss et al., 2010). Mesocosms are closed systems, may represent a limit to their

use over time. They are more significant compared to microcosms and, therefore, require more resources in terms of funding and human resources (Perceval et al., 2009).

Test organisms from different trophic levels and different feeding habits are usually introduced into a microcosm. These organisms can include algae, macroinvertebrates, plants, and vertebrates. Algae are the dominant primary producers in the aquatic food chain. Disruptions to this production base would likely cause effects at higher trophic levels. Similarly, vascular plants such as duckweed (*Lemna gibba*.) are essential for the ecosystem. Along with algae, plants produce oxygen and organic matter that support almost all other life forms.

In toxicity testing, one of the preferred and widely used macroinvertebrate species is the filter-feeding cladoceran, *Daphnia magna* constitutes a significant component of freshwater zooplankton throughout the world and is highly sensitive to certain metals. Freshwater snails, such as *Physa* spp., are commonly found in the interface between the bottom sediment and the water column of streams and lentic systems and form an essential component of the food web. It has been estimated that snails constitute up to 20% of some freshwater fish species (Pennak, 1978; Cheung & Lam, 1998).

Microcosms enable researchers to observe the integrated effects of contaminants on community and ecosystem functions and pathways. According to Szöcs et al. (2015), microcosms are a valuable tool for ecological risk assessments (ERA) since they assess contaminant effects on community structure and functioning. They offer more realistic ecological conditions than individual toxicity bioassays and allow ecotoxicologists to simultaneously perform studies on pollutants' fate and biological effects (Caquet et al., 1996).

2.6 RISK/HAZARD ASSESSMENT

Ecological risk assessment evaluates the likelihood that adverse ecological effects may occur or occur due to one or more stressors. In the study area, oil has historically been pumped into and out of various mines through a series of boreholes. Boreholes are also used to discharge hydrocarbon vapour from high points within the mine and intruded groundwater from low points. The water-oil mixture is passed through an oil separator before the water is discharged into a series of evaporation pans and dams (Oberholster et al., 2016).

PAHs in the aquatic environment is mainly considered to be of four types: derived from fuels (petrogenic), derived from an incomplete combustion process (pyrogenic), generated by organic metabolism (biogenic), and generated by the transformation process in sediment (diagenetic) (Honda & Suzuki, 2019). Several researchers found that crude oil contained PAHs with toxic effects, such as immunotoxicity, embryonic abnormalities, and cardiotoxicity, for wildlife, including fish and benthic organisms, following the Deepwater Horizon Oil Spill and marine vertebrates (Ramesh et al., 2004).

Petroleum-contaminated soil and water are traditionally assessed in Total Petroleum Hydrocarbons (TPH) and targeted individual compounds such as benzene, toluene, ethylbenzene, xylenes, and naphthalene (BTEXN). PAHs in the aquatic environment may endanger aquatic organisms, which can, in turn, lead to the deterioration of the aquatic ecosystem.

PAH inputs to the coastal marine environment are primarily from two sources: (a) the movement of water containing dissolved and particulate constituents derived from watersheds; and (b) atmospheric deposition both in precipitation and dry deposition from airsheds of the coastal ocean (Latimer & Zheng, 2003). PAHs are low lipophilic compounds (log K = 3-8) with very low water solubility, and therefore, their concentrations in water are deficient because they tend to rapidly adsorb on suspended material and sediment (Nasr et al., 2010).

Planktonic filter-feeding organisms have been predicted to be particularly vulnerable to microplastic (MP) pollution due to their feeding modes and the similarity in the size of their natural food and MP. Ingestion of MP has been proven for several species of copepods (Cole et al., 2019). Nasr et al. (2010) found that PAH in fish samples in the exact location was determined at 371.68 ng/g in El-Sarsawia canal to 2019.25 ng/g in Bahr Shebin canal. In fish, PAHs gains entrance into fish by ingestion, dermal absorption and respiration.

2.7 SUMMARY

This chapter has reviewed the literature on oil spillage ass a worldwide environmental concern as far as it results in significant ecological disasters that affect the structure and functioning of aquatic ecosystems. Most of the disasters reported are those occurring in the

marine environment because the transportation of oil tanks is conducted onshore. Very few studies covered oil spillage in freshwater ecosystems. This, however, does not mean that freshwater oil spillages are less likely to occur or have less significant consequences. Furthermore, studies that reviewed oil spillages covered specific elements of the ecosystems (i.e., effects or toxicity on a particular organism or impacts on sediments or vegetation) and did not assess the impacts on organisms from different trophic levels and the potential consequences for a freshwater ecosystem as a result.

The next chapter will draw from the salient features of this chapter by expounding on the approach that has been adopted in this study to establish using microcosm tests, the potential impact of underground bunker crude oil on freshwater aquatic organisms.

CHAPTER THREE: MATERIALS AND METHODS

This chapter provides details about the materials and methods used during this study. It describes the experimental microcosms, the single species toxicity test, the approach followed in executing the experimental work and toxicity testing, and interpretation. All laboratory experiments were conducted at the Ecotoxicity Laboratories of the CSIR's Natural Resources and the Environment, Pretoria.

3.1 STUDY AREA

The proposed research is focused on the Klippoortjie mining area in the Upper Olifants River catchment. The study area is situated in the B11F Quaternary catchment (**Figure 3-1**). The B11F Quaternary catchment is drained by the Tweefonteinspruit and its tributaries, namely the Zaaiwaterspruit and Klippoortjiespruit that flow into the Olifants River which in turn flows into Witbank Dam. Witbank Dam is used for both portable and industrial purposes and recreation. At least three human settlements are situated along the Klippoortjiespruit, namely the Klippoortjie, Saaiwater, and Kamatsheka Clusters. The study area is set in a farming region where maize is the dominant crop (Crafford, 2007).

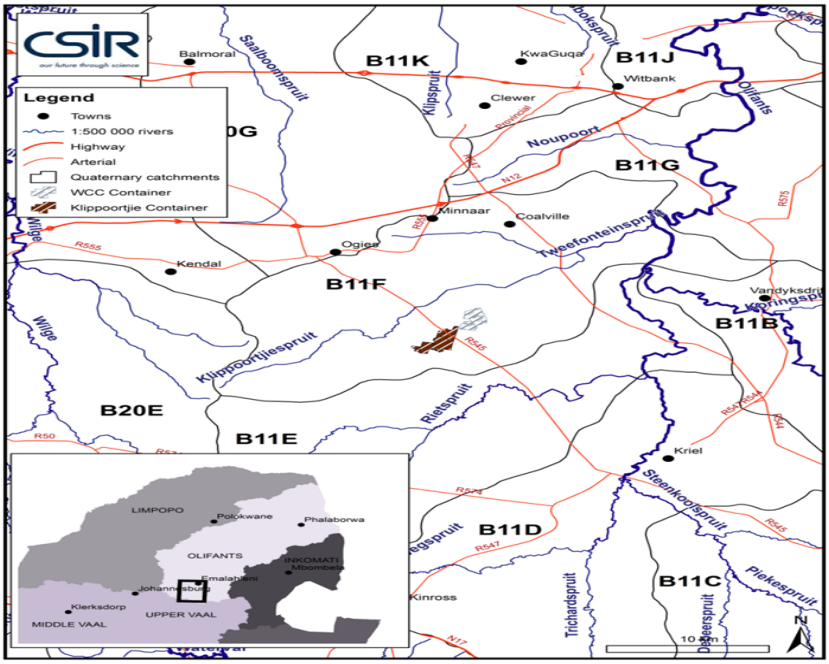

Figure 3-1: Location of the study area, Mpumalanga Province South Africa.

The rivers in the study area are primarily non-perennial, with several wetlands located on or in the vicinity of the mine's premises (**Figure 3-1**). The impact of a crude oil spill on the wetland areas is of concern as these are very important and valuable freshwater ecosystems in a catchment.

3.2 MICROCOSM DESIGN

For the study, indoor microcosms were employed (method adjusted from Clement et al., 2013). Each test microcosm (**Figure 3-1**) consisted of a 3 L glass chamber to which 450 g pre-washed, fine-grained sediment (used in swimming pool filters) was added. The sediments were spiked with different crude oil concentrations, mixed thoroughly before 2.5 L moderately hard dechlorinated, and filtered tap water was carefully added to each chamber. The negative control chambers contained sediment and dechlorinated water. The control and tests samples were performed in triplicate.

The microcosms were each covered with a lid to reduce evaporation. A small hole was drilled into the lid through which silicone tubing (6 mm in diameter) could fit. A small air stone was fitted to the end of the tubing, which was in turn connected to a 220-240V/50Hz electric Dolphin AO 1302 aquarium air pump. Each microcosm was lightly aerated for the duration of the experiment. Since the overall purpose of this experiment was to understand how the structure and function of a simple, mimicked ecosystem will respond to crude oil-contaminated sediment, it was decided not to use groundwater from the bunker or site-specific water which, due to activities in the catchment, is already contaminated with metals and other pollutants and would influence the results and outcome of the study.

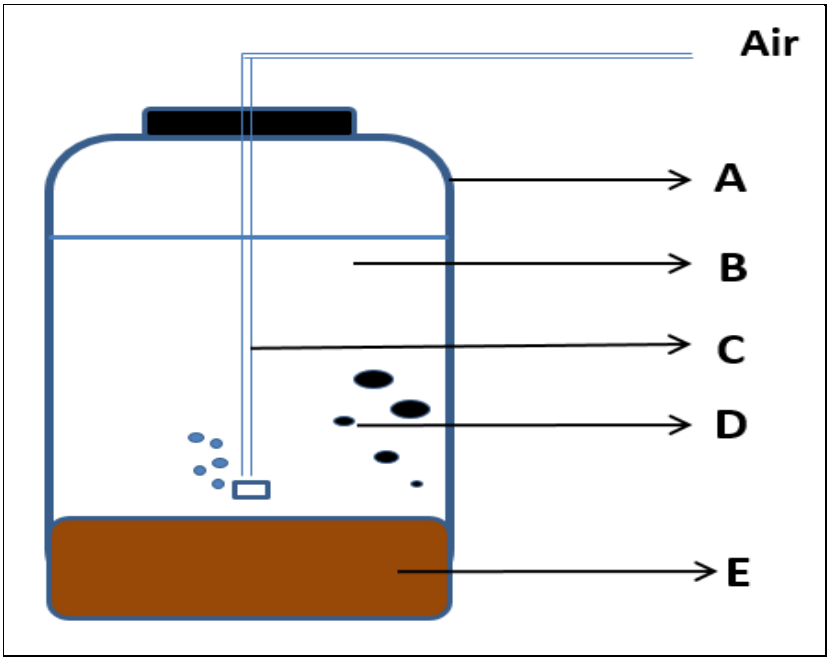

Figure 3-2: Laboratory microcosm: A) 3 L glass chamber; B) overlaying water (dechlorinated tap water) C) light aeration; D) oil released from sediment; E) spiked sediment.

3.2.1 Crude oil introduction into microcosms

For this study, artificial sediments of the experimental microcosms were weighed (450 g per chamber) and washed three times with Milli-Q® deionized water before the sediment was spiked with four concentrations of crude oil. Sediments were spiked with 50 mg/kg, 25 mg/kg, 12.5 mg/kg, and 6.25 mg/kg crude oil per dry weight sediment and mixed well before overlaying it carefully with filtered, dechlorinated tap water. The concentration was chosen using a dilution factor of 0.5 which provides good precision. Test concentrations of 50 mg/kg, 25 mg/kg, 12.5 mg/kg, and 6.25 mg/kg are therefore commonly used in toxicity testing. Each exposure, including the control microcosm, was conducted in triplicate (**Figure 3-2).** Caution was taken not to re-suspend sediments when microcosms were filled with water.

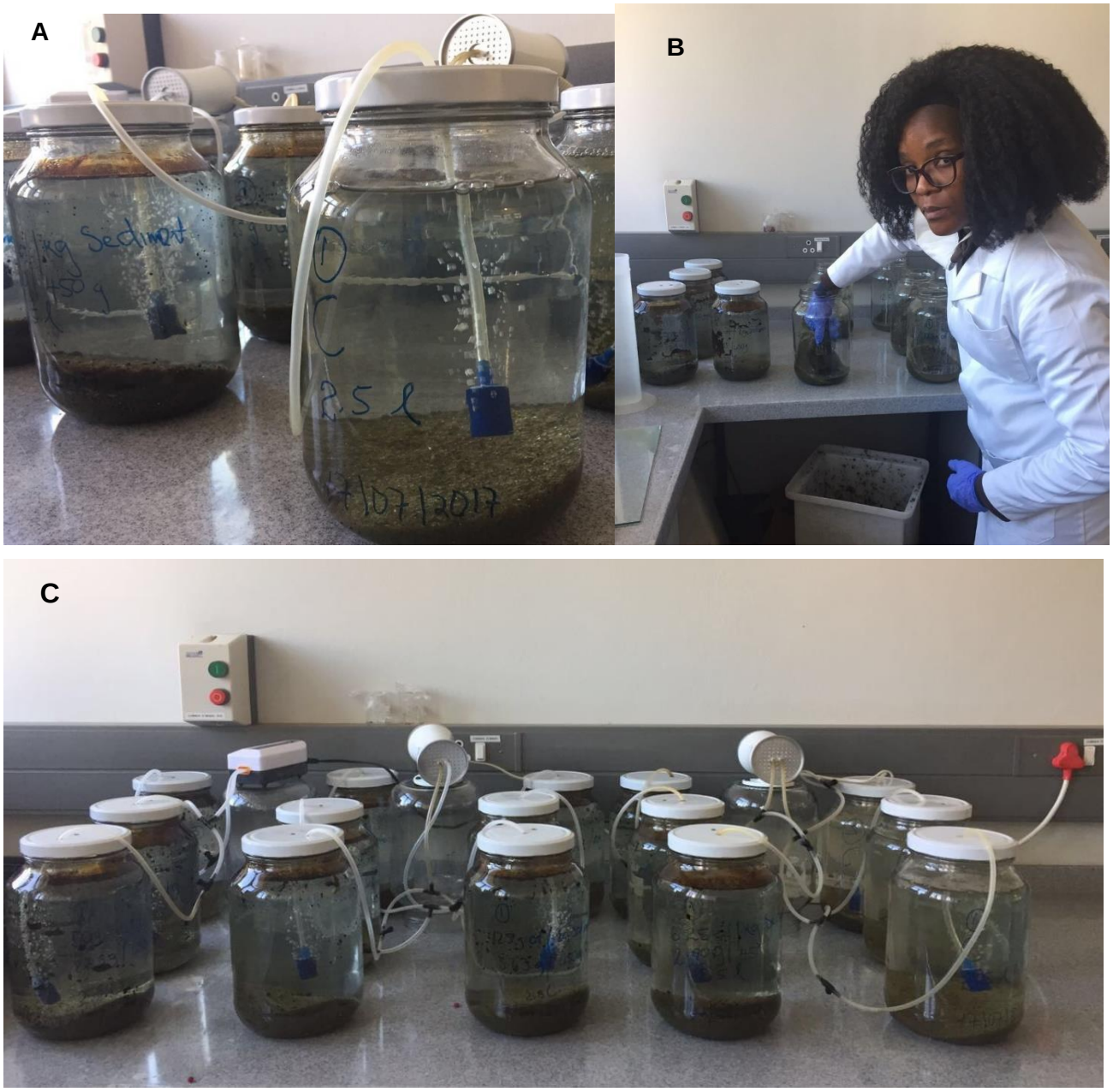

Figure 3-3: Microcosm set-up conducted in triplicate.

A: labelled aerated microcosms; **B**: preparation of the microcosm by putting sediments first at the bottom **C**: Complete set of labelled aerated microcosms with replicates of each concentration

3.2.2 Experimental duration, natural attenuation, and test organism introduction into chambers

Before test organisms were introduced, the microcosms were left undisturbed for one week and allowed to equilibrate. Test organisms from different trophic levels and habitat types were introduced into each control and test chamber.

The following test organisms from different trophic levels into each control and test chamber: *Lemna gibba*. (Aquatic plant); *Daphnia magna* (zooplankton (water flea)); *Physa* spp. (aquatic snail) and *Neocaridina* spp. (freshwater shrimp) (**Table 3-1**). The organisms were reared in the laboratory in aerated aquaria equipped with an aerating system at a temperature of 20±1°C and exposed to a 16-h light / 8-h dark cycle. The organisms were fed with Tetra fish flakes and pellets (TETRA®), 24 hours before the test commenced according to the test protocol and there after every second day. Before their placement in the experimental set-up, test organisms (*D. magna*, *Neocaridin*a spp. and *Physa spp.*) were not fed for 24 h because of possible alteration of the toxicants, the build-up of food and metabolic wastes resulting oxygen demand which are common in static test systems .

Table 3-1: Species used in the microcosm (adapted from Clement et al., 2013).

Type of species	**Species name**	**Trophic level**	**Main habitat**
Floating plant	*Lemna* spp.	producer	Water column / atmosphere
Crustacean (cladoceran)	*D. magna*	Primary consumer	Water column
Crustacean (decapodan)	*Neocaridina* spp.	Primary consumer	Sediment surface
Mollusc (gasteropodos)	*Physa* spp.	Primary consumer	Ubiquitous

The behaviour of the crude oil and the response of the test organisms in each of the chambers were monitored for six weeks in a temperature-controlled room (22±1°C) with an artificial light cycle of 16-h light / 8-h darkness. Qualitative observations were made every week of each chamber and recorded. Physicochemical readings (pH, temperature, electrical conductivity) were also taken and recorded every week using a multiparameter Hach HQ40D handheld meter. At the end of the experimental period (day 42), organisms in each chamber were counted, and where applicable, adults and juveniles were recorded separately. The state of *Lemna* spp fronds and roots were observed and recorded.

3.2.3 Test organisms and measurements

The endpoints assessed in the microcosms included growth potential, survival, and reproduction. This was compared to the negative control chamber that contained the same

organisms. All test organisms came from the CSIR (NRE) Ecotoxicity Laboratory's breeding stock. These organisms not only differ in terms of their trophic status and habitat, but also in terms of their feeding and uptake habits. Organisms were cultured according to standard methods under standard laboratory conditions (USEPA, 2002; OECD, 2006). Test organisms were fed with high-quality fish flakes and pellets (Tetra) every second day for the duration of the experiment. Faeces and excess food were removed every day before feeding.

The test organisms chosen for this assessment are highly prolific and relatively abundant all year round. They were also chosen due to their sensitivity since they are good bioindicators of environmental pollution and can adapt to laboratory conditions. They are a significant source of protein for the region's inhabitants and a principal prey of many larger vertebrates. The microcosm experiment was conducted over six weeks (42 days), at different concentrations of crude oil with three replicates (6.25 mg/kg, 12.5 mg/kg, 25 mg/kg, and 50 mg/kg) and the control.

Qualitative observations were made on a weekly basis with regards to the properties of the oil, as well as the test organism (presence/absence of test organisms and their general observed health status), and the following toxicity responses were monitored and assessed at the end of the study period, guided by methods and protocols referred to below:

i) *Lemna* spp: surviving plants and qualitative description of the condition of the plants (adapted from OECD, 2006).
ii) *D. magna* spp: mortality, reproduction: (adapted from USEPA, 2002);
iii) *Physa* spp.: mortality, reproduction (egg pockets and juvenile snails) (adapted from Turner and Montgomery, 2003); and
iv) *Neocaridina* spp.: mortality, reproduction (adapted from Rubach et al., 2010).

Ten *D. magna* neonates (< 48 hours old), ten juvenile snails (≈ 5 mm in size), five juvenile *Neocaridina* spp (total length ≈ 10 mm), and ten, two-frond stage *Lemna* spp were introduced per microcosm (experimental and control) in triplicate chambers (**Table 3-2**).

Table 3-2: Total number of test organisms used in the experiment over 42 days.

Sample	Organisms introduced per experiment (total in three replicates)			
	***Physa* spp.**	***D. magna* spp**	***Neocaridina* spp.**	***Lemna gibba* (number of plants and in brackets no of fronds)**
Control	30	30	15	30 (60)
50 mg / kg	30	30	15	30 (60)
25 mg/kg	30	30	15	30 (60)
12.5 mg /kg	30	30	15	30 (60)
6.25 mg / kg	30	30	15	30 (60)
Total	150	150	75	150 (300)

A description is provided below of the four test organisms used. These organisms are commonly found in lentic aquatic systems (**Figures 3-3 to 3-6**).

i) *Lemna* spp. *(*aquatic plant)

According to Lewis & Pryor (2013), aquatic plants are essential to the functioning of ecosystems due to their oxygen production, carbon sequestration, and their base position in aquatic food chains. The aquatic plant commonly referred to as duckweed, *L Gibba* spp., belongs to the family *Lemnaceae,* which has several worldwide species in four genera. *L Gibba* spp and *L. minor* (**Figure 3-3**) are species representative of temperate areas and are commonly used for toxicity tests. Both species have a floating or submerged discoid stem (frond), and a fragile root emanates from the centre of the lower surface of each frond. *L Gibba* spp. Rarely produces flowers and the plants reproduce by vegetatively producing new fronds.

Compared to older plants, the younger ones tend to be paler, have shorter roots, and consist of two to three fronds of different sizes. The small size of *Lemna* spp, its simple structure, asexual reproduction, and short generation time makes plants of this genus very suitable for laboratory testing (OECD, 2002).

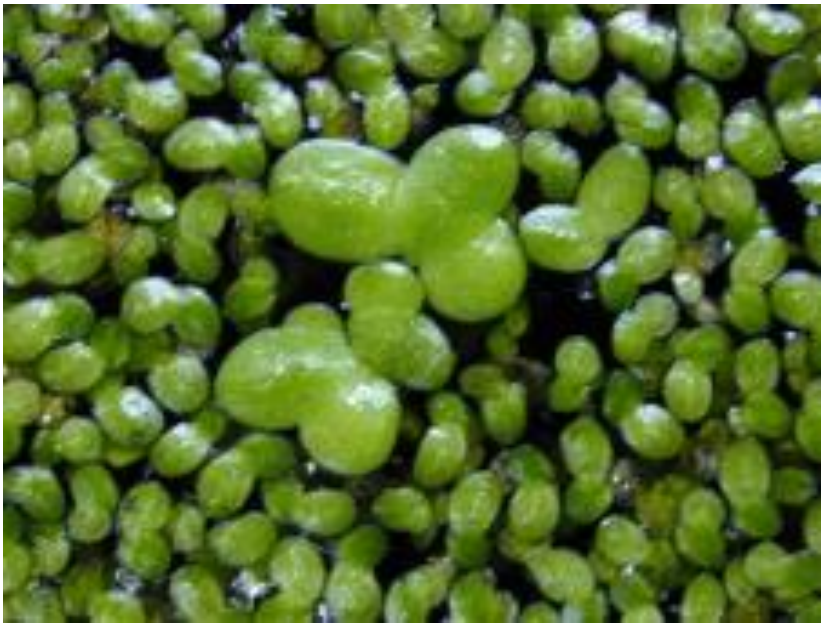

Figure 3-4: *Lemna* spp. (duckweed) (Storey, 2000).

ii) *D. magna* (water flea)

This is a tiny planktonic crustacean. Usually, 2-5 mm long, with an overall shape similar to a kidney bean (**Figure 3-4**). The body is enclosed by a transparent shell-like structure called a carapace that is mainly made of chitin. The life cycle begins when a female produces a clutch of eggs (usually 6-10) released into her body chamber, located under her carapace. The lifespan of these waterfleas in natural environments depends on environmental conditions such as oxygen levels, food availability, temperature and water quality. In general, as temperature decreases, lifespan increases with averages of 40 days at 25˚C and 56 days at 20˚C. Unstable environmental conditions lead to shorter lifespans (Ebert et al., 1993).

D. magna can be found in most freshwater environments where they live as filter feeders, eating small, suspended particles in water. They are mostly found in ponds and calm water in rivers and lakes. Common predators include young and adult fish, hydra, and immature and mature insects (Webber et al., 2010).

Figure 3-5: *D. magna* spp (waterflea) (https://en.wikipedia.org/wiki/Daphnia_magna).

iii) *Physa* spp. (snail)

Snails (*Gastropoda*) make up the class of animals with the most species from the phylum mollusks (*Mollusca*) (**Figure 3-5**). Freshwater snails occur in various habitats, including terrestrial habitats, temporary and permanent ponds, and rivers, where their reproduction is often very intensive (Duft et al., 2007; Woodard, 2005).

The shell height of adult *Physa* spp. Average about 4.3 mm and can reach up to 6 mm. Existing information shows that they may be excellent indicators of ecosystem health and employed in water and sediment quality assessments of different inorganic and organic substances, including metals, metalloids, pesticides, and synthetic detergents (de Freitas Tallarico, 2005; Woodard, 2005).

Figure 3-6: *Physa* spp. (freshwater snail) (Burch, 1982).

iv) *Neocaridina* spp. (freshwater shrimp)

The atyid shrimp, *Neocaridina* spp. is present in all tropical and most temperate waters. Adults of shrimp families always confine to freshwater. This is the only family in the superfamily Atyoidea (**Figure 3-6**). *Neocaridna* spp. is a dwarf species of freshwater shrimps, and adults can grow up to 2.85 cm in body length (Hung et al., 1993). Their functional feeding group are gathering and filtering collectors. These shrimps occur naturally in slow to fast-moving streams in Taiwan and other parts of Asia and are omnivores that feed on algae, biofilm, and detritus but not on vascular plants (Weber & Traunspurger, 2016). Most stomatopods live in temperate or tropical shallow marine habitats, but several species also range into subantarctic waters, and a few tropical species may occur in brackish water (Webber et al., 2010). *Neocaridina* spp. are relatively sensitive to poor water quality conditions and are good sediment and water quality indicators.

Figure 3-7: *Neocaridina* spp. (freshwater shrimp) (Liang, 2002).

3.2.4 Laboratory conditions

The study was conducted indoors at the Ecotoxicity Laboratory of the CSIR's Natural Resources and the Environment. The experiments were performed under static conditions and an ambient temperature of 22 ± 1° C with a day: night cycle of 16:8 hours. Experimental and control chambers were lightly aerated for the duration of the experiment (Clement et al., 2013; López-Mancisidor et al., 2008).

3.3 Single species *D. magna* acute toxicity testing

Furthermore, *D. magna* spp acute toxicity tests were performed at the start, towards the middle, and at the end of the experimental period to assess whether the toxicity of the water column changed over time for each oil concentration tested. 100 ml water was removed from the triplicate chambers and gently mixed before exposing the *D. magna*. The tests were performed according to standard procedures under laboratory conditions (**Table 3-3**).

Table 3-3: Summary of test conditions and acceptability criteria for the *D. magna* acute toxicity test (USEPA, 2002)

Summary of toxicity test	
Test system	*Daphnia* test
Test species	*Daphnia magna*
Age of test organisms	Less than 48h old
Trophic level	Grazer
Toxicity level	Acute toxicity
Test procedure	USEPA, 2002
Summary of test conditions for the Daphnia magna acute toxicity test	
Test type	Static
Water temperature	20 ºC ± 1 ºC
Light quality	Ambient laboratory illumination
Photoperiod	8 hours dark: 16 hours light
Feeding regime	Feed commercial fish flakes while in holding before the test
Aeration	None
Size of the test chamber	50 mℓ
The volume of the test sample	25 mℓ
Number of test organisms per chamber	5
Number of replicate chambers	4
Total number of test organisms per sample	20
Control and dilution water	Moderately hard, de-chlorinated water
Test duration	48 hours
Effect measured	Percentage lethality (no movement on gentle prodding), calculated concerning control
Test acceptability	90% or more remarkable survival in control
Interpretation	Lethality >10% indicates toxicity, provided that control lethality is ≤10%

3.4 CRUDE OIL CHARACTERIZATION

The crude oil was chemically characterized before the exposure period. The oil sample was extracted with hexane and analyzed with GC-MS at the CAF laboratory at Stellenbosch, South Africa. The instrument used for the analysis was an Agilent 6890N GC with CTC CombiPAL Autosampler and Agilent 5975B MS. The column used was a ZB 274305 for Semi Volatiles (30 m, 0.25 mm ID, 0.25 µm film thickness).

3.5 CHEMICAL ANALYSIS

Water column samples were tested at the experiment's start and end for the total petroleum hydrocarbons (TPHs). Total Petroleum hydrocarbons is a term used for any mixture of hydrocarbons found in crude oil. Since there are so many different chemicals in crude oil, it is not practical to measure each one separately. For this study, a “one number value” of TPHs is provided, which does not provide information on the composition (i.e., individual constituents of the hydrocarbon mixture) but was used instead to determine whether the concentration of TPHs in the water column changed from immediately after the sediment was spiked with oil towards the end of the experiment. Also, at the end of the experiment, determine whether there was much difference between the concentration in the die water column and the sediment. The analysis was performed at the Consulting and Analytical Services laboratory at the CSIR campus in Pretoria, South Africa. The laboratory is accredited by the South African National Accreditation System. Analytical procedures are based on “Standard Methods for the Analysis of Water and Wastewater” (APHA, 1992).

3.6 SUMMARY

This chapter has presented the method, design, materials, and facts on how data was collected to address the research questions that this study poses. The chapter has also highlighted a few of the less-than-optimal conditions that they study confronted, how the data was analysed and finally, sets the platform for the presentation and discussion of the study results in Chapter Four

CHAPTER FOUR: RESULTS OF THE STUDY

The aim of the study was identified as being to assess the co-toxicological hazard/risk that sediment contaminated with crude oil (from the bunker area) may pose to freshwater resources, particularly wetlands and pans. Indoor microcosms, under controlled laboratory conditions, were used for this purpose. Chapter Four will present the study findings from this research's experimental lab design and research. The findings are discussed in this chapter in the context of the study aim, objectives, and specific research questions. This chapter will also form the basis for discussion of study conclusions and recommendations to be made on the impact of underground bunker crude oil on freshwater aquatic organisms.

4.1 CHARACTERISTICS OF THE CRUDE OIL

One of the objectives of the study was to determine the characteristics of crude oil found in the bunkers of the study site in Mpumalanga. To do this, measurement of representative crude oil samples originating from Klippoortjie were selected for the microcosm experiment. The analysis reported that the distribution on a-alkanes in the oil sample was like the bunker diesel distribution, a sign of heavy residual fuels such as crude oil. C2-alkylated naphthalene (C_2N) was higher than major PAH, followed by C_3N. No chrysene was present in the oil samples. The results correspond well to the reported chromatograms of the bunker and heavy crude oil reported in the literature by other researchers such as Oberholser et al., 2014).

4.2 MICROCOSM EXPERIMENT

These visual observations of the microcosm experiment are provided in **Table 4-1** and physicochemical readings in **Table 4-2** from the end of week 1 after organisms were introduced to the end of the six weeks (day 42). In **Table 4-3,** the number of surviving organisms (and where reproduction occurred) are summarised.

Measurement of physicochemical variables was done before, during and at the end of the experiment. The experiments showed that the physicochemical (temperature, pH, and EC parameters) levels measured were not found to be significantly different, except for EC at the oil concentration of 50mg/kg.

The objective of the study is to assess the effects of crude oil on freshwater organisms. Crude oil test concentrations (50 mg/kg, 25 mg/kg, 12.5 mg/kg, and 6.25 mg/kg) that were used

revealed to be lethal to *D. magna* and *Neocaridina*, while *Physa* and *L Gibba* showed survival at oil concentrations of 6.25 mg/kg and 12.5 mg/kg respectively. Although Physa and L Gibba showed signs of survival in the two oil concentrations, signs of stress were observed as the Physa movement was slow and that the *L Gibba* fronds were yellow in colour.

In the Controls, the water column remained clear although algal growth was visible on the sediments. On day 42, the algae slightly reduced, probably because the living organisms were feeding on them. In concentrations spiked with oil, sediments were covered with oil, and some developed algal films. Some had dark or black lumpy matter on the sediment showing signs of degradation. Most of the oil that was visible on the water column or on the water surface, settled on the bottom sediments on day 42 of the experiment. This is also why crude oil eventually settles in sediment and the sediment acts as a sink for most hydrocarbons. Most crude oil would most probably have settled on or in the bottom bed over time (**Annexure A**).

4.3 THE VISUAL OBSERVATIONS

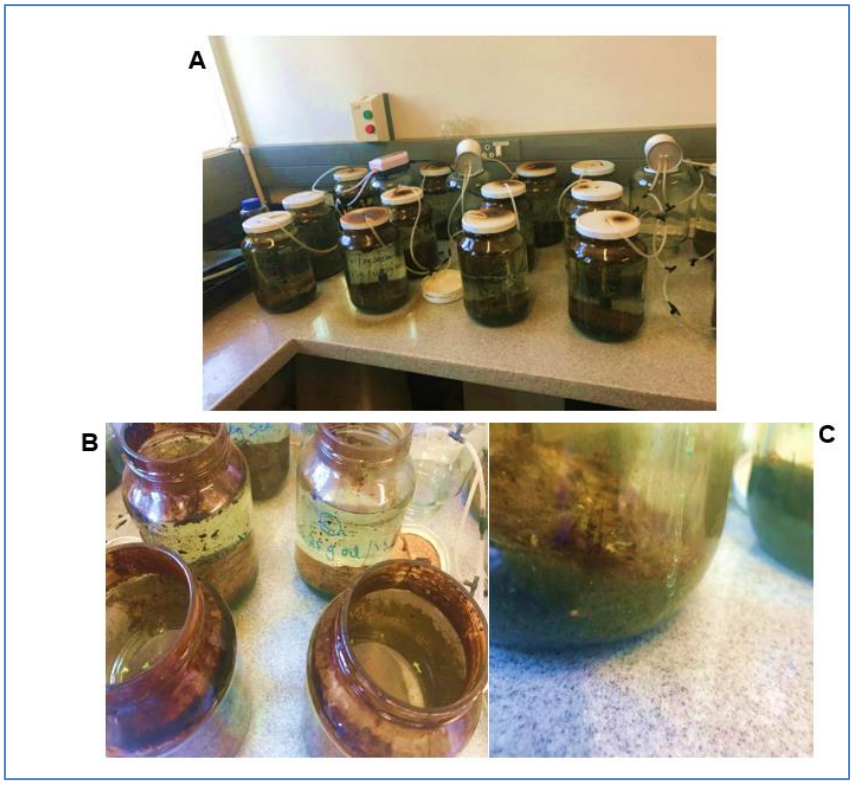

Figure 4-1: Images of the Laboratory Research on Day 42 of the Experiment (Source: Own Research Work, 2021)

Legend: **A**: Microcosms with replicates after 42 days of the experiment; **B**: The water column inside the microcosms after 42 days of the experiment; **C**: The bottom sediments of the microcosms after 42 days of the experiment

Table 4-1: Visual observations over the experimental period

Week	Sample	Visual observation
1	Control	Water was clear; sediment intact; New *Lemna* fronds were visible; *D magna* adults and neonates were observed; All *Neocaridina* and *Physa* introduced were observed.
	6.25 mg/kg	Water column was very turbid with some oil on the surface; Thin layer of oil covered the sediment with some algal growth visible; No *D magna* visible; One adult *Neocaridina* observed; All *Physa* were visible and egg pockets were observed on the air stones.
	12.5 mg/kg	Water column was very turbid with some oil on the surface; Oil layer covered sediment; Few *Lemna* fronds covered in oil were visible; Fronds were small compared to the Control; No *D magna* or *Neocaridina* visible; Few *Physa* visible with some egg pockets on air stones.
	25 mg/kg	Water column very turbid with layer of oil on surface; Oil layer covers sediment; Few *Lemna* fronds covered in oil visible. Fronds were small compared to the Control; No other organisms observed.
	50 mg/kg	Water very turbid with thick layer of oil covering entire water surface; Oil layer covered sediment; no *Lemna* visible; No organisms observed. The water was very turbid.
2	Control	Water column was clear; No algae on sediment was found. fronds increased and smaller *Lemna* started to emerge. Adults and neonates were visible. Five *Neocaridina* were present on sediment surface. All *Physa* introduced were visible and a few egg pockets were present on the side of the aquarium and on the air stone. All the organisms seemed to be thriving.
	6.25 mg/kg	The water column was turbid with between 1 to 5 % oil covering the surface area; Oil had a lumpy appearance with an oil sheen present on the water surface; Oil on sediment looked lumpy as if it was degrading; Algal film in and on sediment increased; Lemna were present, but the number of *Lemna* colonies and fronds did not increase; Lemna roots were covered with oil. *Physa* and egg pockets were present on the air stone and on sides of the aquarium; The water column was turbid.
	12.5 mg/kg	Water column was turbid and 10 to 15% of the water surface was covered in oil; Oil on sediment looked lumpy and as if it is degrading; 2 to 5 *Lemna* plants present per replicate; Although the fronds were green, they were not increasing, and fronds were small compared to the Control; Few *Physa* were present and were very small compared to the ones in the Control; Some egg pockets were present on the air

		stone; Water column was turbid with less oil covering the water surface; Oil on the sediment was brown and appeared to be degrading; Both *Lemna* and *Physa* were much smaller compared to the control and were not thriving.
	25 mg/kg	The water column was turbid, and approximately 50 - 75% of surface water area was covered in oil. Oil and debris settled on the bottom of sediment.
	50 mg/kg	Waterless turbid with a layer of oil covering the entire water surface but appearing to be thinner. More oil settled on the sediment with some algae present. The water column was still turbid. However, the layer of oil that covered the entire water surface area seemed to become thinner.
3	Control	The water column was clear. Some algae were visible on sediment. *Lemna* plants and fronds were increasing. Long healthy roots were present. More *D. magna* adults and neonates were present. Five *Neocaridina* were present on the sediment surface. All *Physa* introduced visible, and a few egg pockets were present on the side of the aquarium and on the air stone. Organisms and *Lemna* seemed to be thriving, indicating a healthy microcosm.
	6.25 mg/kg	Water column slightly turbid with < 5% oil covering the surface area. Sediment covered mainly in oil. Very few *Lemna* observed. Tiny fronds and no new plants and fronds were visible. Roots slightly covered in brown debris. Five big *Physa*, very few juvenile *Physa* were present and egg pockets on the side of the aquarium and on the air stone were observed. Only *Lemna* and *Physa* were present. Adult *Physa* appeared smaller than in Control. Signs of *Physa* reproduction was visible, but not *Lemna.*
	12.5 mg/kg	Water column was quite turbid with very little oil (~ 10%) present on the water surface. Sediment covered in oil which had a dark brown colour. Very few *Lemna* plants visible - roots covered in brown debris, two adult *Physa* observed and a few egg pockets which seemed to detach from the side of the aquarium; no juvenile *Physa* observed. Few *Lemna* present that was in poor condition and only two adult *Physa* that appear to be under stress (smaller than control *Physa*). Water column was quite turbid. But less than 25 mg/L.
	25 mg/kg	Water column was very turbid, especially replicate 2 - chocolate brown colour. 20 to 40% of the surface was covered in oil. Oil on the sediment surface was black and lumpy. Two fronds were visible in two of the replicates - none in the third replicate. Few *Lemna* fronds have survived. But in a deplorable condition. No other organisms were present.

	50 mg/kg	Oil layer of ~ 0.5 cm thick covered the entire water surface in replicate 2 and 3. In replicate 1 oil layer was slightly thinner. Oil appeared less thick and more fluid. Oil was black. Water was slightly turbid. Sediment surface largely covered in oil with oil also present in sediment and was black in appearance. Thinner and more fluid oil covered the entire water surface; water column was less turbid; oil layer present on sediment surface and had settled in the sediment itself. No organisms.
4	Control	The water column was clear; Some algae were visible on sediment. *Lemna* plants and fronds were increasing. Long healthy roots were present. More *D. magna* adults and neonates were present - increased reproduction. Five *Neocaridina* were present on sediment surfaces as well as neonates. All *Physa* introduced visible. Several egg pockets were present on the side of the aquarium and on the air stone—baby *Physa* and adults in various stages. Organisms and *Lemn*a seemed to be thriving and were reproducing and indicating a healthy microcosm.
	6.25 mg/kg	The water column was slight turbid; no oil was visible on the water surface. Sediment covered in an organic type of matter - oil broken down. Few *Lemna* with fronds present were small to medium in size compared to the Control. *Physa* of different sizes were present, as well as egg pockets against the side of the aquaria. No algae present in any of the aquaria
	12.5 mg/kg	The water column was very turbid - light brown colour. Sediment covered in organic matter - oil appeared to have broken down. Few *Physa* present (small in size). Only *Physa* was present - no other organisms or *Lemn*a.
	25 mg/kg	The water column was turbid - light brown in colour; few oil lumps drift on the water surface. Sediment covered with lumpy black oil. Very few *Physa* present. Only *Physa* present - no other organisms or *Lemna*.
	50 mg/kg	Water column was almost clear; 90% of water surface area covered with a thin liquid layer of oil. Sediment surface primarily covered in oil with oil also present in sediment and was black in appearance. No organisms were present.
5	Control	Water column was clear with no visible signs of algae present. Some algae were visible on sediment. *Lemna* increased, and fronds were healthy and big compared to *Lemna* in experimental chambers. Several daphnids adults and neonates visible. Five adult *Neocaridina* (signs of internal eggs present), various stadia of *Physa* (adults and juveniles and egg pockets) were present. Organisms and *Lemna* seemed to be thriving and were reproducing and indicating a healthy microcosm.
	6.25 mg/kg	Water column was slightly turbid with no oil on the water surface. Sediment covered in an organic type of matter - oil broken down. Few *Lemna* were present but had tiny fronds. *Physa* of different sizes present, as well as egg pockets against the side of the aquaria.

	12.5 mg/kg	The water column was turbid (light brown) with some oil present on the surface. Some oil settled on the surface of the sediment. Two small *Lemna* fronds were present. Few *Physa* as well as egg pockets present but was covered in oil.
	25 mg/kg	The water column was turbid (light brown) with some oil present on the surface – covering approximately 40% of the surface area. Oil settled on sediment. No organisms were present.
	50 mg/kg	The water column in the replicates was turbid while altogether clear in the other two replicates. Sediment covered in black oil that seemed to have broken down; some oil lumps were present. No organisms were present.
6	Control	The water column was clear. No visible algae were in or on sediment. *Lemna* proliferates and increase. *D. magna* proliferates, and several adults and neonates were present. Adult and small *Neocaridina* were present. Various sizes of *Physa* as well as egg pockets, were present. *Physa* proliferate. Organisms and *Lemna* proliferate and juveniles and adults present.
	6.25 mg/kg	The water column was clear, with no oil residue on the water surface present. Black matter was present on the sediment and algae was also present in the sediment. *Lemna* fronds were deep green in colour but cover approximately 5% of the water surface area. Fronds were tiny compared to the Control. *Physa* of various sizes were present, as well as egg pockets, but both were covered in a black/grey matter.
	12.5 mg/kg	The water column was turbid. Brown matter covered sediment surface. Very few, small fronds that were light green were present. Very few *Physa* were present and were covered in black residue; egg pockets were present but started to detach from the side of the aquaria.
	25 mg/kg	Water was turbid with oil suspended in the water column and some oil clumps were on the water surface. Sediment covered in thick brown residue in sediment while about 0.5 cm on top of sediment was covered in the black matter. One *Lemna* frond (yellow and small) was present on the surface but covered on oil. Three *Physa* observed, covered in the black matter.
	50 mg/kg	The water column was turbid with oil droplets suspended in the water column.

Source: Own Research Work (2017)

Table 4-2: Physicochemical results over the experimental period

Temperature												
Day	**Control**	**6,25mg/kg**		**Day**	**Control**	**12,5mg/kg**		**Day**	**Control**	**25mg/kg**		**Day**	**Control**	**50mg/kg**
7	22,9	22,7		7	22,9	22,6		7	22,9	23		7	22,9	22,8
14	23,1	22,9		14	23,1	22,9		14	23,1	23		14	23,1	22,8
21	22,9	22,7		21	22,9	22,6		21	22,9	22,4		21	22,9	22,2
28	21,2	21,1		28	21,2	21,2		28	21,2	21,2		28	21,2	21,2
35	22,9	22,9		35	22,9	22,8		35	22,9	22,9		35	22,9	22,8
42	22,8	23		42	22,8	23		42	22,8	22,9		42	22,8	23
Mean	22,63	22,55		Mean	22,63	22,52		Mean	22,63	22,57		Mean	22,63	22,47
SD	**0,65**	**0,66**		**SD**	**0,65**	**0,61**		**SD**	**0,65**	**0,64**		**SD**	**0,65**	**0,62**

pH												
Day	**Control**	**6,25mg/kg**		**Day**	**Control**	**12,5mg/kg**		**Day**	**Control**	**25mg/kg**		**Day**	**Control**	**50mg/kg**
7	7,58	7,82		7	7,58	7,97		7	7,58	7,87		7	7,58	7,9
14	7,72	8,81		14	7,72	7,19		14	7,72	8,1		14	7,72	8,19
21	7,67	7,71		21	7,67	8,06		21	7,67	8,06		21	7,67	8,31
28	7,24	7,64		28	7,24	8,04		28	7,24	7,98		28	7,24	7,89
35	7,35	7,86		35	7,35	7,88		35	7,35	7,81		35	7,35	6,22
42	7,58	7,82		42	7,58	7,97		42	7,58	7,87		42	7,58	7,9
Mean	7,52	7,94		Mean	7,52	7,85		Mean	7,52	7,95		Mean	7,52	7,735
SD	**0,17**	**0,39**		**SD**	**0,17**	**0,30**		**SD**	**0,17**	**0,11**		**SD**	**0,17**	**0,70**

Electronic Conductivity												
Day	**Control**	**6,25mg/kg**		**Day**	**Control**	**12,5mg/kg**		**Day**	**Control**	**25mg/kg**		**Day**	**Control**	**50mg/kg**
7	234	250		7	234	243		7	234	260		7	234	240
14	211	251		14	211	226		14	211	246		14	211	219
21	218	246,6		21	218	244		21	218	245		21	218	219
28	248	255,7		28	248	237		28	248	234		28	248	210
35	223	241		35	223	247		35	223	245		35	223	256
42	272,3	247		42	272,3	249		42	272,3	254		42	272,3	247
Mean	234,38	248,55		Mean	234,38	241		Mean	234,38	247,33		Mean	234,38	231,83
SD	**20,69**	**4,52**		**SD**	**20,69**	**7,68**		**SD**	**20,69**	**8,12**		**SD**	**20,69**	**16,77**

SD is the “Standard Deviation”. Source: Own Research Work (2022)

The standard deviation and the mean of the physicochemical parameters was assessed for all the crude oil concentrations and the controls over a period of 42 days (**Table 4-2**). In terms of the analysis, the results indicated that the there is no difference in the mean and standard deviation for the temperature and pH, while electronic conductivity was different in all crude oil concentrations.

4.4 PHYSICOCHEMICAL READINGS OVER THE EXPERIMENTAL PERIOD

The pH results obtained in this study ranged from 7.5 to 8.5 for the oil concentrations and 7.1 to 8.5 for the controls. However, there was no significant difference in the pH values across the oil concentrations. The temperature ranged from 21˚C to 23˚C and was the same for both the oil concentrations and the controls. The electrical conductivity ranged from 201 µS/cm to 250 µS/cm for the controls and 213 µS/cm to 318 µS/cm (**Figure 4-1 and Figure 4-2**).

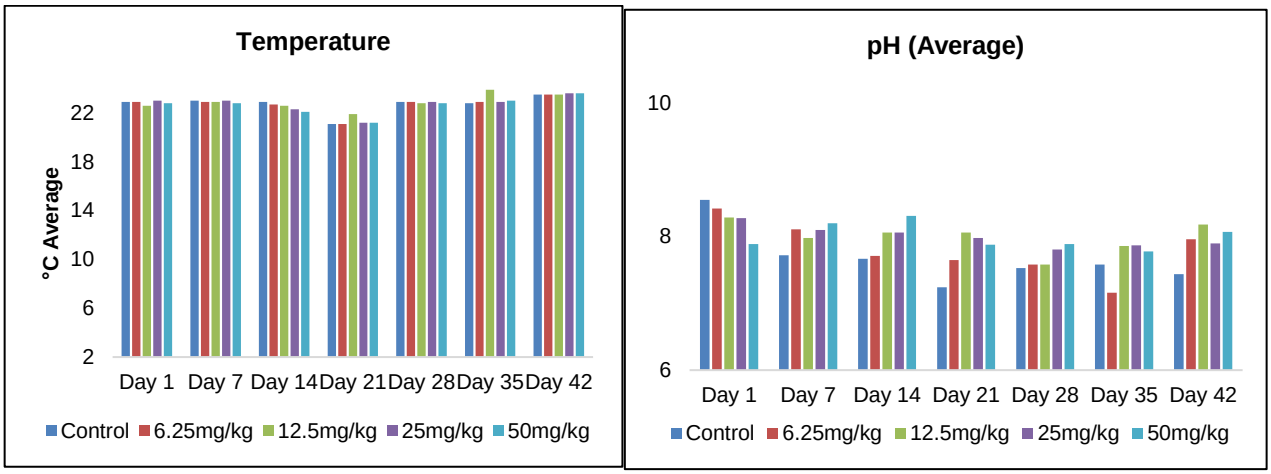

Figure 4-2: Temperature and pH physicochemical results over the 42 days of the experiment.

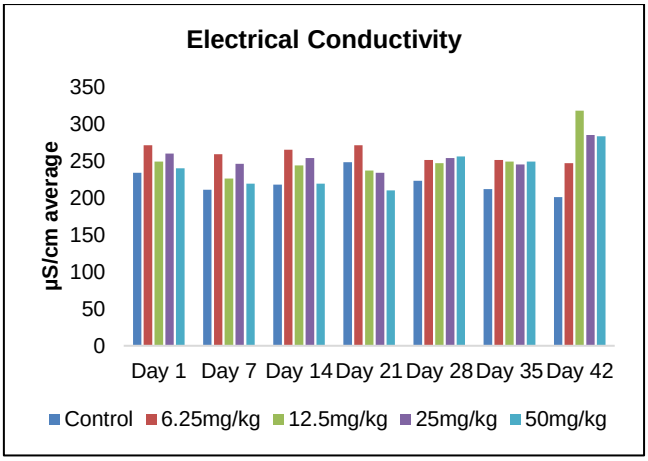

Figure 4-3: Electrical Conductivity results over the 42 days of the experiment.

4.5 RESULTS OF TESTED ORGANISMS 42 DAYS AFTER THE EXPERIMENT

Table 4-3: Average number for the survival and reproduction of the test organisms at the end of the experimental period (day 42) for each microcosm.

Sample	*Physa* spp				*Daphnia*		*Neocaridina*		*Lemna plants (no of fronds in brackets)*
	Egg pockets	Small (2 mm and <)	Medium (>2 and ≤5 mm)	Large (> 5 mm)	Juvenile	Adults	Juvenile	Adults	
Control	25	65	25,6	25	258,6	42,6	15,3	3,6	586,6 (1760)
50 mg/kg	0,66	0,66	0	0	0	0	0	0	0
25 mg/kg	1,6	0,3	0,66	0	0	0	0	0	0,3 (0,6)
12.5 mg/kg	3	1,3	0.66	0	0	0	0	0	3 (12,6)
6.25 mg/kg	15,3	44	9	10,3	0	0	0	0	81 (228,6)

Table 4-3 above summarizes the number of surviving organisms (and where reproduction occurred) in the study. A fair deduction of the results reveals how the *D. magna* and *Neocaridina* spp. organisms were unable to survival for a short period of time in crude oil contaminated water. The *Pyhsa* spp was more able to hold its own survival, and the *L. gibba* plants, in comparison. As filter feeder organisms play a significant role in the aquatic environment's food chain, and the impact of bunker oil on their existences threatens to disrupt the balance of aquatic food chain. **Figure 4-4 and figure 4-5** presents the results of **Table 4-3** even clearer as they show a rapid dying off organisms as a result of contamination by bunker oil in the experiment.

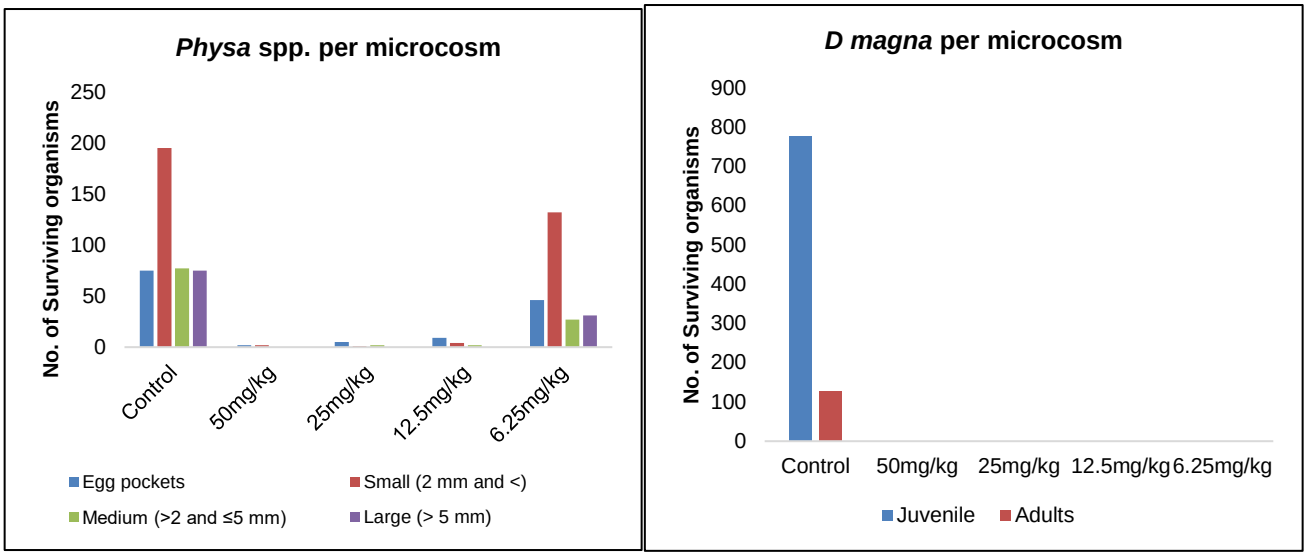

Figure 4-4: Survival of the test species (*Physa spp.* and *D. magna*) after the 42-day period of the experiment.

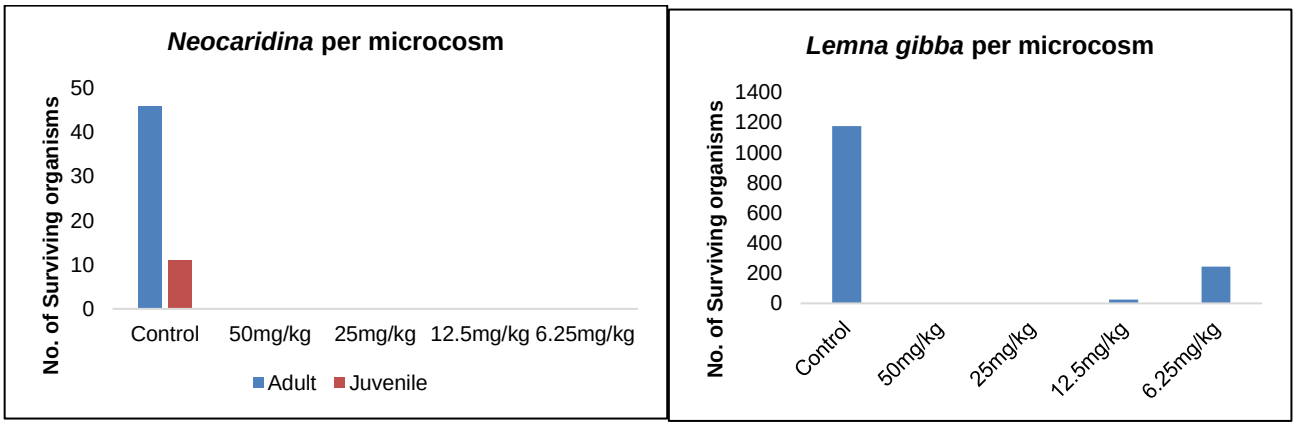

Figure 4-5: Survival of the test species (*Neocaridina* spp. and *L. gibba*) after the 42-day period of the experiment.

4.6 STATISTICAL ANALYSIS

The statistical comparison was conducted through the use of the One-way analysis of variance (ANOVA) for testing for differences. The ANOVA tested whether there is an effect of the crude oil concentrations in the tested organisms. The p value was assumed at 0,05, to determine the effects of crude oil on the organisms. The results of the p values for the tested organisms were greater than 0,05 in that at 6,25mg/kg p value is 0,19, while at 12,5mg/kg, 25mg/kg and at 50mg/kg the p value is 0,18 respectively. The p values obtained indicate that there is an effect of crude oil on the tested organisms.

4.7 SINGLE SPECIES *D. MAGNA* ACUTE TOXICITY TEST

Results of the 48-hour acute *D. magna* toxicity test performed on microcosm water on Day 1, 21 and 42 respectively are summarised in **Table 4-4** below.

Table 4-4: 48 Hour *D. magna* acute toxicity test results summarised as percentage mortality.

Sample name	**% *Daphnia* mortality (48 hrs)**		
	Day 1	**Day 21**	**Day 42**
Control	0	0	5
50 mg/kg	10	90	90
25 mg/kg	0	100	45
12.5 mg/kg	0	100	15
6.25 mg/kg	0	5	0

When 10% or more *D. magna* mortality is observed in a test sample, the sample is regarded to have an acute toxicity potential (Oberholster et al., 2016). In Table 4-4, *D. magna* mortality observed per sample at the end of each test (48 hours), is summarised for the days that samples were collected and tested. Very low toxicity was observed on Day 1 in the three samples including the control except for sample 50mg/kg which showed slight toxicity at mortality of 10%. In Day 21, the samples were highly toxic at there was very high mortality (90% to 100%) in all the samples except for 6,25mg/kg and the control. During day 42, toxicity

was very high in the sample of 50mg/kg because of the records of high mortality of 90% with the second highest mortality of 50% in the concentration of 25mg/kg. Therefore D. magna can survive at the toxicity level of 6,25mg/kg which had very low toxicity values of less than 10%.

4.8 THE CHEMICAL ANALYSIS OF TOTAL PETROLEUM HYDROCARBONS

The Total Petroleum Hydrocarbons (TPHs) measured in the water column of each test sample and the control at the start and end of the experiment and in sediment at the end of the experiment are summarised in **Table 4-5**. Equal amounts of samples were taken from each triplicate and pooled before samples were submitted for analysis.

Table 4-5: Analysis of TPHs analysed in pooled samples of the water column and sediment measured as a once-off

Sample type	**Unit**	**Control**	**6,25mg/kg**	**12.5 mg/kg**	**25mg/kg**	**50mg/kg**
Water column (start)	mg/L	<0.2	0.57	1.2	1.6	3.6
Water column (end)	mg/L	<0.2	0.45	1.8	1.9	180
Sediment	mg/kg	<20	<20	<20	<20	46
SD	-	9,33	9,18	8,72	8,60	75,18

SD is the “Standard Deviation”.

The presence of chemical contaminants and/or TPH in any aquatic environment is a major threat to the water and its organisms (Adeniji et.al., 2017). Petroleum hydrocarbons are known to be one of the major pollutants of aquatic environments. Found in various levels of concentration in sediments which is a habitat for aquatic organisms, petroleum hydrocarbons potentially posing risk of bioaccumulation (Filho et.al., 2013; Muthukumar et.al., 2013; Adeniji et.al., 2017).

Table 4-5 provides the results of this study on the concentration of petroleum hydrocarbons present in the study sample sediments. The findings in **Table 4-5** show that there was gradual variation of concentrations of TPH in the sediment, across the different weights with the statistical tests conducted revealing little to no significant difference in the levels of TPH determined. A significant limitation in the 42-day period was the inability determine the influence of seasonal changes on the concentration of TPH in the sediments sampled

4.9 SUMMARY

This chapter has provided an in-depth discussion of the study findings. The findings reflect the intentions of the study objectives and the research questions that were raised as a basis and justification for conducting this study. The study aimed to assess the co-toxicological hazard/risk that sediment contaminated with crude oil (from the bunker area) may pose to freshwater resources, particularly wetlands and pans. Indoor microcosms, under controlled laboratory conditions, were used for this purpose.

In doing so, this chapter has presented findings that characterise the crude oil present in the bunker; assess the ecotoxicological effect of bottom sediment spiked with bunker crude oil at different concentrations to test organisms over a selected period, namely algae, water fleas, snails, and aquatic plants; and visually assess the behaviour and natural breakdown of the crude oil over the experimental period. This next chapter will present detailed discussions of the findings in order to determine the ecological hazard/risk and make management recommendations based on the study's outcome.

CHAPTER FIVE: DISCUSSIONS

5.1 MICROCOSMS EXPERIMENT WATER

The microcosm experiment showed that different crude oil concentrations have variable toxicity levels to freshwater organisms. Crude oil remained on the water's surface, causing oil to slick in the 25 mg/kg and 50 mg/kg concentrations. Spilt oil will disperse into the water column and the benthic organisms in environments (such as wetlands and pans). Crude oil in the water column will bind with the suspended particles while the oil moves along the water column. For example, the laboratory tests conducted by Doelling et al. (2014) at Louisiana State University using E2MS 303 oil from the spill of Bakken crude from a barge collision into the lower Mississippi River, indicated that the “oil will quickly adhere to suspended solids in the water column, forming unstable emulsions”. As a result, the colour of the water was light brown because the natural cleaning process was retarded. This was also observed in the different experimental mesocosms over different stages of the experiment.

5.2 PHYSICOCHEMICAL MEASUREMENTS

The physicochemical measurements (pH, temperature, electrical conductivity, oxygen demand) were measured throughout the experiment (**Figure 4-1; Figure 4-2 and Table 4-2**). The pH values ranged from 7.16 to 8.3 in all concentrations, including the control, and were within the detectable limits described by DWAF (2011). The electrical conductivity (EC) values varied between 240 to 201 µS/cm in the Controls and varied between 210 µS/cm and 318 µS/cm in the 6.25 mg/kg to 50 mg/kg experimental concentrations. The EC values in this study were below the World Health Organisation (WHO) limit of 1000 µS/cm. These values compared well with results obtained by Edema (2019). Kadiri (2006) indicated that high levels of EC in water is a sign of pollution. The EC has a linear relationship with total dissolved solids (TDS), as the higher the TDS, the higher was the EC (Olayinka et al., 2020). The electrical conductivity results were higher in the 50 mg/kg test concentration. These results were still higher than those reported by Oberholster et al. (2016).

The statistical analysis indicated that the physicochemical water quality parameters were not influenced by the crude oil in the water column (**Figure 4-2 and Figure 4-3**). No definite trend was observed in the measured physicochemical parameters (**Annexure B**).

The colour (and hence turbidity) of the water column varied in the different experimental concentrations over the study period. The colour of the water varied from light brown to dark brown. Ololade & Lajide, (2010) indicated that the presence of colour in some of the concentrations indicates more organic and inorganic matter in suspension.

5.3 TEST ORGANISMS: *D. MAGNA*, *PHYSA*, *NEOCARIDINA* AND *LEMNA GIBBA*

The test organisms showed pathological changes in terms of mortalities in a concentration dependant manner. *Neocaridina* spp and *Daphnia magna* spp were more sensitive to crude oil contamination with 100% mortality after 24h compared to snail and *L gibba* which showed survival (although limited) in all concentrations except at the 50 mg/kg (**Figures 4-3 and 4-4, Table 4-3**) compared to the Controls.

Neocaridina and *D. magna* are most sensitive to crude oil**,** and *Physa* spp and *L Gibba* spp appeared to be more resistant to the exposure to oil (**Figure 4-3 and Figure 4-4**). *Physa* spp. and *L Gibba* spp. were affected by higher oil concentrations (25 mg/kg and 50 mg/kg) and displayed gradual lower toxicity effects and survived up to end of the experiment (**Figure 4-3 and Figure 4-4**). The organisms in the Controls seemed to thrive with no stress observed. Others observed this same sequence, Ren et al. (2015) reported that the total hydrocarbons concentration of about 6 to 7 ppm caused about 50% mortality in white *Neocaridina* after 96 hours. Tudararo-aherobo et al. (2013), found that freshwater shrimp is more sensitive to crude oil than brackish water shrimp. The quality of the water can also influence the toxicity of the hydrocarbons. This has been reported by Asadi & Khoiruddi (2017) where decreasing pH (8.5 to 6.5) modified the toxicity of crude oil WAF to whiteleg shrimps, *Litopenaeus vannamei*. The pH of 6.5 slightly, but significantly increased the toxicity of crude oil WAF. Bioaccumulated constituents of the oily sludge move up the food chain, and since they are recalcitrant, and can lead to mutations and cancers, among other conditions, in man and other higher organisms.

5.3.1 Physa spp.

In this study, *Physa* spp. survived in all the concentrations; however, at a lower rate in 25 mg/kg while the 50 mg/kg concentration experienced high mortality (**Figure 4-3**; **Table 4-3**). The findings of this experiment are similar to the study by Abdul-sam et al. (1996), which mentioned that *Physa* spp. exposed to high concentrations of crude oil displayed acute

toxicity symptoms and died within two weeks, while snails exposed to lower concentrations showed gradual toxicity symptoms and survived up to 6 weeks of the experiment. *Physa* spp. were able to tolerate crude oil exposure throughout this experiment. In the study by Robinsons and Rabalais (2019), it was revealed that snails encountered PAHs through direct contact with WAFs and volatile PAHs. There were reproduction and growth of *Physa* spp. in the experiment in that small, medium, and larger *Physa* spp. were observed in the concentrations 6.25 mg/kg, 12.5 mg/kg and 25 mg/kg. *Physa* spp. egg pockets were observed in the aquarium, which presents signs that reproduction took place during the experiment's duration. In the 50 mg/kg concentration, the growth rate was also affected as the surviving *Physa* spp. were very few. The feeding habits of the organisms are also influenced by the impacts of crude oil on snails. For example, Lee et al. (2001) found differences in sensitivity between the mystery snail (*Viviparus georgianus*) and the mimic pond snail (*Pseudosuccinea columella*) in a controlled oil spill experiment at a wetland site along the St. Lawrence River (Ste. Croix, QC) that were attributed to feeding habits. *V. georgianus*, a detritivore, assimilated contaminants directly from sediments, while *P. columella*, an herbivore, assimilated contaminants indirectly, presumably from oiled vegetation.

5.3.2 Lemna gibba.

In the current study, the crude oil affected the *L Gibba* spp. in all the concentrations by changing the fronds from green to light green / yellow (**Figure 4-4; Table 4-3**). The roots were not visible as they were covered in oil and debris compared to the Control. Duckweed has been reported to have an ability for pollutant uptake (e.g., metals such as cadmium, lead, and zinc) from water. It is also being researched for use as a tool for phytoremediation (Latour et al., 2015). Kosesakal et al. (2015), however found that crude oil adversely influenced the growth of *L. minor*. Different *L Gibba* species may therefore respond differently to crude oil contamination.

Ekperusi et al. (2020) indicated that several studies supported the significant removal of hydrocarbons by various macrophytes, including duckweed, from contaminated wetlands. Duckweed may therefore play an important role in crude oil remediation. In Taiwan, duckweed is used as food for pigs and poultry (FAO, 2001). In fish feed, *L Gibba* spp. are usually used in the fresh state. There is a growing interest in this free-floating macrophyte in

aquafeed (Chakrabarti, 2018). Therefore, crude oil impacts will result in environmental and economic impacts.

5.3.3 Daphnia magna

During the experiments of this study, the *D. magna* neonates died within 24h of being introduced into the experimental chambers (**Figure 4-3, Table 4-3**). It was therefore observed that crude oil is harmful to *D. magna* young. This is in contrast to the findings of the study by Lennuk et al. (2015), which revealed that among different size groups, the medium-sized Cladocerans were the most sensitive to crude oil pollution than the small and large ones. Fereidouni et al. (2013) found that the effects of oil inhibited glutamic oxalacetic transaminase activity, gas exchange inhibition, direct feeding, and absorption of oil residues by the organism. In the study by Lennuk et al. (2015), the impacts of crude oil observed included immobilizing of organisms by the insoluble surface layer of crude oil, the whole carapace of an animal removed because of oil pollution and Cladocerans stuck together due to crude oil. Sodani et al. (2011) concluded that acute toxic effects of crude oil on *D. magna* are linked with physical and chemical characteristics of water such as the turbidity and TDS. It is likely the physical stress caused by the crude oil in this experiment, affected the *D. magna* (e.g., movement) and may have also potentially affected their breathing (e.g., by clogging their gills) and feeding ability.

5.3.4 Neocaridina (freshwater shrimp)

In the current experiment, the mortality of *Neocaridina* spp. was 100% within 24 hours of the study in all the experimental concentrations (**Figure 4-4, Table 4-3**) compared to the Control, suggesting that crude oil e attributed to the effect on the organisms. *Neocaridina* spp. is relatively short-lived creatures. Dwarf shrimps usually live for only 1-2 years. All freshwater shrimps are omnivorous and feed on dead organic material of all kinds, plus anything else that offers. The study by Asadi et al. (2017), indicated that when the experimental *Litopenaeus vannamei* were acutely exposed to crude oil WAF for 72 h, the biological activities of individuals decreased, and the body balances were gradually lost leading to comatose and even death.

Other studies have shown that sublethal WSF exposure affects lipid anabolism and catabolism, biological membranes fluidity, increased vitellogenins, and induced antioxidant defence systems (Lavarías et al., 2005, 2006, 2007, 2011).

In the statiscal analysis conducted, the correlation was found between the crude oil and the tested organisms (**Annexure A**). Crude oil was found to be toxic to the organisms as *D. magna* and *Neocaridina* showed 100% mortality within 24 hours at the low oil concentration of 6.25 mg/kg of crude oil. Some brown adhesive materials and flocs that were observed around their carapaces, mainly the gills suggests that the organisms were physically stressed.

Crude oil also impacted the population of *Physa* and *L Gibba* spp, although the organisms demonstrated some tolerable level of the pollutant. Therefore, crude oil levels as low as 6.25 mg/kg can impact the organisms at lower trophic levels and the impacts can be catastrophic as they can result in foodweb destruction.

5.4 CRUDE OIL CHARACTERIZATION

Representative crude oil samples originating from Klippoortjie were selected for the microcosm experiment. The analysis reported that the distribution on a-alkanes in the oil sample was like the bunker diesel distribution, a sign of heavy residual fuels such as crude oil. C2-alkylated naphthalene (C_2N) was higher than major PAH, followed by C_3N. No chrysene was present in the oil samples. The results correspond well to the reported chromatograms of the bunker and heavy crude oil reported in the literature by other researchers.

5.5 SINGLE SPECIES *D. MAGNA* ACUTE TOXICITY TEST

The *D. magna* single acute toxicity tests that were conducted on the water column, resulted in mortality of the test organisms on Day 21 and 42 of the experiment with higher and more acute toxicity observed on Day 21, compared to Day 42. This could be attributed to the turbidity of the water column, or contaminants that are associated with the crude oil that may have been more pronounced on Day 21. The water column was only slightly toxic to *Daphnia*

on all Days of the experiment for the 50 mg/kg concentration and can be ascribed to the fact that the oil had significantly dispersed into the water column.

5.6 CHEMICAL ANALYSIS

Total petroleum hydrocarbon (TPH) concentrations should also be considered in the experiment to better understand the overall toxic impacts of an oil spill. TPHs were detected in the water column of all experimental concentrations with high TPH levels observed in the 50 mg/kg concentration in both the water column and the sediments during the start and end of the experiment (**Table 4-5**).

The concentration of TPH ranged from 0.45 mg/l to 180 mg/l which is way higher than the concentration range of <0.01 mg/l to 7.6 mg/l reported by Tekere et al., 2016 in the study of carwash in South Africa, Gauteng Province. The results of the current studies implies that the crude oil spill of the 50mg/kg may results in environmental disaster as they possess high level of TPH that may be carcinogenic, environmentally persistent and toxic to the receiving environment and organisms. Edjere et al., 2020 indicated that when TPH enter an aquatic environment, they may remain in water or accumulate in organisms and migrate as water flows. Meanwhile, sediment acts as a local scale collector for environmental contaminants. TPHs adsorbed on the sediment would be retained in the sediment for a long time or will be released into the water column causing secondary pollution (Zhang et al., 2018). TPHs were found to be readily adsorbed onto particulate matter and settle to the bottom sediment, which ultimately acts as a reservoir for hydrophobic contamination (Rao et al., 2019).

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study which investigated the impacts of crude oil on a freshwater ecosystem was critical in establishing the impact of crude oil spillage research on the marine environment. This study therefore provided insights in terms of how freshwater ecosystems may respond.

The present study presents evidence that crude oil is very harmful to the survival of aquatic organisms in that the tested organisms used, showed mortality due to exposure to crude oil. *D. magna* and freshwater *Neocaridina* spp. were the most sensitive of the organisms and died within 24 hours of the experiment at all the concentrations. While *Physa* spp. and *L. Gibba* spp. survived at lower concentrations of 6,25mg/kg and 12,5 mg/kg, their survival was lower compared to the Control experiment. Organisms exposed to crude oil exposure experienced physical stress, and it was observed that *D. magna*, *Neocaridina* and *Physa* spp., were covered by oil. In the *L. Gibba* spp poor growth was observed in the concentrations 12,5mg/kg in terms of the reduced number of fronds and the colour of the fronds turned yellow compared to the Control. The roots were furthermore visibly covered by oil. The change of colour and dying fronds was a sign of stress. The test organisms in the Controls were visibly healthier as growth and reproduction were observed in the *D. magna*, *Neocaridina* and *Physa* spp. adults and neonates. Therefore, the oil concentrations of 6,25mg/kg, 12,5mg/kg, 25mg/kg and 50mg/kg are harmful to the *D. magna* and *Neocaridina* spp, while the oil concentrations of 25mg/kg and 50mg/kg are harmful to *Physa* and *L. Gibba spp*.

The tested organisms are key organisms in aquatic ecosystems being an essential link between primary production and many important fish species. Extinction of these organisms in the ecosystem due to crude oil spillage, will result in significant negative impacts in the ecosystem and will affect the structure and functioning of these systems. Also, ecosystem services such as removal of suspended solids and large quantities of fine-grained sediments in the water column will be impacted on as the filter feeder organisms (*D. magna*, *Neocaridina* and *Physa* spp.) responsible for this function would have been destroyed. *L. Gibba* spp. play an important role in aquatic ecosystems as it is a source of food for certain species and could

play an important role in the bioremediation of a contaminated system due to the uptake of pollutants. Therefore, oil spillages which may result from the proposed mining activities in the study area may result in severe environmental consequences as the area is surrounded by water courses and there are communities still depending on ecosystem services such as grass harvesting around the water resources.

6.2 RECOMMENDATIONS

The results of the study revealed that crude oil has impacted the tested freshwater organisms over a period of 42 days. Mortality (at 100%) of *D. magna* and *Neocaridina* spp. was observed and recorded within 24hrs of the experiment in all the crude oil concentration. At the same time, reduced toxicity was observed for *Physa* and *L Gibba* as the mortality was low for both organisms in the crude oil concentrations of 6,25mg/kg and 12,5mg/kg. Although, *Physa* and *L. Gibba* spp. survived in the two oil concentrations, signs of stress where observed which included slow movements of *Physa* spp, 2 jeveniles while *L Gibba* spp. had few yellow fronds, and which is the opposite as compared to the control. Based on the above, it is recommended that future studies should be conducted to examine the stress and resilient of the *Physa* and *L Gibba* spp. when exposed to crude oil.

There are many methods for treating and mitigating petroleum contaminated sites and include naturally (e.g., evaporation, photooxidation), physically (mechanical removal, booming, skimming, etc.), chemically (e.g., dispersants) and bioremediation (e.g., biostimulation, bioaugmentation, phytoremediation, microbially) (US EPA, 2001). The chemical and physical methods generally are expensive and often have limited effectiveness (Bayat et al., 2015). The main disadvantage of applying non-biological methods is that additional chemicals are required to increase removal, which escalates the total cost, making it difficult to follow in low-income countries (Marzan et al., 2017). Dave & Ghaly (2011) indicated several physical, chemical, thermal, and biological remediation technologies for oil spills, booms, skimmers, sorbents, dispersants, in situ burning and bioremediation. Each technique has its advantages and disadvantages, and the choice of a particular technique will depend on: type of oil, physical, biological and economic characteristics of the spill, location, weather and environmental conditions, amount spilt and rate of spillage, depth of water column, time of the year and effectiveness of the technique.

Therefore, the following treatment methods may be implemented to manage and treat the crude oil spillages at the Klippoortjie underground bunkers. Such treatments methods include the following:

- Use of sorbents materials that can absorb (or adsorb) the spilled oil are often used to collect oil, especially if the spill occurred on hard, and otherwise, non-absorbent surfaces. Mineral particulates (such as sand and soil mixtures) are inexpensive and can be easily applied, with straw bales also being a good option. Straw can be floated on water to collect small amounts of floating oil in dams or pans (Hill et al., 2016).

- The water pumped from the pit mixed with oil must be pumped to a dam or chambers for separation of water and oil using the oil skimming technique. The separated oil can be moved by a reputable company registered with the recycling oil saves environment foundation (ROSE) and this will ensure proper final disposal or management of crude oil waste.

- Use of conveyer belts to move oil contaminated coal from the pit to the plant. This will prevent coal trucks (articulated dump truck) to move coal out of the pit as it will increase the footprint of the crude oil contamination (Gudani Consulting, 2015).

- An emergency oil response plan must always be in place to ensure that major spillages into the aquatic environments are immediately stopped and controlled. An emergency response plan must include external service providers with expertise and technology on how to clean the crude oil spillages. Establish an internal emergency response plan consisting of trained personnel on how to clean the spillages.

- Water monitoring programme must be developed to monitor the possibility of oil migration into the nearby water resources including groundwater. This action will provide an indication whether the proposed measures are sufficient or not and what other actions must be put in place.

- Use of micro-organisms that have the ability to breakdown crude oil spillage could be considered. These microbes are widespread and often occur naturally at oil spill sites. The effectiveness of microbial biodegradation is dependent on four key factors, namely: nutrients, oxygen availability, ambient temperatures, and the extent of oiling. Adequate oxygen and nutrients are required by the microbes to allow them to degrade the oil, and the biodegradation process is enhanced by higher ambient temperatures. In contrast high oil concentrations inhibit biodegradation and often lead to anaerobic conditions and the eutrophication of waterbodies (Hill et al., 2016, Matara 2015).

- Investigate the restoration of nearby impacted wetlands and grow macrophytes that can remediate nutrients load, heavy metals and hydrocarbons. Macrophytes have been used extensively for the phytoremediation of pollutants. This is due to their invasive nature, wide distribution, simple structure and sporadic growth pattern and the ability to thrive in diverse habitats and tolerate high levels of contaminants in the environment (Ekperuse et al., 2020). The microcosm experiment showed that different crude oil concentrations have variable toxicity levels to freshwater organisms. Crude oil remained

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ANNEXURE A

Sample	DAYS	Replicate	Temperature (°C)	pH (–log10H+)	Electrical conductivity (EC) (µS/cm)	Dissolved oxygen (mg/L)	QUALITATIVE OBSERVATIONS						
							Water column	Sediment	Lemna	Daphnia	Shrimps	Snails	General
Control	7	1	22.9	8.59	232	6.61							
		2	22.8	8.51	240	7.01	Water column is clear	No algae on sediment	fronds increased and small Lemna starting to emerge	Original number of adults and neonates visible	Five shrimps present on sediment surface	All snails introduced visible and a few egg pockets are present on the side of the aquarium	Slight presence of algae on side of aquarium
		3	23.1	8.53	231	6.98							
50 mg/L		1	23.1	7.59	234								
		2	22.8	8.01	235		Water very turbid with thick layer of oil covering entire water surface	Oil layer covers sediment	No Lemna visible	No alive Daphnia visible	No alive shrimps visible	No alive snails visible	100% mortality of all test organisms introduced
		3	22.4	8.38	252								
25 mg/L		1	23.2	8.12	252								
		2	23.1	8.06	272		Water column very turbid with layer of oil on surface	Oil layer covers sediment	Few green Lemna fronds covered in oil visible. fronds appear small	No alive Daphnia visible	No alive shrimps visible	No alive snails visible	All the organisms died except for a few oil covered Lemna fronds that are visible
		3	22.7	8.47	258								
12.5 mg/L		1	22.3	8.41	239								
		2	23.0	8.26	260		Water column very turbid with some oil on surface	Oil layer covers sediment	Few green Lemna fronds covered in oil visible. fronds appear small	No alive Daphnia visible	No alive shrimps visible	Few snails visible with some egg pockets on airstone	Few Lemna fronds covered in oil are present as well as a few snails and some snail egg pockets. No shrimp or Daphnia visible
		3	22.6	8.19	250								
6.25 mg/L		1	23.1	8.32	271								
		2	23.2	8.35	239		Water column very turbid with some oil on surface	Thin layer of oil covers the bottom sediment with some algae starting to grow	Lemna fronds are mostly green with yellow fronds also visible. Lemna are not thriving and fronds appear small	No alive Daphnia visible	One shrimp adult visible	All introduced snails visible as well as egg pockets on air stones.	Lemna appear to be under stress with fronds yellowing. One shrimp and all the introduced snails as well as snail egg pockets are visible. 100% Daphnia mortality.
		3	21.9	8.58	241								
Control	14	1	23.1	7.66	209	6.55							
		2	23.0	7.79	211	7.01	Water column is clear	No algae on sediment	fronds increased and more small Lemna starting to emerge	Adults and neonates visible	Five shrimps present on sediment surface	All snails introduced visible and a few egg pockets are present on the side of the aquarium and on the airstone	Organisms and Lemna seem to be thriving.
		3	23.1	7.71	214	7.09							
50 mg/L		1	22.8	8.35	231								
		2	22.8	8.12	209		Water less turbid with thick layer of oil covering entire water surface	Some oil present on sediment	-	-	-	-	Although the water column is becoming slightly less turbid, a thick layer of oil covers the entire water surface area
		3	22.9	8.11	219								
25 mg/L		1	22.9	8.19	249		Water column very turbid, about 90% of surface water area covered in thick layer of oil.	Some oil on sediment with algae starting to form on the sediment	-	-	-	-	Water column very turbid; no more Lemna present.
		2	23.1	8.09	251								
		3	23.0	8.01	239								
12.5 mg/L		1	22.8	8.7	228		Water column is turbid and approximately 30% of water surface area covered in oil	Oil layer covers sediment	Very few green Lemna fronds covered in oil visible. fronds appear small	-	-	Few snails visible with some egg pockets on airstone	Water column is turbid; few snails and egg pockets are visible and Lemna is present but fronds are covered with oil.
		2	23.9	7.98	219								
		3	23.9	7.8	231								
6.25 mg/L		1	23.1	8.29	244		Water column is turbid and 10 to 15% of water surface area is covered with oil	Some oil present on sediment	Very few green Lemna fronds covered in oil visible. fronds appear small	-	-	Few snails visible with some egg pockets on airstone	Water column is turbid; few snails and egg pockets are visible and Lemna is present but fronds are covered with oil.
		2	22.8	8.03	251								
		3	22.8	8.01	259								
Control	21	1	23.2	7.69	218	6.65							
		2	22.8	7.59	224	7.11	Water column is clear	Some algae are visible on sediment	Lemna plants and fronds are increasing, long healthy roots are present.	More Daphnia adults and neonates are present	Five shrimps present on sediment surface	All snails introduced visible and a few egg pockets are present on the side of the aquarium and on the airstone	Organisms and Lemna seem to be thriving indicating a healthy microcosm.
		3	22.7	7.74	214	7.09							
50 mg/L		1	22.6	8.31	228		Water less turbid with layer of oil covering entire water surface. but appearing to be thinner	More oil seem to have settled on the sediment with some algae present	-	-	-	-	Water column is still turbid. however the layer of oil that covers the entire water surface area seem to become thinner.
		2	21.9	8.28	213								
		3	22.1	8.31	218								
25 mg/L		1	22.7	8.16	258		Water column is turbid and approximately 50 - 75% of surface water area is covered in oil.	Oil and debris settled on bottom of sediment	-	-	-	-	Water column is turbid and oil covering surface of water is becoming slightly less.
		2	22.4	8.03	259								
		3	23.0	8.00	245								
12.5 mg/L		1	22.5	8.33	239		Water column turbid and 10 to 15% of water surface is covered in oil.	Oil on sediment looks lumpy and as if it is degrading	2 to 5 Lemna plants present per replicate, however although the fronds are green, they are not increasing and fronds are very small compared to the Control.	-	-	Few snails are present. although very small compared to the snails in the Control. Some egg pockets are present on te airstone.	Water column still turbid with less oil covering the water surface; oil on the sediment is brown and appear to be degrading. Both Lemna and snails are much smaller compared to the Control and are not thriving.
		2	22.7	7.94	249								
		3	22.6	7.9	245								
6.25 mg/L		1	22.9	7.61	265		Water column is turbid with between 1 to 5 % oil covering the surface area. Oil have a lumpy appearance with oil sheen on surface	Oil on sediment looks lumpy and as if it is degrading. Algal film in and on sediment is increasing.	Lemna plants are present, but do not increase and fronds also don't increase. Roots are covered with oil.	-	-	Snails and eggs pockets present on airstone and ona sides of the aquarium.	Water column is turbid; few snails and egg pockets are visible and Lemna is present but fronds and roots have oil on.
		2	22.7	7.64	239								
		3	22.6	7.89	236								
Control	28	1	21.1	7.16	250.1		Water column is clear;	Some algae are visible on sediment	Lemna plants and fronds are increasing. Long healthy roots are present.	More Daphnia adults and neonates are present - increased reproduction	Five shrimps present on sediment surface as well as neonates	All snails introduced visible. several egg pockets are present on the side of the aquarium and on the airstone.	Organisms and Lemna seem to be thriving and are reproducing. indicating a healthy microcosm.
		2	21.2	7.32	251.8								
		3	21.2	7.23	244.5		Oil layer of ~ 0.5 cm thick covers entire water surface in replicate 2 and 3. In replicate 1 oil layer is slighty thinner. Oil appears less thich and more fluid. Oil is black. Water slightly turbid.						
50 mg/L		1	21.2	7.87	207.8			Sediment surface largely covered in oil with oil also present in sediment and is black in appearance	-	-	-	-	Thinner and more fluid oil covering entire water surface; water column less turbid; oil layer present on sediment surface and has settled in sediment itself. No organisms.
		2	21.2	7.91	211.2								
		3	21.3	7.89	213.5								
25 mg/L		1	21.2	7.95	233		Water column very turbid, especially replicate 2 - chocolate brown colour. 20 to 40% of surface is covered in oil.	Oil on sediment surface is black and lumpy.	2 fronds visible in two of the replicates - none in 3rd replicate.	-	-	-	Few Lemna fronds have survived, but in a very poor condition. No other organisms present.
		2	21.2	7.98	235.1								
		3	21.1	8.02	235.4								
12.5 mg/L		1	21.2	8.08	238.7		Water column quite turbid with very little oil (~ 10%) present on the water surface.	Sediment covered in oil which has a dark brown colour.	Very few lemna plants visible - roots covered in brown debris	-	-	Two adult snails observed and a few egg pockets which seem to detach from the side of the aquarium; no juvenile snails.	and only two adult snails that appear to be under stress (smaller than Control snails). Water column quite turbid, but less than 25 cm.
		2	21.3	8.01	236.3								
		3	21.2	8.04	237.2		Water column slightly turbid with < 5% oil covering the surface area.	Sediment laregly covered in oil.	Very few Lemna. very small fronds and no new plants and fronds visible. Roots slightly covered in brown debris.	-	-	Five big snails a very few juvenile snails present and egg pockets on the side of the aquarium and on the airstone.	Only lemna and snails present. Adult snails appear smaller than in Control. Signes of snail reproduction visible, but not Lemna.
6.25 mg/L		1	21.2	7.56	271.3								
		2	21.1	7.63	252.3								
		3	21.2	7.75	243.7								
Control	35	1	22.9	7.52	217		Water column clear with no visible signs of algae present	Some algae are visible on sediment	Lemna increased and fronds are healthy and big compared to lemna in experimental chambers.	Several daphnids adults and neonates visible	Five adult shrimps (signs of internal eggs present)	Various stadia of snails (adults and juveniles and egg pockets) present.	Organisms and Lemna seem to be thriving and are reproducing. indicating a healthy microcosm.
		2	23.9	7.29	222								
		3	22.8	7.78	231								
50 mg/L		1	22.8	7.82	261		Water column almost clear; 90% of water surface area covered with thin liquid layer of oil.	Sediment surface largely covered in oil with oil also present in sediment and is black in appearance.	-	-	-	-	No organisms present.
		2	23.8	7.93	257								
		3	22.7	7.91	252								
25 mg/L		1	23.9	7.89	255		Water column is turbid - light brown in colour; few oil lumps drift on water surface.	Sediment covered with lumpy black oil.	-	-	-	Very few snails present	Only snails present - no other organisms or Lemna.
		2	23.1	7.77	252								
		3	22.8	7.78	229								
12.5 mg/L		1	23.9	7.79	250		Water column very turbid - light brown colour.	Sediment covered in organic matter - oil appears to have broken down.	-	-	-	Few snails present (small in size)	Only snails present - no other organisms or Lemna.
		2	22.8	7.82	244								
		3	22.8	8.03	248								
6.25 mg/L		1	23.1	7.99	244		Water column slight turbid; no oil visible on water surface.	Sediment covered in organic type of matter - oil broken down.	Few Lemna with fronds present that are small to medium in size compared to the Control.	-	-	Snails of different sizes present, as well as egg pockets against the side of the aquaria.	No algae present in any of the aquaria.
		2	22.9	7.81	238								
		3	22.9	7.8	241								
Control	42	1	22.9	7.55	208		Water column is clear.	No visible algae in or on sediment.	Lemna proliferate and increase.	Daphnida proliferate and a number of adults and neonates are present.	Adult and small shrimps present.	Various sizes of snails as well as egg pockets are present. Snails proliferate.	Organisms and Lemna proliferate and juveniles and adult present.
		2	22.8	7.61	211								
		3	22.8	7.59	219								
50 mg/L		1	23.2	7.95	261		Water column in dbe replicate turbid while altogether clear in the other two replicates.	Sediment covered in black oil that seem to have broken down; some oil lumps are present.	-	-	-	-	No organisms present.
		2	23.1	7.91	259								
		3	23.8	7.85	249								
25 mg/L		1	22.9	7.89	241		Water column is turbid (light brown) with some oil on surface present - covers about 40% of the surface water.	Oil settled on sediment.	-	-	-	-	No organisms present.
		2	23.9	7.85	239								
		3	22.8	7.88	255								
12.5 mg/L		1	23.1	7.99	255		Water column is turbid (light brown) with some oil present on surface.	Some oil settled on surface of sediment.	Two small lemna fronds present.	-	-	Few snails as well as egg pockets present, but is covered in oil.	
		2	23.2	8.1	251								
		3	22.8	7.82	241								
6.25 mg/L		1	23.1	7.81	251		Water column slightly turbid - no oil on water surface.	Sediment covered in organic type of matter - oil broken down.	Few Lemna present, but fronds very small.	-	-	Snails of different sizes present, as well as egg pockets against the side of the aquaria.	
		2	22.9	7.79	244								
		3	22.9	7.85	246								
Composite samples taken at 49 days													
	49						Water column is clear with slight presence of algae visible.	Slight presence of alae observed on surface of sediment.	Healthy Lemna fronds (deep green colour)of various sizes over about 60% of water surface.	A number of Daphnia adults and neonates present.	Adult and few juvenile shrimps present.	A number of snails of various sizes and stages (especially small juveniles) present as well as a lot of egg pockets on the side of the aquaria.	Organisms and Lemna proliferate and various adults and juvenile are present.
Control		1	23.5	7.44	201.6								
							Water column is turbid with oil droplets suspended in the water column.	Sediment covered in black residue.	-	-	-	one to two very small snails visible that are covered in oily residue. Egg pockets visible, but are detaching from the side of the aquaria.	
50 mg/L		1	23.6	8.07	283								
							Water turbid with oil suspended in the residue in sediment which about 0.5 cm on top of sediment is covered in black matter.	Sediment covered in thick brown residue in sediment which about 0.5 cm on top of sediment is covered in black matter.	One Lemna front (yellow and small) present on surface, but covered on oil.	-	-	Three snails observed, covered in black matter.	
25 mg/L		1	23.6	7.9	285								
							Water column is turbid.	Brown matter covers sediment surface	Very few, small fronds that are light green in colour are present.	-	-	Very few snails (approximately 6 in total) are present, but are covered in black residue; egg pockets are present, but start to detach from the side of the aquaria.	
12.5 mg/L		1	23.4	8.18	318								
							Water column is clear with no oil residue on water surface present.	Black matter present on sediment and algae present in sediment.	Lemna fronds are deep green in colour, but cover approximately 5% of the water surface area. fronds are very small compared to the Control.	-	-	Snails of various sizes are present, as well as egg pockets, but both are covered in a black / grey matter.	
6.25 mg/L		1	23.5	7.96	247								