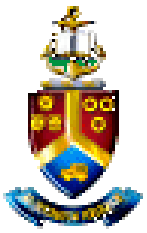

Evaluation of biotic succession in the Con Joubert Bird Sanctuary wetland after a vegetable oil spill

By

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- National Research Foundation for the funding.

DEDICATION

To my grandparents and my family.

DECLARATION

I declare that *Evaluation of biotic succession in the Con Joubert Bird Sanctuary wetland after a vegetable oil spill* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Mapurunyane Callies Selala

March 2013

Signed……

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ABBREVATIONS

APHA	**A**merican **P**ublic **H**ealth **A**ssociation
CSIR	**C**ouncil for **S**cientific and **I**ndustrial **R**esearch
DGGE	**D**enaturing **G**radient **G**el **E**lectrophoresis
DNA	**D**eoxyribo**n**ucleic **a**cid
DO	**D**issolved **o**xygen
DOC	**D**issolved **O**rganic **C**arbon
dNTPs	2'-**d**eoxy**n**ucleoside 5'-**t**ri**p**hoshate
Eh	redox potential
EMM	**E**kurhuleni **M**etropolitan **M**unicipality
EPA	**E**nvironmental **P**rotection **A**gency
et al.	**et al**ibi
H′	Shannon diversity index
mg/l	**m**illi**g**ram per **l**itre
ml	**M**illi**l**itre
mS/cm	**m**illi**s**iemens per **c**enti**m**etre
NCA	**N**ational **C**onservation **A**gency
NOAA	**N**ational **O**ceanic and **A**tmospheric **A**dministration
NWA	**N**ational **W**ater **A**ct
PCR	**P**olymerase **C**hain **R**eaction
RDA	**R**e**d**undancy **a**nalysis
SANBI	**S**outh **A**frican **B**iodiversity **I**nstitute
TAE (1x)	40 mM **T**ris-**a**cetate, 1mM **E**DTA, pH 8.0
TDS	**T**otal **d**issolved **s**ubstances
TE	10 m **T**ris-HCL, 1mM **E**DTA, pH 8.0
UNEP	**U**nited **N**ations **E**nvironment **P**rogramm**e**
UNESCO	**U**nited **N**ations **E**ducational, **S**cientific and **C**ultural **O**rganisation
UV	**U**ltra**v**iolet
µl	**M**icro**l**itre

µg/l	**M**icro**g**ram per **l**itre
v/v	**V**olume per **v**olume
WHO	**W**orld **H**ealth **O**rganization
WRC	**W**ater **R**esearch **C**ommission

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CHAPTER 1

Introduction

Wetland degradation has been ever increasing due to agricultural practice, urbanization and industrial development to the point where today it is estimated that 50 % of the wetlands in countries such as South Africa and United State are destroyed (van Dam *et al*., 1998; Dickens *et al*., 2003; Zhu *et al*., 2004). Pollution arising from oil spills happens frequently due to the need to transport oil, as well as oil-production refineries. These spills consequently have impacts on the environment by causing loss of species diversity, richness, habitat and destruction of breeding environment of aquatic organisms. Oil spills in wetlands directly affect the essential role that these systems play in ecosystems health and function (Mitsch and Gosselink, 2000). Increasing pressures on wetlands because of increasing anthropogenic pollutions, as well as scarceness of the water resources and increasing demand, has prompted many research bodies to turn their focus in wetlands studies (Turner *et al*., 2000).

The Con Joubert Bird Sanctuary wetland is a municipal reserve in Randfontein on the West Rand of Gauteng, South Africa (Fig. 1.1a). The sanctuary is approximately 30 ha wide and is comprised of a freshwater wetland with a maximum depth of 1.2 m (in the rain season) and marginal vegetation, which are dominated by *Phragmites australis* and *Typha capensis* (Fig. 1.1b) (Oberholster *et al*., 2010). The bird sanctuary provides a wide range of habitats from well established reed beds to shallow open water, patches of short emergent vegetation and mudflats (Fig. 1.1a). The sanctuary is bounded to the south and east by an industrial area.

Figure 1.1(a): The Con Joubert Bird Sanctuary freshwater wetland (Downloaded from google maps 16 October 2012).

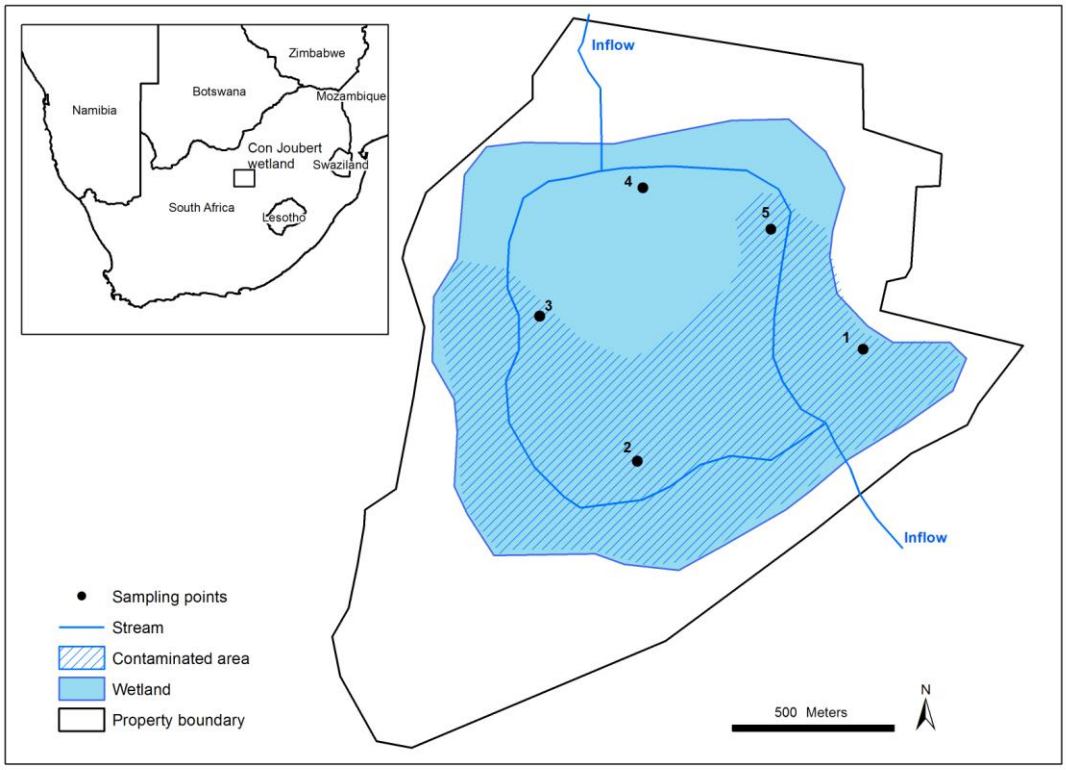


Figure 1.1(b): Map of the Con Joubert Bird Sanctuary freshwater wetland showing the location of five sampling sites and the stormwater inflow inlets. Insert shows the location of the map area in South Africa (Oberholster *et al*., 2010).

The Con Joubert Bird Sanctuary wetland can be classified as a transitional open freshwater wetland type with an open water zone (Morant, 1983). The water budget of the wetland area is governed by evaporation, precipitation and the inflow of stormwater inlets, making the wetland a low-energy budget aquatic system with reduced flushing especially during the dry season months April-August (Oberholster *et al*., 2010).

In September 2007, a spill of 250 ton sunflower oil occurred at a nearby vegetable oil production facility, when a sunflower oil storage tank collapsed. The spilled oil followed the stormwater drains from the facility into the wetland area. This oil spills pose a major

threat to ecological system of the wetland and to our knowledge is the latest vegetable oil spill in a freshwater wetland in the world (Oberholster *et al*., 2010).

Figure 1.1(c): Floating booms were used to respond to the oil spill in the Con Joubert Bird Sanctuary freshwater wetland.

The succession of biota in the Con Joubert Bird Sanctuary wetland after a vegetable oil spill using bioindicators and molecular tools was investigated, which enabled the determination of the adverse effects of the vegetable oil spill and the recovery of the wetland after pre-spill conditions. Therefore, the main objectives of this study were firstly, to determine the applicability of biostimulation by adding organic fertilizer to the freshwater wetland for remediation of the impacted aquatic ecosystems. Secondly, to

determine the effects and responses on biostimulation by aquatic organisms such as phytoplankton, macroinvertebrates, protozoan and microbial consortia within the water column and sediment of the selected pilot sampling sites before and after biostimulation. Thirdly, to determine the post spill wetland condition by using planktonic phytoplankton, macroinvertebrate assemblage and a battery of biotests using different aquatic organisms under controlled laboratory conditions and in the field.

For experimental approach, three selected sampling sites were used for the pilot study to determine the effectiveness of biostimulation as a remediation technique. Five different post spill sampling sites were selected for the battery of biotests, physicochemical parameters analysis, planktonic phytoplankton and macroinvertebrate assemblage over a period of one year. Water column and sediment samplings were performed on two monthly intervals over a period of one year period (23 October 2008 to 21 October 2009). All analyses were carried out within one week after sample collection. The water column and sediment samples were collected in triplicate using a grab bottle sampler (2 liters for each specific test) and sediment corer at all five sampling sites.

This thesis is a compilation of research chapters, in dependent of each other and each chapter addressing a specific aspect.

Outline of Chapters

Chapter 2

In Chapter 2, literature review evaluated wetland types in South Africa as well Ramsar site areas. The impacts of oil spill on aquatic biota, degradation and wetland recovery process.

Chapter 3

In Chapter 3, physicochemical parameters and succession of aquatic biota were used as a measure of the impact of the sunflower oil spill in the Con Joubert Bird Sanctuary freshwater wetland. A pilot study was performed at three selected sites in the vicinity of the stormwater inlet from where the vegetable oil entered the wetland. These sites were heavily contaminated by the spilled sunflower oil.

The objectives of this pilot-scale study chapter were (1) to determine the applicability of biostimulation to the contaminated freshwater wetland, by adding organic fertilizer to the wetland. (2) To determine the effect and responses of biostimulation on aquatic organisms such as planktonic phytoplankton, macroinvertebrates, protozoan and microbial assemblage within the water column and bottom sediment in each of the selected sites before and after biostimulation.

Chapter 4

In Chapter 4, the adverse effects of the sunflower oil spill on different aquatic organisms were evaluated and investigated. A battery of biotests was performed using aquatic biota from different trophic levels such as *Daphnia pulex*, *Spirodela punctata*, *Physa acuta* and *Raphidocelis subcapitata*. Exposure of the biota to different water samples contaminated with sunflower oil has focused on evaluating individual species under laboratory conditions mimicking environmental conditions. This method is known as "laboratory microcosm ". Microcosm is defined as laboratory model of a natural ecosystem in which certain environmental variables can be manipulated to observe the response. The model test results are not always applicable to an actual ecosystem because the microcosm is, of necessity, a simplified collection of selected physical, chemical, and biological ecosystem components (Vennie, 2007).

The objective of chapter 4 was to use survival, growth reduction and chlorophyll *a* and *b* concentrations as endpoints at different trophic levels. In this chapter, I also made use of field experimental data in addition to laboratory toxicity tests e.g. chlorophyll concentrations generated in the laboratory bioassay tests were compared with chlorophyll concentrations analyses from the field study over a period of one year.

Chapter 5

In Chapter 5, planktonic phytoplankton assemblage and biomass were used to determine the succession of planktonic phytoplankton over a period of one year after the oil spill. Both diversity and richness of planktonic phytoplankton community were measured to

determine wetland recovery. The study also studied the interrelationsphip between different planktonic phytoplankton genera and vegetable oil pollution.

The objectives of chapter 5 were: Firstly, to determine the response of planktonic phytoplankton genera as indicators of physicochemical water column change in the wetland over a period of one year. Secondly, to determine planktonic phytoplankton diversity and dominance, chlorophyll *a* (as surrogate phytoplankton biomass), and physicochemical parameters under post spill conditions. 3) To investigate if there was an increase in planktonic phytoplankton diversity, which serves as an indicator of the recovering of the phytoplankton communities in the wetland over the period of one year.

Chapter 6

In Chapter 6, aquatic macroinvertebrate organisms were used as bioindicators in order to assess and monitor toxicological response after the sunflower oil spill. The composition of these aquatic macroinvertebrates, as well as diversity and richness were used as a measure of recovery of the wetland. The interrelationships between pollutant tolerant and susceptible aquatic macroinvertebrates in the wetland are also discussed in detail.

The main aim of chapter 6 was to determine the temporal aquatic macroinvertebrate community structure changes at various sites within a wetland at the Con Joubert Bird Sanctuary after a sunflower oil spill. Through the information generated by this study, it endeavors to improve the current knowledge base of the aquatic environmental effects of vegetable oil spills in wetlands on macroinvertebrate assemblage.

In each chapter of this thesis physicochemical monitoring of the wetland were integrated with biological measures to assess the prevailing status of the wetland ecosystem. Generally, chemical monitoring assists in identifying the toxic components in the wetland, however by itself it is unable to provide adequate information on toxicity (van Dam *et al*., 1998).

Chapter 7

Chapter 7, summarizes the entire thesis.

References

Dickens, C., Kotze, D., Mashigo, S., MacKay, H. and Graham, M., (2003). Guidelines for integrating the protection, conservation and management of wetlands into catchment management planning. *WRC Report No. TT 220/03*, pp. 1-104.

Mitsch, W. J. and Gosselink, J. G., (2000). The values of wetlands landscape and institutional perspectives. The value of wetlands: importance of scale and landscape setting. *Ecol. Econ* **35**: 25-33.

Morant, P. D., (1983). Wetland classification: towards an approach for Southern Africa. *Limnol. Soc. South Afr*. **9**: 76-84.

Oberholster, P. J., Blaise, C. and Botha, A.-M., (2010). Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland. *Ecotoxicol*. **19**: 1426-39.

Turner, R. K., van den Bergh, J. C. J. M., Söderqvist, T., Barendregt, A., van der Straaten, J., Maltby, E. and van Ierland, E. C., (2000). Ecological-economic analysis of wetlands: scientific integration for management and policy. *Ecol. Economics* **35**: 7-23.

van Dam, R. A., Camilleri, C. and Finlayson, C. M., (1998). The potential of rapid assessment techniques as early warning indicators of wetland degradation: A review. *Environ. Toxicol. Water Quality* **13**: 297-312.

Zhu, X., Venosa, A. D., Suidan, M. T. and Lee, K., (2004). Guidelines for the bioremediation of oil-contaminated salt marshes. *US EPA. Cincinnati, OH* **45268**, pp. 1-61.

Vennie, J., (2007). Water-words glossary: North American Lake Management Society.

CHAPTER 2

Literature Review

Introduction

2.1. General characteristics and dynamics of wetlands

According to the South African National Water Act (Act 36 of 1998) a wetland is defined as land which is transitional between terrestrial and aquatic systems where the water table is usually at or near the surface, or the land is periodically covered with shallow water, and which under normal circumstances supports vegetation typically adapted to life in water saturated soil. Bunn *et al*. (1997) recognized the difficulty of defining wetlands because it tends to form intermediate zones along the margins while interacting significantly with terrestrial and aquatic ecosystems.

Semeniuk and Semeniuk (1995) stated that there is no general global definition applicable to the term "wetland", as well as classification thereof in practical or even achievable terms. However, for water body to be called a wetland, it should adhere to certain criteria to conform to a definition of the wetland. The environment should be permanently inundated (e.g. lake), seasonally inundated (e.g. creek) or seasonally waterlogged (e.g. dampland) by water table rise (Semeniuk and Semeniuk, 1995). The Ramsar Convention has adopted a generalized definition of the term "wetland" for international use which is define as an area of marsh, fen and peatland. The wetland might be natural or artificial, permanent or temporary, with water that is static or flowing, brackish or salty including areas of marine water with the depth, which at a low tide does not exceed six meters (Scott and Jones, 1995; van Dam *et al*., 1998, Ramsar Convention, 2002).

Winter (1988) reported that wetlands persist due to the lower permeability of the compact and humidified organic matter, together with underlying silts and clay soil that virtually restrict vertical movement of the water. Wetland classification indices that are based on geomorphic and hydrologic measurement of landforms were regarded as more consistent than any other criteria (Finlayson *et al*., 2002). However, wetlands physical framework forms various settings for their existence. The majority of wetlands occur in topographic depressions or in lowlands with minimal topographic slope. However it is also common to find wetlands on steep slopes, topographic hights, or drainage divides. In many inland wetlands, depression and minimal slopes could be attributed to slow permeability of soil, and thus contribute to the groundwater seepage. Groundwater modeling studies have indicated that water movement has an upward break in slope (Winter, 1988), which favours the existence of wetlands with no inlet or outlet channels. Figure 2.1 shows the characteristics of physical settings and hydrologic landform of various wetlands as set out by Guntensergen *et al*. (2002). Table 2.1 shows various wetland hydro-geomorphic types.

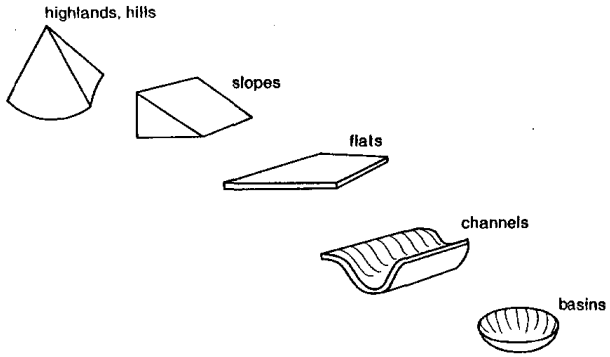
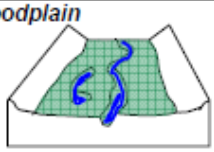
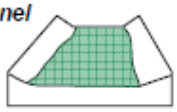
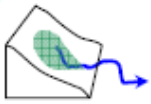


Figure 2.1: Types of geomorphic and hydrologic landforms that are hosts to wetlands (Guntensergen *et al*., 2002).

Ewart-Smith *et al.* (2006) define wetlands as inland systems which are permanently or periodically inundated or saturated and have no existing connection to the ocean. These were characterised by the complete absence of marine exchange and inter-tidal influence. All the wetlands described fall within the South African wetland perspective of international importance (Fig. 2.2). Ewart-Smith *et al*. (2006) distinguished between wetlands that are non-isolated and those that are hydrologically isolated in terms of their surface flow. Non-isolated systems are those that have a hydrological connection to a surface drainage network, whereas isolated systems are inland systems that are hydrologically isolated in terms of surface flows as described in the "Wetlands inventory for the Ekurhuleni Metropolitan Municipality (EMM) report, South Africa" (Naledzi Environmental Consultants cc, 2007). Hence, precipitation is an important water source and evapotranspiration an important output in all settings outlined in figure 2.2.

Table 2.1: Wetland hydro-geomorphic types typically supporting inland wetlands in South Africa adopted from the EMM wetlands inventory report (Naledzi Environmental Consultants cc, 2007).

Hydro-geomorphic types	Description	Source of water maintaining the wetland[1]	
		Surface	Sub-surface
Floodplain 	Valley bottom areas with a well defined stream channel, gently sloped and characterized by floodplain features such as oxbow depressions and natural levees and the alluvial (by water) transport and deposition of sediment, usually leading to a net accumulation of sediment. Water inputs from main channel (when channel banks overspill) and from adjacent slopes.	***	*
Valley bottom with a channel 	Valley bottom areas with a well defined stream channel but lacking characteristic floodplain features. May be gently sloped and characterized by the net accumulation of alluvial deposits or may have steeper slopes and be characterized by the net loss of sediment. Water inputs from main channel (when channel banks overspill) and from adjacent slopes.	***	*/ ***
Valley bottom without a channel 	Valley bottom areas with no clearly defined stream channel usually gently sloped and characterized by alluvial sediment deposition, generally leading to a net accumulation of sediment. Water inputs mainly from channel entering the wetland and also from adjacent slopes.	***	*/ ***
Hillslope seepage linked to a stream channel 	Slopes on hillsides, which are characterized by the colluvial (transported by gravity) movement of materials. Water inputs are mainly from sub-surface flow and outflow is usually via a well defined stream channel connecting the area directly to a stream channel.	*	***
Isolated Hillslope seepage 	Slopes on hillsides, which are characterized by the colluvial (transported by gravity) movement of materials. Water inputs mainly from sub-surface flow and outflow either very limited or through diffuse sub-surface and/or surface flow but with no direct surface water connection to a stream channel	*	***
Depression (includes Pans) 	A basin shaped area with a closed elevation contour that allows for the accumulation of surface water (i.e. it is inward draining). It may also receive sub-surface water. An outlet is usually absent, and therefore this type is usually isolated from the stream channel network.	*/ ***	*/ ***

Water source: * Contribution usually small
*** Contribution usually large

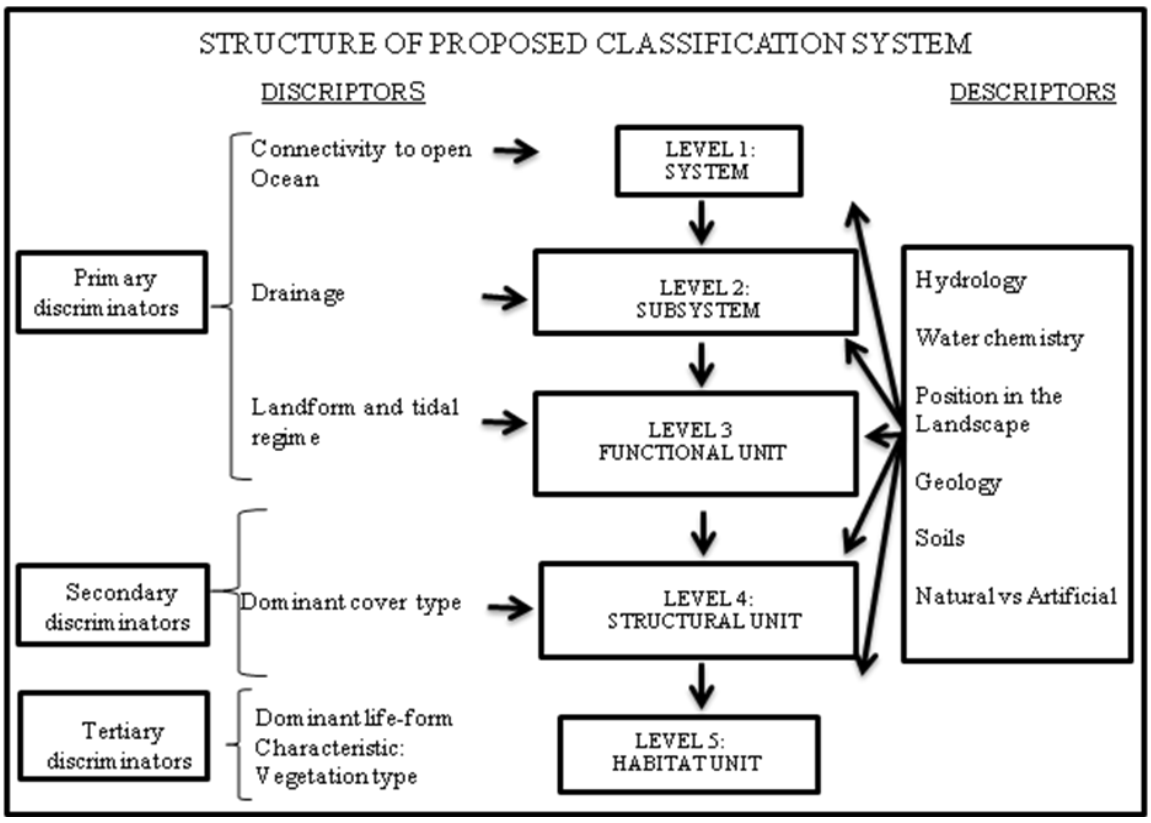


Figure 2.2: Wetland classification system structure (Ewart-Smith *et al*., 2006).

Freshwater wetlands are protected by the National Water Act, which recognized them as an integral part of the physical and biological aquatic ecosystem. Even though, the water from wetlands is not necessarily used for drinking water (Dickens *et al*., 2003). Previous studies reported an increase in man-made wetlands due to their use as wastewater treatment for petroleum oil removal (Greer *et al*., 2003). These man-made wetlands are used as many industrial sites to assist in absorption of harmful chemicals by aquatic vegetation and act as passive treatment options.

Wetlands function like “kidneys”, they filter water, reduces floodwater velocity, and therefore reduce the damage caused by floods, particularly soil erosion (US EPA, 2002). Since wetlands have diverse ecological attributes, they provide important ecosystem

services such as water storage, biogeochemical cycling and maintenance of biodiversity and biotic productivity (Wang *et al*., 2006).

2.2. Wetlands in South Africa

According to Dickens *et al*. (2003), wetlands world-wide constitute approximately 6 % of land surface and they are found in every climate zone, from the trophics to the frozen tundra. In South Africa alone, as described in a previous study by Breen and Begg (1989), almost 35-50 % of the wetlands were lost or severely destroyed due to unsustainable social and economic pressures where these ecosystems were viewed as excellent systems for water abstraction, drainage, grazing, sewage waste disposal, mining and cultivation. These natural water resources have been affected by anthropogenic activities such as infrastructure development, industrial effluents and urban sewage effluents (Oberholster *et al*., 2008).

South Africa being a water scarce country, experiences low rainfall volumes (average annual rainfall = 460 mm) and as a result wetlands receive a reduced volume of influent water. Ground water availability varies between seasons throughout the year and constitutes only 15 % of the total water resource of South Africa (Ashton, 2007). The loading capacity of wetlands varies seasonally, which is altered by the addition of nutrients and affects biological integrity during winter in temperate regions (Howard-Williams, 1985). Wetlands form an integral part of the water resources covered by the National Water Act (NWA) of South Africa (Dickens *et al*., 2003).

In South Africa, the majority of the existing freshwater wetlands are man-made, for example from road construction and other infrastructure developments that resulted in aquatic vegetation growth. According to the international body (Ramsar) responsible for wetland protection and conservation (Ramsar Convention, 2002), 17 South African natural wetlands have been designated in the list of national importance across the country (Fig. 2.3). The majority of these freshwater wetlands are situated in protected areas, which excluded them from high risk of human land use impacts. Two of the listed 17 wetlands are situated in National Parks, 12 in Provincial Game Reserves and the other two wetlands reside under the National Conservation Agency (NCA) while the remaining one is privately owned. Other known wetland areas, that are not listed in the Ramsar Wetland Classification are regarded as pans and depend mainly on the amount of seasonal rains available. This fits well into the Howard-Williams (1985) definition of wetland as "an area where the water table is at or above the land surface for a long enough period of time each year to promote the formation of waterlogged soils and to support growth of aquatic vegetation much of which is emerged". Wetland classification in the South African perspective was developed to ensure the potential extrapolation of results between sites and regions, as it would be difficult to monitor all wetlands in the country (Ewart-Smith *et al*., 2006). This has become a useful classification system since each country has got its own unique geographical landscape.

The South African National Biodiversity Institute (SANBI) (2010) stated that wetlands play a vital role in human health and well-being and thus should be protected. However, of the more than 114 000 wetlands that have been mapped all over the country, many are

either modified or destroyed due to human impact. To date, about 40 wetlands rehabilitation projects are in progress all over South Africa. This has provided thousands of people with employment opportunities and also made an impact on skills development. In 2009 alone, 95 wetlands in all nine provinces created employment for more than 1 500 people and made use of 250 small businesses (Rebelo and Gull, 2012).

One such wetland under rehabilitation is the Con Joubert Bird Sanctuary freshwater wetland located near Randfontein on the West Rand, Gauteng Province. This wetland is situated at lowland and is surrounded by an industrial area in the east and residential area in the west. This wetland occupies depressions in the landscape that are typically above the water table and the dynamics of the water table tends to be vertical in relation to water inputs and outputs (Lemly, 1997). The main source of the water supply comes from storm water through a manmade drainage system, which makes the wetland more vulnerable to contamination due to drainage stormwater inlets into the wetland. The constant presence of ground water in this wetland resulted in the growth of a variety of aquatic vegetation which attracted different bird species to this area (Oberholster *et al*., 2010). If evaporation-transpiration exceeds precipitation, depression wetlands tend to dry during the warm season if it depends only upon a groundwater source (Lemly, 1997).

Figure 2.3: South African wetlands of international importance (Dickens *et al*., 2003).

2.2.1. Main causes of wetlands degradation

The degradation and loss of wetlands have been scrutinized in recent years (van Dam *et al*., 1998). Broadly, wetlands loss and degradation have been attributed to the fact that the aquatic environments are freely utilized without consideration of their natural well-being (Gardiner, 1994). The five major causes of wetland degradation as identified by Bunn *et al*. (1997) were as follow: i) altered water regime, ii) habitat modification, iii) pollutants, iv) exotic species, and v) exploitation of biological products. The lists of causes also apply to the loss or degradation of wetlands in South Africa. Generally, human settlement, industrial expansion, agricultural practice, mining and horticulture, pose a serious threat to wetlands (van Dam *et al*., 1998).

2.2.2. Pollutant impacts on wetlands

Major pollutant types impacting on wetlands could be associated with anthropogenic activities from which they originate as summarized in Table 2.2. According to Bunn *et al*. (1997) processing and extraction of mineral operations produce large volume of pollutants such as lead, zinc, copper, arsenic, and cyanide that could have posed a threat to wetlands. In addition, oil manufacturing industries (vegetable and crude oil) also produce by-products that are associated with water pollution.

Table 2.2: A summary of human activities and major pollutants impacting on wetlands, and the types of pollutant sources (modified from van Dam *et al*., 1998**)**.

Anthropogenic activity	Major pollutants	Source
Mineral extraction and processing	Heavy metals, arsenic, cyanide	Point
Agriculture	Nutrients, insecticides, herbicide	Diffuse/point
Food production	Vegetable oils	Point
Oil exploration/transport/refining	Hydrocarbons, heavy metals, crude oil, oil products	Diffuse/point
Pulp and paper industry	Chlorinated compounds	Point
Boating/shipping	Hydrocarbons, antifoulants	Diffuse
Urbanization and related activities	Land and road runoff, sewage effluent	Point/diffuse

Regardless of the source of the pollutants, the entry of contaminants to the aquatic environment can be via point source or diffusion (nonpoint source). Adverse effects on wetland habits could be caused either indirectly or directly by the pollutant, or else via pollutant-induced alterations to the process linking to the habitats, such as changes in nutrient cycling and loss of migratory species (van Dam *et al*., 1998).

2.2.3. Early warning indicators of wetland degradation

Early warning indicators were defined as "the measurable biological, physical or chemical responses to a particular stress, preceding the occurrence of potentially significant adverse effects on the system of interest" (van Dam *et al*., 1998). This concept has been addressed in previous studies by Cairns and van der Schalie (1980); Cairns *et al*. (1993); McCormick and Cairns (1994) and van Dam *et al*. (1998). The characteristics of early warning indicators are summarized in Table 2.3.

Table 2.3: To have the potential to act as an early warning indicator, the indicator needs to respond as follows:

anticipatory	it should occur at levels of organisation, either biological or physical, that provide an indication of degradation, or some form of adverse effect, before serious environmental harm has occurred;
sensitive	in detecting potential significant impacts prior to them occurring, an early warning indicator should be sensitive to low levels, or early stages of the problem;
diagnostic	it should be sufficiently specific to a problem to increase confidence in identifying the cause of an effect
broadly applicable	it should predict potential impacts from a broad range of problems;
environmentally correlated effects/ecological relevance	an understanding that continued exposure to the problem, and hence continued manifestation of the response, would usually or often lead to significant environmental (ecosystem-level) adverse effects;
timely & cost-effective	it should provide information quickly enough to initiate effective management action prior to significant environmental impacts occurring, and be inexpensive to measure while providing the maximum amount of information per unit effort;
Regionally/nationally relevant	it should be relevant to the ecosystem being assessed;
socially relevant	it should be of obvious value to, and observable by stakeholders, or predictive of a measure that is socially relevant;
easy to measure	it should be able to be measured using a standard procedure with known reliability and low measurement error;
constant in space & time	it should be capable of detecting small change and of clearly distinguishing that a response is caused by some anthropogenic source, not by natural factors as part of the natural background (that is, high signal to noise ratio);
Non-destructive	measurement of the indicator should be non-destructive to the ecosystem being assessed;

2.3. Vegetable oil production and processing in South Africa

2.3.1. Production of the raw product

In the late eighties South Africa had ±16 edible oil processing plants, which produced approximately 250 000 tons of edible oil per year (Steffen *et al*., 1989). Accept for 1981, when a record in maize crop and oil seeds production (982 000 tons) enhances the industry's record braking production of 275 000 tons of oil (Dudrow, 1983). Sunflower oil remains a strong and sturdy product in South Africa, and a record sunflower seed crop of 872 000 tons was produced 2010, while the crop production was estimated at 900 000 tons for 2011 (The Baker, 2011). Other oils that are produced include canola oil (40 000 tons per annum), palm, peanut, rape seed and olive oil. Palm oil is emerging as the most popular oil in the food industry and from the baking industry to the fast food industry where it is used for frying, while peanut, rape seed and olive oil are also been used for baking (The Baker, 2011). Imports of soybean oil and palm oil have soared over the last decade, while the imports of sunflower oil and palm kernel oil have remained fairly stable. In 2007, about 60 000 tons of soft oil and palm products were imported each month. Despite the increased sunflower seed crop, South Africa did import 800 000 tons of cooking oil by 2011 to provide for the increasing consumer demand. Although there is plenty of suitable arable land available in South Africa for oilseed cultivation, farmers generally prefer to plant sunflower as a rotational crop, due to the higher yields of maize and wheat (The Baker, 2011). South African production remains small compared to the large producing areas in the international commercial market such as the EU, Ukraine and South America (The Baker, 2011). Different vegetable crop seeds are used for the

extraction of vegetable oil such as cottonseed, soya beans, linseeds, olives and coconuts. The use of these seed crops depends on the climatic conditions that favour their geographical production. For instance, in South Africa the common seed crops used for vegetable oil production are sunflower, groundnuts and maize. The other seeds such as cotton and soya are also processed for oil production. Although a large quantity of vegetable oil is produced for the local market, fish oil and some animal fat are also refined and imported to international markets (Steffen *et al*., 1989).

2.3.2. Vegetable oil production, water use and effluents from the oil processing industry

The industry collectively has eight crushing facilities and a crushing and refining capacity of over 90 000 tons/month, including cold pressers. Vegetable oil production involved two stages, namely oil extraction that happens in an oil mill and then the oil processing conducted at the refinery. These two production stages are performed in the same refinery plant with the exception of marine oil and animal fats. The resulting products are purchased as refined and used as they are. The end products from processed vegetable oil are liquid oil and are used for cooking and other commercial products. The other products manufactured from vegetables include margarine, mayonnaise, baker's fat and peanut butter (Steffen *et al*., 1989).

The vegetable oil refiner or processing plant releases around 35 % of water used in the beginning of process and the remaining 65 % evaporate through a series of cooling systems or are released as secondary by-products (Steffen *et al*., 1989). The effluents that are discharged from the refinery contain large quantities of oil, fats, sodium, sulphates,

phosphates and other toxic pollutants. These inorganic and organic compounds also formed part of the toxic effluents that pose a serious threat to municipal resources (Steffen *et al*., 1989).

Water consumption from these industries is estimated to be around 1.5 million cubic liters a year (Steffen *et al*., 1989). The primary water pollutants from an oil plant are oil and fat, which are associated with changes in oxygen demand of water ecosystems. The largest proportion of the effluent (oils and fats) discharged from a vegetable plant are attributed to 80 % of the refining operation (Steffen *et al*., 1989). Detergents used in the washing operations often emulsify oils and fats leading to high levels of effluents constituting mainly of fatty acids and acid water. The effluents containing high levels of fatty acids are subjected to further treatment before releasing it into the storm water drainage system to comply with established final effluent quality requirements as set by the US EPA (2000). The effluent quality guidelines for the edible oil industry as recommended volumes are as follow: BOD_5 (200 mg/l), suspended solids (200 mg/l), temperature (65.5 ^{o}C), pH (6.5) and oil (9 mg/l) and fats (100 mg/l) (US EPA, 2000).

2.4. Cases of impacts of vegetable and petroleum oil spills on the aquatic environment

Vegetable, petroleum and non-petroleum oil spills can cause substantial damage to the environment, and as a consequence of oil spills, macroinvertebrates, fish, benthic macroorganisms, aquatic plants and bird life could suffer from the contamination

(Mudge, 1999; Ji *et al*., 2007). In addition, the chemical composition and physical properties of these agents determines their fate in the environment (US EPA, 1997). Edible oils are perceived harmful to environmental ecosystems due to the various conditions such as temperature and physicochemical conditions prevailing in the affected environment (Pereira *et al*., 2002; Pereira *et al*., 2003b). Mudge (1995) and Pereira *et al*. (1998) suggested that the higher the demand for vegetable oil production, the greater increase the risk of accidental spillage during transportation. Although reported cases of vegetable spills are few, impact of these spills on the aquatic ecosystem can be devastating. Table 2.4 summarizes a few reported cases of previous vegetable oil spillages that occurred in different parts of the world, as well as the quantity of spilled oil in those incidences (Bucas and Saliot, 2002).

Table 2.4: Major vegetable oil spills history (modified from Bucas and Saliot, 2002)

Location	**Year**	**Amount of oil spill**	**Product oil**
Minnesota River (USA)	1962-1963	3 500-5 500 tons	Soyabean
Vancouver Harbour	1974-1978	5 tons	Soyabean & Rapeseed
Fanning Island	1975	10 000 tons	Palms and Coconut
Vancouver Harbour	1989	1.6 tons	Rapeseed
Vancouver Harbour	1991	1 500 tons	Sunflower
Lake Lanier (USA)	1994	20 tons	Soybean
Monterey Bay (USA)	1997	8 tons	Unknown vegetable
English Channel	1997	900 tons	Palmnut
Gulf of Mexico (USA)	1998	50 tons	Unknown vegetable
Vancouver Harbour	1998	unknown	Rapeseed
Hong-Kong	1998	400 tons	Rapeseed
Mississippi River	1998	460 tons	Palm
Vancouver Harbour	1999	200 tons	Rapeseed

If oil in general is spilled in an aquatic environment, fatty acids constituents may float on the water surface and become soluble or emulsify in the water column (Crum-Wiesner and Jennings, 1975; US EPA, 1997). The impact of vigorous wave action could break down oil slicks to small droplets and dispersed them, or may form water-in-oil emulsions with an oil content estimated at around ±30 %. These emulsions remain on the surface for considerable periods of time and when they aggregate, they form tar-balls that are sometimes carried ashore by the tide in marine environments. Emulsion droplets, if adsorbed onto particulates or detritus, which will end up on the bottom of the wetland (Tong *et al*., 1999), and eventually settles on the sediment as sludge, depending on the physical and chemical properties of the oil (US EPA, 1997).

2.5. Impacts of petroleum and non-petroleum oils

Spillages of petroleum and vegetable oils cause adverse environmental impacts directly or indirectly to freshwater wetland landscape ecosystems. Biological diversity could be used as a measure responding to the impact of oil onto the wetland ecosystem (Oberholster *et al*., 2010). Blaise *et al*. (2004) reported that vegetable oil spills into freshwater wetland is a cause of great concern because the structure and function of the ecosystem would be altered, and thus impact negatively on long term biodiversity. Wetland degradation has huge impacts on biological composition due to their rich diversity of macroinvertebrates, which indicate a change in food quality and physical composition in the water (Oberholster *et al*., 2008).

One of the common difficulties associated with impact assessment after an oil spill and interpretation of data obtained from a contaminated area is the lack of pre-spill data (i.e., site characterization and the absence of a reference site), which is key to the analysis of data from contaminated environments (Singer *et al*., 1996; Pezeshki *et al*., 2000). Fano *et al*. (2003) reported that chemical water quality analyses alone are inadequate to predict or reflect the condition of all aquatic resources which has let to the development of measures of biological integrity, expressed by biological indicators.

It is evident from previous studies that aquatic vertebrates and invertebrates are negatively affected by petroleum oil because it can remain in the wetlands for longer periods of time depending on the type of wetland and flow regime (Vinson *et al*., 2008). The effects of petroleum oil in particular depend on the type of oil because oil varies from refined to crude heavy oil that can not float on water. Heavy petroleum oil settles on the sediment and suffocates benthic organisms immediately after a spill.

The short-and long term toxicity of vegetable and petroleum oils on an aquatic ecosystem is the reduction in transpiration and carbon dioxide fixation that is harmful to wetland life (Mudge, 1995; Pereira *et al*., 2002; Shafir *et al*., 2007). Once the water body is contaminated with oil, dissolved oxygen (DO) in the water becomes depleted and aquatic life is threatened (Mudge, 1995; Campo *et al*., 2007). Oxygen depletion reduced oxygen exchange across the air water surface and below spilled oil or from the high biochemical oxygen demand (BOD) produced by microorganisms degrading the oil (Mudge, 1995; US EPA, 1997). Pezeshki *et al*. (2000) and Pereira *et al*. (2003a) showed that the

mechanisms of oxygen reduction in plants grown in petroleum oil contaminated wetlands are the direct result of stress that develops due to the blockage of stomata in the leaves. Petroleum hydrocarbons are also implicated in damaging the root membrane system of aquatic plants, which affects the ionic balance in aquatic plants and their ability to tolerate salinity (Pezeshki *et al*., 2000).

2.6. Bioassays and organism sensitivity to oil spills

The potential toxicity impacts of vegetable oil on the bottom sediment and water quality could be chronic and acute to the structure and function of natural aquatic ecological systems. Ingestion of large quantities of vegetable oil serves as a laxative and results in diarrhea and lipid pneumonia, which also decrease the ability of birds to escape predators (US EPA, 1997; Wincele *et al*., 2004). According to Mudge (1999) and Ji *et al*. (2007) vegetable oils lack acute toxicity compounds as compared to harmful aromatic hydrocarbons in crude and refined petroleum oils.

Hui (1996) and US EPA (1997) found that free fatty acids are among the products formed from vegetable oil in the environment and also found canola and sunflower oil as more acutely lethal at 96 hours test period than the 24 hours test period. Various forms of biota differ in their sensitivity to toxicants, and it is important that a bioassay test approach include species from different trophic levels during the assessment of adverse effects (Zhu *et al*., 2004). US EPA (1997) has been using the acute lethality tests to evaluate acute toxicity of corn oil, cotton oil and petroleum-derived mineral oil. The outcome of these tests showed that vegetable oil in water induce unfavourable conditions that are

toxic to macroinvertebrate fauna, phytoplankton, vertebrates and aquatic plants resulting in high mortality rates (Mudge, 1995; Pereira *et al*., 2002). These conditions are associated with the depletion in dissolved oxygen (below 4 mg/l) in the water column and have a potential impact on aquatic biodiversity (Campo *et al*., 2007).

2.7. Effects of spilled oil on wildlife

2.7.1. Birds

The aquatic organisms such as birds could be affected by oil spills (e.g. vegetable oil) in the contaminated water environment. The risks of vegetable oils are characterized by their lack of repugnant smell or variable colours to frighten wildlife away (Bucas and Saliot, 2002). These oils started fouling when undergoing chemical change or when in contact with organic matter in the water column, and thus impact wildlife. Oil slick of both petroleum and vegetable oil coat surfaces with which they come into contact with including plants, animals and man-made structures (Hartung, 1995; Wincele *et al*., 2004). Figure 2.4 shows a bird covered with oil residue at the Con Joubert Bird Sanctuary wetland, Randfontein (South Africa) after the vegetable oil in 2007. These oily residues result in the disruption of the structural arrangement of the birds feathers leading to a reduction of air entrainment and causing loss of buoyancy and thermal insulation (Frink and Miller, 1995; Hartung, 1995). Mortality of birds after being exposed to floating spilled vegetable oils can also have the consequence of the reduction of their ability to fly (Mudge, 1998; Ji *et al*., 2007).

Figure 2.4: An example of the impact of the vegetable oil spill on the bird population at the Con Joubert Bird Sanctuary wetland.

2.7.2. Fish communities

Fish absorbs oil from the water through their respiratory systems. In general, oil contaminants attach to the epithelial surface of fish, accumulate on gills and obstruct gaseous exchange (Mudge, 1995). These oils will not be removed from the fish's bodies as quickly as they are being received and so the poisonous substances (such as mercury and lead in crude oil) tend to build up, but fish mortalities are also associated with the smothering and clogging of their digestive tracts (Mudge, 1995).

In addition, toxic substances may accumulate in fish tissues that may be preyed upon by larger predators causing these substances to bioacculate in the food web. Concentrations of 0.3 mg/l or more of crude oil can cause toxic effects on fish communities (UNESCO/WHO/UNEP, 1992, 1996). Furthermore, an increase in BOD and oxygen depletion particularly in shallow waters where there is low water flow and dilution can lead to starvation of fish due to coating of their surfaces (US EPA, 1997).

2.7.3. Macroinvertebrate responses to oil contamination

The measurement of the response of macroinvertebrate community structure to point and non-point pollution is vital for impact assessment, which forms the basis of various aquatic biomonitoring methods (Cao *et al*., 1997; Thiere and Schulz, 2004). Poulton *et al*. (1997) and Barron *et al*. (1999) reported that threshold concentrations of approximately 500 µg/g petroleum oil residues is toxic to aquatic organisms. Macroinvertebrate taxons such as Trichoptera and Ephemeroptera were reported sensitive groups to petroleum oil aromatic hydrocarbons. However, epithelial and aqueous modes of oil exposure to various species are relatively unknown for aquatic macroinvertebrate (Poulton *et al*., 1997). According to Marques and Barbosa (2001) bioaccumulation of toxins can change the population composition and abundance of functional feeding groups. However very little is known from the literature on macroinvertebrates and vegetable oil spills.

2.7.4. Phytoplankton response to contamination

Phytoplankton organisms are primary photosynthetic, free-floating or attached organisms with vast ranges of shapes, size and pigments, structural complexities and life cycles.

Phytoplankton is sensitive to the slightest change in the aquatic environment and could be used as bioindicators of water quality (Megharaj *et al*., 2000; Willén, 2001), e.g. benthic phytoplankton can assist to regulate water quality in the wetland particularly with regard to phosphorus (P) and nitrate (N) loading from urban effluent (McCormick and Stevenson, 1998). Water with low nutrient concentrations usually supports minimal phytoplankton biomass with low species diversity (e.g. Chryophyceae and Cryptophytes) (Willèn *et al*., 1990). For instances, a highly eutrophic content of nutrients in the environment support high level of phytoplakton biomass growth mostly dominated by cyanobacteria and euglenophyceae (Padisak and Dokulil, 1994).

2.7.5. Mitigation techniques for wetlands with special reference to bioremediation

The conventional countermeasure methods after crude and vegetable oil spills include various physical, chemical and biological techniques. Physical methods include booming, skimming, manual removing, mechanical removal, water flushing, sediment relocation and tilling (Zhu *et al*., 2001; Shafir *et al*., 2007). Biological techniques include for example bioremediation.

Bioremediation is defined as the addition of nutrients to water resource that enhances the conversion of toxigenic compounds of oil to nontoxic products through the increase of microbial organisms. After spillage of any type of oil, oil floats on the water surface before the process of degradation starts (French-McCay, 2004). US EPA (1997) reported that the environmental factors such as pH, temperature, oxygen concentration, dispersal of oil, and the presence of other chemicals, bottom soil characteristics, nutrient quantities,

and diversity of various microorganisms at the location wherein the spill occurred influences biodegradation of oil in the freshwater. There are two types of bioremediation namely: (i) the addition of bacterial cells (bioaugmentation) to a freshwater wetland to supplement the existing microbial population, and (ii) addition of nutrients such as nitrate and phosphorus to stimulate natural microbial consortia. In the latter instance, the basic requirement for bioremediation is to add nutrients that would be in contact with the sediment. A schematic representation of remediation of floating vegetable oil spills by coagulant is shown by the model of Wincele *et al*. (2004) (Fig. 2.5). This technique of remediation is also faced with challenges such as the competition for nutrients between oil-degrading bacteria and non-oil degrading microbes; and predation of these microorganisms by protozoan. For example, a previous study by Oberholster *et al*. (2010) highlighted the effect of within taxa competition, e.g. *Chlamydomonas africana* that out-compete other phytoplankton species and become dominant in the oil polluted environmental water.

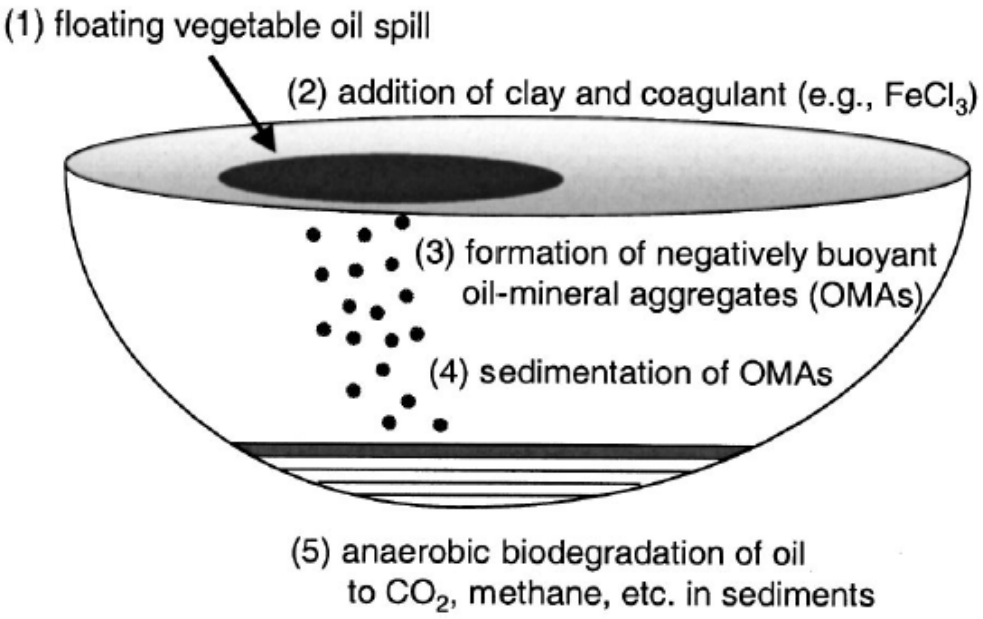


Figure 2.5: Schematic representation model for remediation of a vegetable oil spill by clay oil flocculation (Wincele *et al*., 2004).

Pereira *et al*. (1998) demonstrated the role of aerobic and anaerobic bacteria in the degradation of vegetable oils through the observation of the variations between linseed and sunflower oil degradation rates. The authors showed that aerobic bacteria are also able to assist in the reduction of vegetable oils in the presence of anaerobic conditions. According to Sueiro *et al*. (2011) the presence of sulphate reducing bacteria is indicative of the reduction of sunflower oil to oil by-products. Howarth (1993) reported that sulphate reducing bacteria can't breakdown and utilize pure oil for growth, but can only use the end products of fermentation. These sulfate-reducing bacteria slowly degrade materials that are rich in cellulose in anaerobic environments such as seawater, sediment, or water rich in decaying organic material.

Bioremediation with fertilizer application (i.e. addition of fertilizers into the polluted environment) as means to biostimulate freshwater wetlands after an oil spillage would promote growth of indigenous plants and their associated microbial population (Greer *et al*., 2003). This stimulation results in an increase in metabolic activity and the potential to degrade oil.

2.8. Microbial consortium response to oil contamination

Microbial biomass is an important component in pristine or contaminated water ecosystems and also vital for sediment health (Megharaj *et al*., 2000; Venosa and Zhu, 2003). Naturally, oils form a layer on the water surface a few days after a spill occurred, which is biodegraded by aerobic bacteria in surface water (Greer *et al*., 2003). These bacteria utilize free fatty acids that they use as carbon source. Sedimentation of larger volumes of oil creates anaerobic aquatic environmental conditions by blocking oxygen access to microbial organisms that normally will degrade vegetable oil (Foght *et al*., 1998; Wincele *et al*., 2004; Li *et al*., 2005). However, previous studies by Li *et al*. (2005) have showed that high levels of vegetable oil spillage cause reduction of microbial biomass in the freshwater sediment.

2.9. References

Ashton, P., (2007). Riverine biodiversity conservation in South Africa: current situation and future prospect. *Aquatic Conserv: Mar. Freshwater Ecosyst*. **17**: 441-445.

Barron, M. G., Podrabsky, T., Ogle, S. and Ricker, W. C., (1999). Are aromatic hydrocarbons the primary determinant of petroleum toxicity to aquatic organisms? *Aquatic Toxicol.* **46**: 253-268.

Blaise, C., Gagné, F., Chévre, N., Harwood, M., Lee, K., Lappalainen, J., Chial, B., Persoone, G. and Doe, K., (2004). Toxicity assessment of oil-contaminated freshwater sediments. *Environ. Toxicol.* **19**: 267-273.

Breen, C. M. and Begg, G. W., (1989). Conservation status of southern African wetlands. In: Biotic diversity in southern Africa: concepts and conservation, ed. B. J. Huntley. Oxford University Press, Cape Town, South Africa, pp. 254-263.

Bucas, G. and Saliot, A., (2002). Sea transport of animal and vegetable oils and its environmental consequences. *Mar. Poll. Bull.* **44**: 1388-1396.

Bunn, S. E., Boon, P. I., Brock, M. A. and Schofield, N. J., (1997). National wetlands R&D program: Scoping review. Occasional paper 01/97, Land and water resources research and development corporation, Canberra: pp. 1-29.

Cairns, J. and van der Schalie, W. H., (1980). Biological monitoring Part I-Early warning systems. *Water Res.* **14**: 1179-1196.

Cairns, J., McCormick, P. V. and Niederlehner, B. R., (1993). A proposed framework for developing indicators of ecosystem health. *Hydrobiologia* **263**: 1-44.

Campo, P., Zhao, Y., Suidan, M. T., Venosa, A. D. and Sorial, G. A., (2007). Biodegradation kinetics and toxicity of vegetable oil triacylglycerols under aerobic conditions. *Chemosphere* **68**: 2054-2062.

Cao, Y., Bark, A. W. and Williams, W. P., (1997). Analysing benthic macroinvertebrate community changes along a pollution gradient: a framework for development of biotic indices. *Water Res*. **31**: 884-892.

Crump-Wiesner, H. J. and Jennings, A. L., (1975). Properties and effects of nonpetroleum oils. In "Pro. of 1975 conference on prevention and control of pollution". American Petroleum Institute, Washington, DC, pp. 29-32.

Dickens, C., Kotze, D., Mashigo, S., MacKay, H. And Graham, M., (2003). Guidelines for integrating the protection, conservation and management of wetlands into catchment management planning. *WRC Report No. TT 220/03*, pp. 1-104.

Dudrow, F. A., (1983). Deodorization of edible oil. *J. Amer. Oil Chem. Soc.* **60**: 224-226.

Ewart-Smith, J. L., Ollis, D. J., Day J. A. and Malan, H. L., (2006). National Wetland Inventory: development of a wetland classification system for South Africa. *SA WRC Report* No. KV 174/06.

Fano, E. A., Mistri, M. and Rossi, R., (2003). The ecofunctional quality index (EQI): a new tool for assessing lagoonal ecosystem impairment. *Estuar. Coast. Shelf Sci.* **56**: 709-716.

Finlayson, C. M., Begg, G. W., Howes, J., Davies, J., Tagi, K. and Lowry, J., (2002). A Manual for an Inventory of Asian Wetlands: Version 1.0. Wetlands International Global Series 10, Kuala Lumpur, Malaysia, pp. 1-87.

Foght, J., Semple, K., Westlake, D. W. S., Blenkisopp, S., Sergy, G., Wang, Z. and Fingas, M., (1998). Development of a standard bacterial consortium for laboratory efficacy testing commercial freshwater oil spill bioremediation agents. *J. Ind. Microbiol. Biotechnol.* **21**: 322-330.

French-McCay, D. P., (2004). Oil spill impact modelling: Development and validation. *Environ. Toxicol. Chem.* **23**: 2441-2456.

Frink, L. and Miller, E. A., (1995). Principles of oiled bird rehabilitation. Wildlife and oil spills. *Tri-State Bird Rescue and Res*, Inc., Newark, DE, pp. 61-68.

Gardiner, J., (1994). Pressures on wetlands. Edited by Falconer, R. A and Goodwin, P. *Wetland Manage.* pp. 47-74.

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

Guntensergen, G. R., Peterson, S. A., Leibowitz, S. G. and Cowardin, L. M., (2002). Indicators of wetlands condition for the prairie pothole region of the United States. *Environ. Monit. Assess*.**78**: 229-252.

Hartung, R., (1995). Assessment of the potential for long term toxicological effects of the Exxon Valdez oil spill on birds and mammals. In "Wells, P. G., Butler, J. N and Hughes, J. S. (eds), Exxon Valdez Oil Spill". *Fate and Effects in Alakan Waters. ASTM, Philadelphia, PA*, pp. 693-725.

Howarth, R. W., (1993). Microbial processes in salt-marsh sediments. In "Ford, T. E., (eds), Aquatic Microbiology. An ecological approach". Blackwell Scientific Publications, Oxford, pp. 239-260.

Howard-Williams, C., (1985). Cycling and retention of nitrogen and phosphorus in wetlands: a theoretical and applied perspective. *Freshwater Biol*. **15**: 391-431.

Hui, Y. H., (1996). Bailey's industrial oil and fat products, edible oil and fat products:Gennral application. John Wiley and Sons, Inc., New York 1: fifth edition, pp. 1-280, 397-439.

Ji, G., Sun, T. and Ni, J., (2007). Impact of heavy oil-polluted soils on reed wetlands. *Ecol. Eng.* **29**: 272-279.

Lemly, A. D., (1997). Risk assessment as an environmental management too: Considerations for freshwater wetlands. *Environ. Manage.* **21**: 343-358.

Li, Z., Wrenn, B. A. and Venosa, A. D., (2005). Anaerobic biodegradation of vegetable oil and its metabolic intermediates in oil-riched freshwater sediments. *Biodegrad.* **16**: 341-352.

Marques, M. M. and Barbosa, F., (2001). Biological quality of waters from an impacted tropical watershed (middle Rio Doce basin, southeast Brazil), using benthic macroinvertebrate communities as an indicator. *Hydrobiol*. **457**: 69-76.

McCormick, P. V. and Cairns, J., (1994). Algae as indicators of environmental change. *J. Appl. Phycol.* **6**: 509-526.

McCormick, P. V. and Stevenson, R. J., (1998). Periphyton as a tool for ecological assessment and management in the Florida Everglades. *J. phycol.* **34**: 726-733.

Megharaj, M., Singleton, I., McClure, N. C. and Naidu, R., (2000). Influence of petroleum hydrocarbon contamination on microalgae and microbial activities in a long term contaminated soil. *Arch. Environ. Contam. Toxicol*. **38**: 439-445.

Mudge, S. M., (1999). Shoreline treatment of spilled vegetable oils. *Spill Sci. Technol. Bull.* **5**: 303-304.

Mudge, S. M., (1998). Vegetable oil spills-pollution or over-cautiousness. *Chem. Ecol.* **14**: 259-263.

Mudge, S. M., (1995). Deleterious effects from accidental spillage of vegetable oils. *Spill Sci. Technol. Bull.* **2**: 187-191.

Naledzi Environmental Consultants cc prepared for Ekurhuleni Metropolitan Municipality (EMM)., (2007). Identification, classification, assessment and delineation of wetlands within Ekurhuleni Metropplitan Mucipality. *EMM Wetland Inventory Report: May 2007*, pp 1-92.

Oberholster, P. J., Blaise, C. and Botha, A.-M., (2010). Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland. *Ecotoxicol*. **19**: 1426-39.

Oberholster, P. J., Botha, A.-M. and Cloete, T. E., (2008). Biological and chemical evaluation of sewage water pollution in the Rietvlei nature reserve wetland area, South Africa. *Environ. Poll.* **156**: 184-192.

Padisak, J. and Dokulil, M., (1994). Contribution of green algae to the phytoplankton assemblage in large, turbid shallow lake (Neusiedlersee, Austria/Hungary). *Hydrobiol.* **49**: 571-579.

Pereira, M. G., Mudge, S. and Latchford, J., (1998). Bacterial degradation of vegetable oils. *Chem. Ecol.* **14**: 291-303.

Pereira, M. G., Mudge, S. and Latchford, J., (2002). Consequences of linseed oil spills in salt marsh sediment. *Mar. Poll. Bull.* **44**: 520-533.

Pereira, M. G., Mudge, S. and Latchford, J., (2003a). Vegetable oils pills on salt marsh sediments: comparison between sunflower and linseed oils. *Mar. Environ. Res.* **56**: 367-385.

Pereira, M. G., Mudge, S and Latchford, J., (2003b). Polymerisation versus degradation of sunflower oil spilled in the marine environments. *Mar. Poll. Bull.* **46**: 1078-1081.

Pezeshki, S. R., Hester, M. W., Lin, Q. and Nyman, J. D., (2000). The effects of oil spill and clean-up on dominant US Gulf coast marsh macrophytes: a review. *Environ. Poll.* **108**: 129-139.

Poulton, B. C., Finger, S. E. and Humphry, S. A., (1997). Effects of a crude oil on the benthic invertebrate commnuty in the Gasconade River, Missouri. *Arch. Environ. Contam. Toxicol.* **33**: 268-276.

Ramsar Convetion., (2002). The Ramsar Strategic Plan 2003-2008, Ramsar Convention on wetlands. (htt/http://www.ramsar.org/key%20strat%20plan%202003%20e.htm; 10/03/2009).

Rebelo, A. and Gull, G., (2012). Policy brief asset tips 4-urban water use March 2012. http://www.tips.org.za/files/policy_brief_asset_research_tips_4_urban_water_use_march _2012.pdf, pp 1-4.

SANBI, (2010). Working for wetlands. http://wetlands.sanbi.org/resource.php?id=254.

Scott, D. A. and Jones, T. A., (1995). Classification and inventory of wetlands: A global overview. *Plant Ecol.* **118**: 3-16.

Semeniuk, C. A. and Semeniuk, V., (1995). A geomorphic approach to global classification for inland wetlands. *Plant Ecol.* **118**: 103-124.

Shafir, S., van Rijn, J. and Rinkevich, B., (2007). Short and long toxicity of crude oil and oil dispersants to two representative coral species. *Environ. Sci.Technol*. **41**: 5571-5574.

Singer, M. M., George, S., Jacobson, S., Lee, I., Weetman, L. L., Tjeerdema, R. S. and Sowby, M. L., (1996). Comparison of acute aquatic effects of the oil dispersant Corexit 9500 with those Corexit series dispersants. *Ecotoxicol. Environ. Safety* **35**: 183-189.

Steffen., Robertso. and Kirsten Inc., (1989) "prepared for the Water Research Commission". Water and waste-water management in the edible oil industry. SA WRC *Project NO.145*: *TT 40/89*, pp. 1-51.

Sueiro, R. A., Garrido, M J. and Araujo, M., (2011). Mutagenic assessment of Prestige fuel oil spilled on the shore and submitted to field trials of bioremediation. *Sci. Total Environ*. **409**: 4973-4978

The Baker (2011) Vegetable oil production. [htt://www.thebaker.co.za/ad_ vol13no8fats.html; 16/05/2011.]

Thiere, G. and Schulz, R., (2004). Runoff-related agricultural impact in relation to macroinvertebrate communities of the Lourens River, South Afica. *Water Res.* **38**: 3092-3102.

Tong, S.L., Goh, S. H., Abdulah, A. R., Tahir, N. M. and Wang, C.W., (1999). ASEAN Marine water quality criteria for oil and grease. *Cooperative Programme Mar. Sci:*1-28.

UNESCO/WHO/UNEP., (1992, 1996). Water quality assessments- a quide to use of biota, sediments and water in environmental monitoring, 2nd edition. In "Chapter 5-the use biological material" (eds) by Friedrich, G., Chapman, D and Beim, A.

US EPA, (2002). Short term methods for estimating the chronic toxicity of Effluents and receiving waters to freshwater organisms, pp. 1-335.

US EPA., (2000). Environmental assessment of final effluent limitations guidelines and standards for synthetic-based drilling fluids and other non-aqueous drilling fluids in the oil and gas extraction point source category, pp. 1-184.

US EPA., (1997). Oil pollution prevention; non-transportation related onshore facilities. *Federal Register* **62**: 54508-54543.

van Dam, R. A., Camilleri, C. and Finlayson, C. M., (1998). The development of potential rapid assessment techniques as early warning indicators of wetland degradation: A review. *Commonwealth of Australia*: 297-312.

Venosa, A. D. and Zhu, X., (2003). Biodegradation of crude oil contamination marine shorelines and freshwater wetlands. *Spill Sci. Technol. Bull.* **8**: 163-178.

Vinson, M. R., Dinger, E. C., Kotynek, J. and Dethier, M., (2008). Effects of oil pollution on aquatic macroinvertebrate assemblages in Gabon wetlands. *Afr. J. Aquat. Sci.* **33**: 261-268.

Wang, Y.-K, Stevenson, R. J., Sweets, P. R. and DiFranco, J., (2006). Developing and testing diatom indicators for wetlands in the Casco Bay watershed, Maine, USA. *Hydrobiologia* **561**:191-206.

Willén, E., Hajdu, S. and Pejler, Y., (1990). Summer phytoplankton in 73 nutrient poor Swedish lakes. *Limnologica* **20**: 217-227.

Willén, E (2001). Phytoplankton and water quality characterization: experiences from the Swedish large lakes Mälaren, Hjälmaren, Vätten and Vänern. *Ambio.* **30**: 529-537.

Wincele, D. E., Wrenn, B. A. and Venosa, A. D., (2004). Sedimentation of oil-mineral aggregates for remediation of vegetable oil spills. *J. Environ. Eng*. **130**: 50-58.

Winter, T. C., (1988). Conceptual framework for assessing cumulative impact on the hydrology nontidal wetlands. *Environ. Manage.* **12**: 605-620.

Zhu, X, Venosa, A. D., Suidan, M. T. and Lee, K., (2004). Guidelines for the bioremediation of oil-contaminated salt marshes. *US EPA. Cincinnati, OH* **45221**, pp. 1-4

Zhu, X., Venosa, A. D., Suidan, M. T. and Lee, K., (2001). Guidelines for the marine shorelines and freshwater wetlands. *US EPA. Cincinnati, OH* **45268**, pp. 1-136.

CHAPTER 3

A pilot study: Responses of selected biota after biostimulation of a vegetable oil spill in the Con Joubert Bird Sanctuary wetland.

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3.1. Introduction

Con Joubert Bird Sanctuary wetland can be classified as a transitional open freshwater wetland type with a transitional open water zone (Morant, 1983). The water budget of the wetland is governed by evaporation, precipitation and the inflow of stormwater inlets, making the wetland a low-energy budget wetland with reduced flushing especially during the dry season. This wetland served as habitat for of 250 different bird species, amongst these nearly 400 Greater flamingos. In September 2007 a spill of 250 ton vegetable oil occurred in a nearby vegetable oil production facility when an old vegetable oil storage tank collapsed. The spilled vegetable oil followed the stormwater drains into the adjacent Con Joubert Bird Sanctuary wetland area. Inflatable booms were used to isolate the contaminated area of vegetation from the open water zone to prevent further contamination, while free oil between reeds and vegetation on the eastern side of the wetland was collected by means of absorbent material and inflatable booms (Oberholster *et al*., 2010).

Vegetable oils lack the acute toxicity components that are present in petroleum and its refinery products such as aromatic and hydrocarbons due to its long persistence in the sediment (Mueller *et al*., 2003; Li *et al*., 2005). However, non petroleum products, including vegetable oils and animal fats, share common physical properties in water and produce similar environmental effects (US EPA 1997), such as insolubility, low density, low specific gravity and high viscosity relative to water (Campo *et al*., 2007). These behavioral characteristics of oils in water lead to the formation of an oily layer on the

surface of the water that results in a decrease in dissolved oxygen and increase in biochemical oxygen demand (BOD). This depletion of dissolved oxygen negatively impacts aquatic ecosystems, which is exacerbated by high BOD when microbial biodegradation occurred (Groenewold *et al*., 1982). The extent of the damage to the aquatic system is dependent on the chemical composition of the oil (Poulton *et al*., 1997). From the literature, it is evident that spilled vegetable oil has a huge impact on sensitive aquatic organisms and wild fauna such as birds (Mudge, 1995; Bucas and Saliot, 2002), and the immediate effects include coating of bird feathers and animals' fur (US EPA, 1997).

Vegetable oil degradation and reduction of toxicity can be achieved through bioremediation, *i.e*. by the addition of organic nutrients to a wetland. Conventional methods such as excavation and mechanical removal of oil from the water often increases ecological damage by destroying vegetation and natural wetland biota habitat (Mueller *et al*., 2003). Bioremediation, on the other hand increase microbial activity with little physical/mechanical impact on the treated area (Venosa *et al*., 1996; Shuhong *et al*., 2006). Balba *et al*. (1998) and Shuhong *et al*. (2006) demonstrated that bioremediation is a cost-effective and environmentally friendly methodology depending on the optimal and limiting factors required in the freshwater wetland's ecosystem. However little is known about the effects of biostimulation on protozoa, macroinvertebrates and phytoplankton as well as the bacterial assemblage.

Most of the microorganisms responsible for vegetable oil degradation in freshwater sediment are anaerobic bacteria (Pereira *et al*., 2002; Li *et al*., 2006). However, extremely high concentrations of vegetable oil present in the sediment may reduce biodegradation and this could be relieved by the presence of ferric hydroxide (Li *et al*., 2005). According to Mudge (1999) and Li *et al*. (2005) vegetable oils are transformed to methane and carbon dioxide by a complex of microbial consortia involving several groups of microorganisms. The ester bonds of vegetable oil can be hydrolyzed to yield free fatty acids and glycerol. Hydrolysis of vegetable oil is initiated by enzymatic activity of triglycerides to glycerol and long fatty acids, which serves as the growth substrates for several members of the microbial consortia (Aluyor *et al*., 2009; Dutta *et al*., 2009).

Bioremediation, however can also have a negative impact on a targeted area, since results are dependent upon the prevailing environmental physical/chemical status, as the addition of organic nutrients to on already autotrophic environment could further damage the wetland (Mueller *et al*., 2003). The addition of nutrients would cause an increment of primary producers such as algae and other phytoplankton that would produce high concentrations of nuisance toxic compounds to macroinvertebrate and vertebrate species (Oberholster *et al*., 2010).

Wetland restoration is defined as the returning of pre-disturbed wetland conditions and efforts to restore the biodiversity and biological functioning of the ecosystem (Winter, 1988). There is limited information available on the impact of vegetable oils spilled on freshwater wetlands, and most of the studies on abiotic and biotic factors that control

freshwater ecosystem responses to vegetable oil contamination come from marine environments (Poulton *et al*., 1997). Because of the difficulties associated with restoration and the lack of pre-spill data on the wetland (Oberholster *et al*., 2010), a decision was made to conduct a pilot study before full-scale bioremediation of the wetland. A pilot-study presents a series of steps to be followed that assists in designing a monitoring program to be undertaken in wetlands before full-scale restoration. The objectives of this pilot-scale study were (1) to determine the applicability of biostimulation to the contaminated freshwater wetland, by adding organic fertilizer to the wetland. (2) To determine the effect and responses of biostimulation on aquatic organisms such as phytoplankton, macroinvertebrates, protozoan and microbial assemblage within the water column and sediment in each of the selected sites before and after biostimulation.

3.2. Materials and methods

3.2.1. Study area description

This study was conducted on the Con Joubert Sanctuary Bird area (24.69 ha), located near Randfontein, South Africa. The 12.96 ha wetland has a maximum depth of 1.2 m (during the rainy season) and is dominated by the following emergent macrophytes vegetation (*Typha capensis*, *Schoenoplectus brachyceras*, *Phragmites australis*, *P. mauritianus* and *Persicaria lapathifolia*) and free-floating plants (*Azolla pinnata*, *Spirodela* spp. and *Wolffia arrhiza*).

3.2.2. Site selection for pilot study

Due to the lack of an uncontaminated pre-spill site or pre-pilot data, it was difficult to correlate data generated from this study with a particular reference site. However, three experimental sites (each approximately 2 m^2 in extent) were selected in the most highly contaminated areas where different concentrations of oil were measured during diagnostic risk assessment (Oberholster *et al*. 2010). These sites are located in close proximity to the stormwater inlet into the freshwater wetlands (Fig. 3.1) and their respective locations are as follows: Site 1 is located at 26° 11’ 13” S, 27° 41’ 16” E; Site 2 (26° 11’ 13” S, 27° 41’ 19” E) and Site 3 (26° 11’ 17” S, 27° 41’ 18” E). Samples were collected from the sediment and water column at these sites.

Figure 3.1: Aerial photograph of Con Joubert Bird Sanctuary wetland study area, including the three (1-3) sampling sites. Site 1 = 26° 11’ 13” S, 27° 41’ 16” E; Site 2 = 26° 11’ 13” S, 27° 41’ 19” E and Site 3 = 26° 11’ 17” S, 27° 41’ 18” E.

3.2.3. Biostimulation of the wetland

Data obtained for oil concentrations and the presence of microbial organisms within the sediment and water column before and after biostimulation were compared for each site to determine the degree to which vegetable oil degradation had occurred. The concentrations of vegetable oil, redox potential (Eh), BOD, total nitrogen (TN), total phosphorous (TP), dissolved oxygen (DO), and pH were measured before biostimulation and one month after biostimulation with three different concentrations of a slow-release fertilizer in relationship with the low or high concentration of vegetable oil according to Oberholster *et al*. (2010). The fertilizer used during the study had the following nutrient content: ratio (3:1:5) 87.0 g/kg N: 29.0 g/kg P; 144.0 g/kg K 3:1:5. The fertilizer was added to the respective sites as follows: site 1 (200 g/m^2); site 2 (400 g/m^2); and site 3 (800 g/m^2). Pre-biostimulation data generated at each site was used as control for biostimulation responses.

3.2.4. Physical and chemical parameters

Integrated water column samples collected at site(s) 1-3 were used to assess the extent of oil concentrations, as well as the overall water quality through chemical analyses before and after biostimulation. Sediment samples were collected according to Oberholster *et al*. (2005) at all three sites with a sediment core (5 cm in diameter) to investigate the spatial extent of sediment contamination before and after biostimulation with organic fertilizer over a period of one month. Physical and chemical parameters such as phosphates and nitrates were performed to determine the condition of the wetland using classical

spectrophotometric methods (American Public Health Association, American Water Work Association and Water Pollution Control Federation, 1989). Other *in situ* water quality parameters such as temperature, pH, DO and conductivity were measured with a Hach™ sension 156 portable multiparameter (Loveland, CO, USA).

3.2.5. Chlorophyll measurements

Chlorophyll (Chl) as surrogate of phytoplankton biomass was measured using sub-samples (50 ml) of the water column sample (collected at the surface and at depths of 0.5 meters and 1.0 meters) of each site before and after biostimulation. Chlorophyll was extracted using 80 % acetone and left overnight for incubation. The chlorophyll *a* and *b* content were determined spectrophotometrically at 664 nm and 647 nm wavelengths respectively according to Porra *et al*. (1989). A PerkinElmer™ Lambda 25 spectrophotometer was used for absorbance determination.

3.3. Sampling methods of biota

3.3.1. Macroinvertebrates

Sampling of macroinvertebrates at the three selected sample sites were conducted using a hand net (300 x 300 mm frame, 1000 µm mesh). All available biotopes were identified, according to Minnesota Pollution and Control Agency (2000), as well as de Klerk and Wepener (2011) and sampled at the sampling sites before and after biostimulation. Loose substratum was agitated by kicking to dislodge organisms, which were collected in the net. Aquatic and marginal vegetation was swept with the net (for 2 m^2); while sand and

mud were agitated by kicking and swept for 30 seconds. A random sampling procedure was used to reduce hydrobiological variability between sites (Voeltz and Ward, 1991). Samples were immediately preserved in 70 % ethanol and later washed through a 75 µm mesh sieve to remove fine particles. The samples were then sorted and identified according to Merrit and Cummins (1996) to the lowest possible taxonomic category under an Olympus dissection microscope. The collected organisms were identified to the appropriate taxonomic level (mostly to family level, except for Oligochaeta, Hydrachnellae, Amphipoda and Porifera for which a higher taxonomical level was used). Sorting continued until at least 300 individuals were counted, or the entire sample was sorted. Invertebrate diversity was calculated using the Shannon's diversity index (Shannon, 1948).

3.3.2. Phytobenthos and phytoplankton sampling

For sampling of phytobenthos a corer was used (diameter 5 cm) after which the sediment water that was passed through a 75 µm mesh sieve, where after the sample was fixed with buffered 5 % (v/v) formaldehyde for determination of phytobenthos composition, community structure and identification of the algal species present. A total of 50 to 100 ml of each of the samples were concentrated in chambers and analyzed under an inverted microscope at 1250 x magnification using the strip-count method (American Public Health Association, 1989). Diatoms were identified after clearing in acid persulfate. The biovolumes of the more abundant taxa were estimated by measuring cell dimensions of at least 20 individuals and using the closest geometric formulae (Willen, 1976). Integrated water column samples from the surface up to one meter depth of the littoral zone were

collected at each of the sampling sites as previously described (Oberholster *et al*., 2010). The duplicate samples were preserved in the field by addition of 5 % formaldehyde to a final concentration of 2.5 %. All identifications were made according to Van Vuuren *et al*. (2006) and Taylor *et al*. (2007). The total number of phytoplankton and phytobenthos taxa and their frequency of occurrence at each sampling site were categorized according to Hörnström (2002): 1 ≤ 250, 2 = 251-1000, 3 = 1001-5000, 4 = 5001-25 000 cells l^{-1}. The Berger-Parker dominance index (Berger and Parker, 1970) was used to measure the evenness or dominance of organisms at each site. In all cases samples were collected in triplicate and subsequently processed.

3.3.3. Protozoa

A 100 ml sub-sample of the integrated water column phytobenthos sample (5 L) was used for determination of protozoa assemblage before and after biostimulation. Protozoa assemblage was determined and quantified using the live counting technique at 400 x or 1,250 x magnification with a compound microscope Olympus™ BX40 and identification was based on the quantitative protocol (QPS) method of Montagnes and Lynn (1987a, b) and Skibbe (1994). Triplicate samples were taken and subsequently processed.

3.4. Denaturing gradient gel electrophoresis (DGGE)

3.4.1. DNA extraction

Bacterial genomic DNA was extracted from the integrated water column and core sediment samples from each of the three sampling sites before and after biostimulation. The samples were kept at 4 ^{o}C before genomic DNA extraction was performed. A ZR

Soil Microbe DNA Kit™ (Zymo Research CORP) was used to extract the genomic DNA from both water (1 ml) and sediment (0.5 g) samples according to manufacturer's description. DNA concentrations were quantified using a NanoDrop® ND-100 spectrophotometer. Samples were analyzed using a 1 % agorose gel (v/v) and electrophoresed in 1×TAE buffer (Tris-acetate-EDTA) at 80 volts. The DNA was visualized by staining with GoldView™ nucleic acid, illuminated under UV light and photographed on a UVP image system.

3.4.2. Polymerase chain reaction (PCR) amplification

PCR products obtained from all sites (1 to 3) were analyzed on DGGE according to Muyzer (1999) as modified by Surridge (2007). The thermal cycling protocol was included with an initial denaturation at 96 ^{o}C for 5 min, followed by 35 cycles. Each cycle began with 30 seconds at 94 ^{o}C followed at annealing temperature at 58 ^{o}C for the 16S rDNA DGGE primer pair (PRUN518r- ATTACCGCGGCTGCTGG, Muyzer, 1999; pA8f-GC-AGAGTTTGATCCTGGCTCAG, Fjellbirkeland *et al*., 2001), then an elongation step of 1 min at 72 ^{o}C. The amplification reactions contains a 10 × amplifications buffer with 1.5 mM $MgCL_2$, 0.2 mM dNTPs, 20 pmol of each primer and 1 unit Taq DNA polymerase, and 3-5 ng purified DNA in the final volume of 25 µl reaction. The cycles were followed by incubation at 4 ^{o}C. The PCR products were again analyzed using agarose gel electrophoresis to verify the fragment size before DGGE.

After excision of PCR fragments from the DGGE gels, the PCR products were cleaned through ethanol precipitation and cloned (pGEM-T Easy Vector, Promega) before

bidirectional sequencing (ABI BigDye v3.1. System, Applied Biosystems) by Inqaba Biotech (Pretoria, South Africa). Putative sequence identities were obtained with BLAST (Altschul *et al*., 1990; 1997) analysis against the non-redundant Genbank database (NCBI, http://www.ncbi.nlm.nih.gov/).

All phylogenetic analyses were done with PAUP 4.0b10 (Swofford, 2002) and Bayesian analysis with MrBayes 3.1.1 (Huelsenbeck and Ronquist, 2001; Ronquist and Heulsenbeck, 2003). Multiple sequence alignments were done with ClustalW version 2 (Larkin *et al*., 2007) and manually evaluated before further analysis. Ambiguous characters and uninformative nucleotides were excluded from data prior to analysis and all characters were re-weighted to the consistency index. Heuristic searches using random sequence additions were preformed with the tree-bisection-reconnection (TBR) branch-swapping algorithm and MaxTrees set to auto increase. Phylogenetic signal that is consistency index (CI) and retention index (RI), was assessed by evaluating the tree length distributions in each dataset after 100 random generated trees. Only groups with a 70 % or more support were retained in bootstrap analyses over a 1 000 replicates.

3.5. Statistical analysis of data

All data were recorded on standard Excel spreadsheets for subsequent processing and the statistical analysis was conducted using the SYSTAT® 7.0.1 software package (SYSTAT, 1997). Statistical differences were analyzed through a *t* test using the Sigma Plot (Jandel Scientific) program. Values of $p \leq 0.05$ were regarded as significant in the study.

To determine changes in the biotic (macroinvertebrates, algae and protozoa) community compositions on a temporal and spatial scale, the most appropriate univariate and multivariate statistical analyses were used. Univariate analysis, such as diversity, was used to describe macroinvertebrate species-abundance relations with the help of the software program PRIMER version 6.0 (Clarke and Gorley, 2006). This included the use of the Shannon diversity index (*H'*) (Shannon, 1948). Multivariate analysis was used to express the results of the diversity and abundance of the different biota as an ordination pattern, with the different water quality parameters overlaid, which resulted in the placements of the respective sampling sites (before and after stimulation) reflecting certain (dis)similarities between each other (Shaw, 2003). In an ordination plot the arrows point in the direction of the steepest increase, whilst the angles are used to indicate correlation between variables. These types of ordination plots are called Redundancy Analysis (RDA) plots and are derived from Principle Component Analysis (PCA) plots, but the values used in the analysis are the best-fit data that is estimated from multiple linear regressions between each variable in turn and a second matrix of environmental data. Hence, an RDA was used to determine the relationship between biotic community structures and selected environmental variables with the help of the software program CANOCO version 4.5 (Ter Braak and Šmilauer, 2002).

3.6. Results

3.6.1. Physical and chemical conditions of the wetland

The chl *a* increase was highest at sampling site 2 after applying 400 g/m^2 of fertilizer as compared to site 1 (200 g/m^2) and site 3 (800 g/m^2). The most polluted site amongst the 3

sites was sampling site 3 with a vegetable oil content of 20 970 mg/kg in the sediment before and 20 985 mg/kg after biostimulation in the sediment (Table 3.1). An increase in TP and TN concentrations can be observed after stimulation (Table 3.1). Similarly an increase in chlorophyll *a* and BOD levels was also noticed at all three sites after stimulation. From Table 3.1 it can be seen that the oil concentrations decrease in the sediment and water column at site 1 and site 2, whilst the oil concentration in both the sediment and water remained similar after stimulation at site 3.

Table 3 1: Physical and chemical parameters before and after biostimulation.

Chemical parameters	Before biostimulation			After biostimulation		
	Site 1	**Site 2**	**Site 3**	**Site 1**	**Site 2**	**Site 3**
DO water column (mg/l)	0.51 (± 0.014)	0.63 (± 0.031)	0.31 (± 0.470)	0.51 (± 0.071)	0.63 (± 0.005)	0.34 (± 0.110)
pH sediment	6.26 (± 0.11)	6.39 (± 0.23)	6.10 (± 0.90)	6.26 (± 1.94)	7630 (± 0.12)	6.10 (± 0.98)
pH water column	7.42 (± 0.2)	7.46 (± 0.31)	7.11 (± 0.16)	7.42 (± 0.41)	7.46 (± 0.28)	7.11 (± 0.30)
Conductivity (µS/cm)	259 (± 21)	298 (± 34)	278 (± 28)	259 (± 18)	298 (± 41)	278 (± 61)
Chlorophyll *a* (µg/l)	3.9 (± 0.10)	3.3 (± 0.11)	3.1 (± 0.09)	28 (± 9)	39 (± 8)	31 (± 5)
Oil sediment 5 cm deep (mg/kg)	1,295 (± 211)	2,900 (± 334)	20,970 (± 421)	450 (± 63)	160 (± 11)	21,985 (± 2,177)
Oil in water column (mg/l)	30 (± 2)	90 (± 6)	60 (± 16)	10 (± 4)	19 (± 11)	58 (± 2)
Total phosphorus (µg/l)	1,800 (± 310)	2,200 (± 416)	1,200 (± 110)	12,600 (± 421)	16,500 (± 1,100)	20,000 (± 428)
Total nitrogen (µg/l)	5,800 (± 210)	4,700 (± 661)	789,200 (± 510)	12,600 (± 900)	19,200 (± 2,100)	269,000 (± 43,700)
BOD	25.1 (± 3)	25.7 (± 8)	24.8 (± 7)	41.2 (± 5)	48.0 (± 3)	27.6 (± 11)
Eh	-189 (± 18)	-164 (± 24)	-263 (± 39)	-179 (± 31)	-170 (± 24)	-291 (± 28)
Temperature (^{o}C)	11.5 (± 2.0)	12.3 (± 3.0)	13.6 (± 4.0)	11.1 (± 2.0)	11.7 (± 4.0)	12.3 (± 2.0)

3.6.2. Response of biota after biostimilation

Phytoplankton and phytobenthos

Phytoplankton species diversity recorded at all 3 sites under investigation prior to biostimulation showed low diversity before and after biostimulation in both water column and sediment (Fig. 3.2). Four phytoplankton divisions were the main representatives after vegetable oil contamination. The diatom *Craticula ambique* has been dominant at site 2 and site 3 before biostumilation, respectively. The diatom species *Criticula ambigue* were the most dominant (Berger and Parker Index. 0.413; 0.361; 0.211) of the algal Class Bacillariophyceae at all three chosen sites after biostimulation. High numbers of filamentous cyanobacteria *Oscillatoria princep* (1001-5000 cells/l) and the green algal *Chlamydomonas africana* appeared to increase in biomass at site 3. At site 2 there were significant increases in the biovolume (from 8.3 mm^3/l, 1 001 cells/l to 15 mm^3/l, 1 001 – 5 000 cells/l) of *Chlamydomonas africana* after biostimulation despite the low water column temperature (11±2 ^oC). The expression of phytoplankton biomass by average chl *a* (average 3.7 µg/l) remained relatively low at all sites before biostimulation in comparison with levels (average of 27 µg/l) after biostimulation. After biostimulation site 2 had the highest content of chl *a* (39 µg/l). Figure 3.2 shows diverse phytoplankton assemblage among the 3 sampling sites before and after biostimulation.

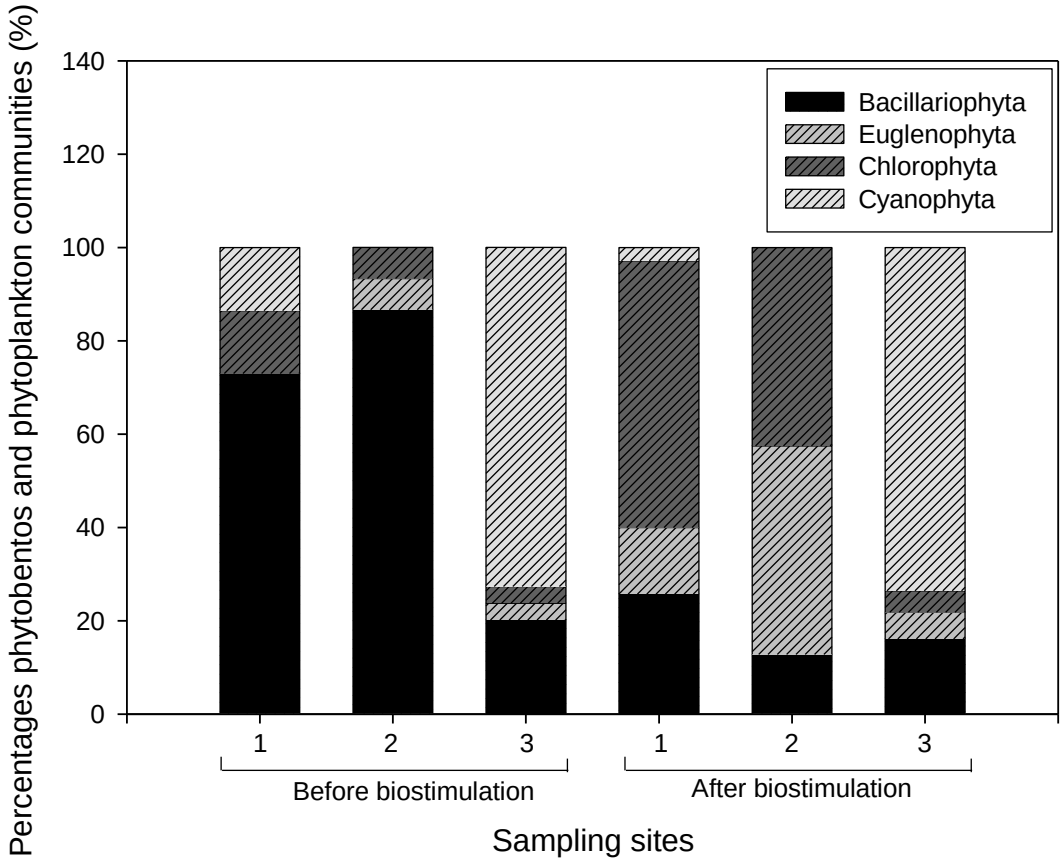

Figure 3.2: Percentage of phytobenthos and phytoplankton communities before and after biostimulation.

Protozoa

Before biostimulation the observed water column protozoa at all sampling sites were low in numbers (50-100 specimens/L) and consisted mainly of large ciliate species, such as *Pseudomicrothorax agilis*, *Paramecium caudatum* and *Didinium nastum* (Fig. 3.3). However, after biostimulation the total numbers of smaller protozoa taxa and their corresponding frequency at site 2 increased. At site 1 the percentage protozoa taxa were very low before and after biostimilation.

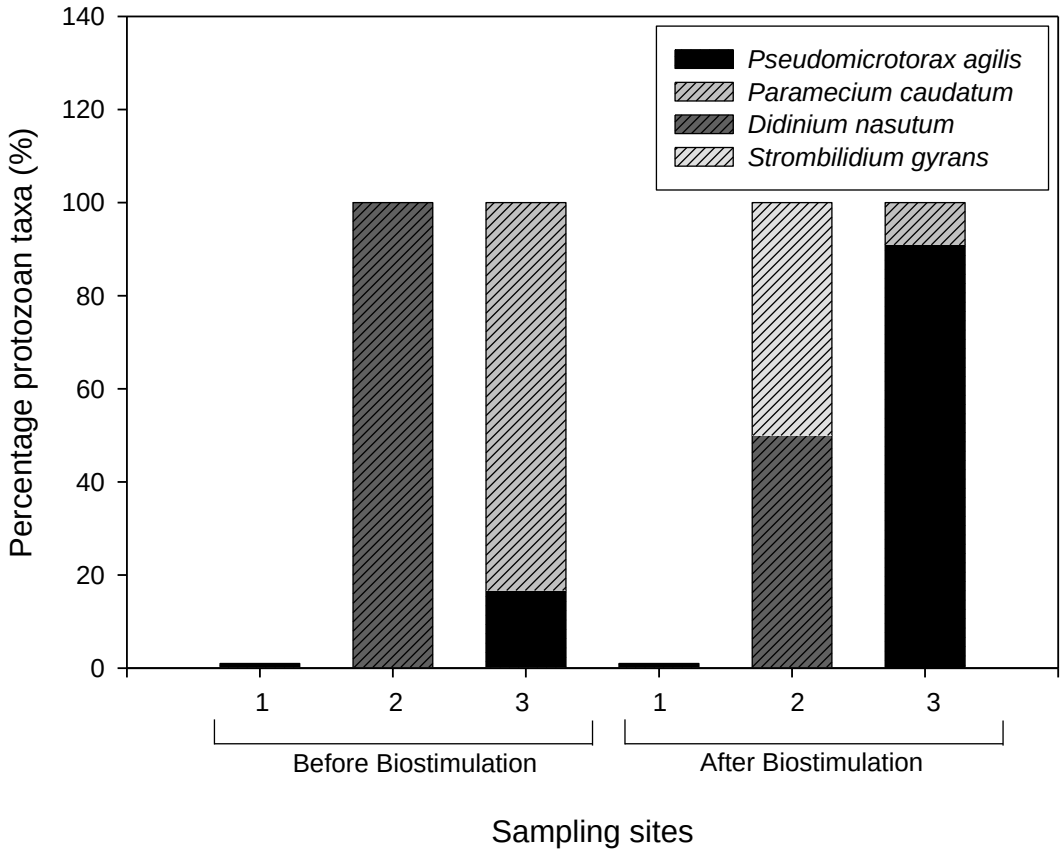

Figure 3.3: Percentage of protozoa species composition before and after biostimulation.

Macroinvertebrates

The highest numbers of macroinvertebrate families (eight and six) occurred at site 2 before and after biostimulation. An average of four families was accounted for at sites 1 and 3 before biostumulation. In these families the dominant class in Annelida was Oligochaeta, which constituted around 50 % of the total species at all the sites (Table 3.2). Diptera was another order that were well represented with families such as Chironomidae and Culicidae present at sites 1 and 3 before biostimilation. However, the numbers of these families drop after biostimilation at both sites.

Table 3.2: Macroinvertebrate families and orders before (180 days after oil spill) and after (211 days after oil spill) biostimulation.

	180 days after oil spill (*n* = 3)			211 days after oil spill (*n* = 3)		
Macroinvertebrate families and orders	Site 1	Site 2	Site 3	Site 1	Site 2	Site 3
Annelida						
Oligochaeta	0	5	0	0	1	4
Hirudinae	1	0	0	1	0	0
Hemipteria	0	0	0	1	0	0
Coleptera						
Hydraenidae	1	0	0	1	0	0
Hydrophilidae	0	0	0	0	0	0
Diptera						
Chironomidae	10	2	5	6	1	1
Culicidae	8	0	41	4	7	0
Psychodidae	0	0	4	0	4	1
Muscidae	0	0	7	2	3	0
Syrphidae	0	0	0		2	2
Blepharoceridae	0	0	0	0	0	1
Ordonata						
Gomphidae	0	1	0	0	1	0
Trichoptera						
Ecnomidae	0	1	0	0	0	0
Hemiptera						
Corixidae	0	1	0	0	0	0
Ephemeroptera						
Baetidae	0	1	0	0	0	0
Mollusca						
Planorbidae	0	0	0	0	0	0
Total No. of families	4	6	4	6	7	5
Shannon Index (H)	0.97	1.27	0.89	1.53	1.63	1.13

Orders ≡ bold
Families≡ standard

3.6.3. Influence of water quality parameters on the selected biotic community structures

Based on the RDA triplot (Fig. 3.4), distinct differences can be seen between the respective sites before and after stimulation. This triplot describes 93.9 % of the variation in the data, with 86.7 % described on the first axis and 7.2 % on the second axis. At site 1, for example, a decrease in more tolerant invertebrates (e.g. Hirudinea and Chironomidae) (Fig. 3.4) and an increase in the overall invertebrate diversity at site 1 (Fig. 3.5) can be seen after stimulation when compared to the same site prior to stimulation. The overall diversity of invertebrates at site 2 remained relatively similar before and after stimulation (H'=1.70 and H'=1.69 respectively), whilst the diversity at site 3 increased (H'=0.89 and H'=1.43 respectively) (Fig. 3.5). Phytoplankton diversity increased at site 1 after stimulation (from H'=2.04 to H'=2.27), whilst the diversity at sites 2 and 3 remained relatively similar before (H'=2.15 and H'=2.43, respectively) and after stimulation (H'=2.01 and H'=2.45 respectively) (Fig. 3.5). In contrast to the trend noticed with the macroinvertebrates and phytoplankton assemblages, no difference in protozoan diversities was noticed at site 1 after stimulation. Site 3 also showed no change in protozoan diversity (from H'=0.67 to H'=*0*.68), whilst the protozoan diversity increased at site 2 after stimulation (from H'=0 to H'=*0*.69). When relating these biotic changes to water quality changes (Fig. 3.4 and Fig. 3.5) it can be noticed that the changes in TP and TN concentrations after stimulation affected the chlorophyll *a* and BOD levels. Changes in oil concentrations within the sediment and water were also noticed after stimulation. All of these changes influenced the different biotic community structures due to two of the three biotic components being observed to have a noticeable change after

stimulation at sites 1 and 2. This is in contrast to site 3 where only the macroinvertebrates showed a change after stimulation.

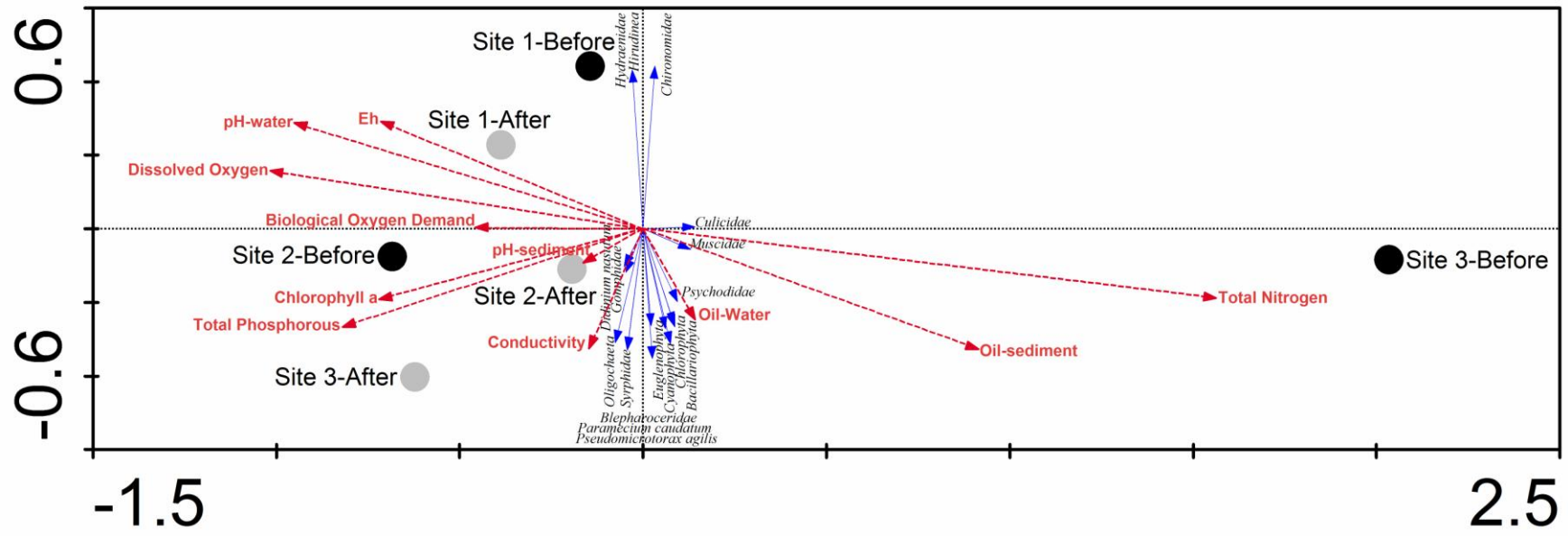

Figure 3.4: An RDA plot showing the (dis)similarity between the different sampling sites before and after stimulation based on the macroinvertebrate, freshwater algae and protozoan abundance data with water quality variables superimposed.

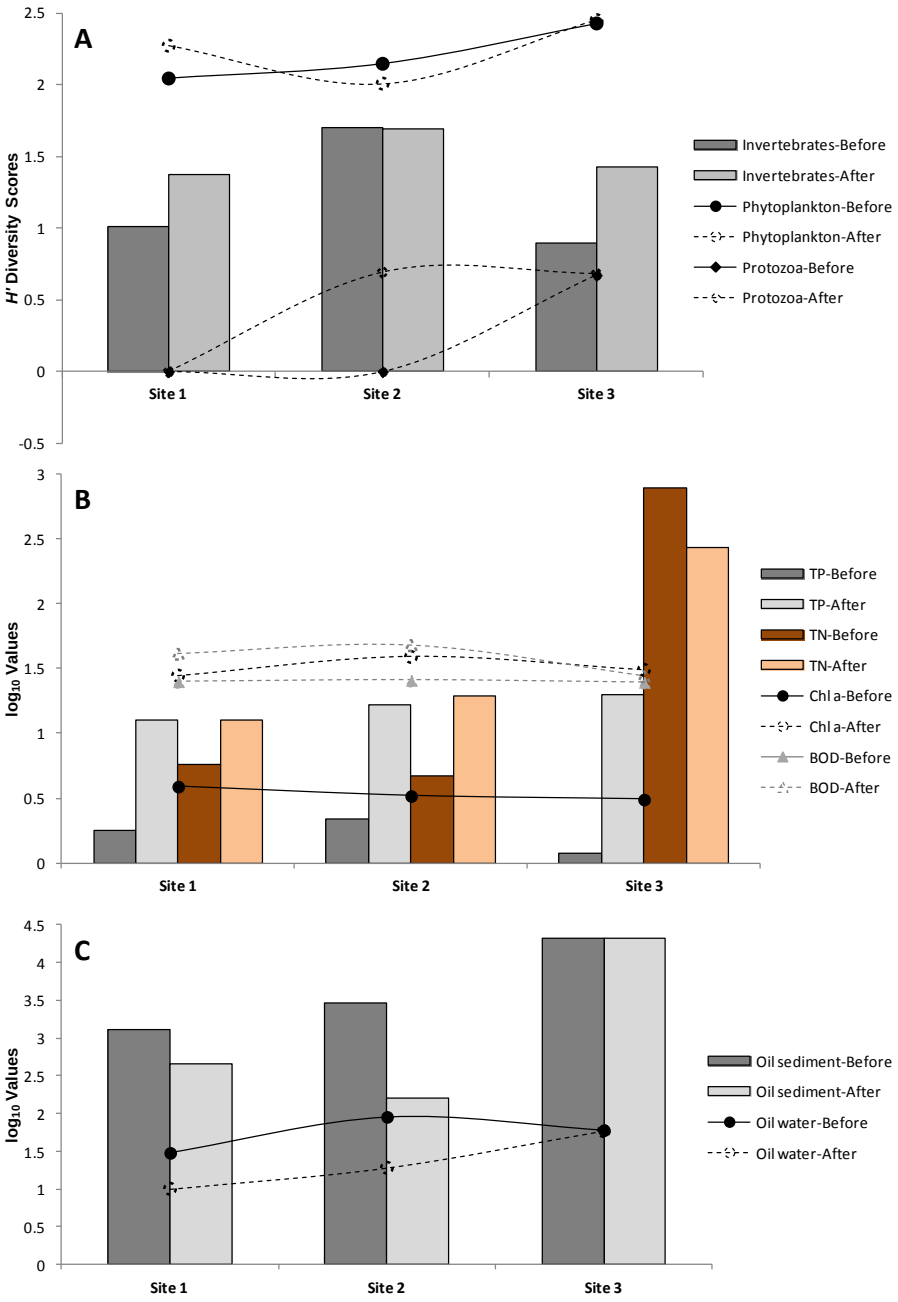

Figure 3.5: The observed spatial and temporal (before and after stimulation) changes observed for a variety of different endpoints, namely changes in the diversity of the macroinvertebrate, freshwater algae and protozoan communities (**A**), the changes in total phosphorous (TP), total nitrogen (TN) and chlorophyll *a* concentrations, as well as the biological oxygen demand (BOD) values after being $\log_{10}$ transformed (**B**); the changes in the oil concentrations within the sediment and water after being $\log_{10}$ transformed (**C**).

3.6.4. PCR-DGGE analysis for microbial diversity

The integrated water and sediment samples were analyzed on the 1 % agarose gel and also on DGGE to determine dissociation patterns of microbial diversity biomass (Fig. S1, S2, Appendix). Figure 3.6 shows species simple matching based on the DGGE band pattern similarity before and after biostimulation. The microbial composition in the sediment was highest at site 2 and the lowest at site 3 (Fig. 3.6, S2, Appendix). The microbial composition increased after biostimulation (Fig. S2, Appendix). Analysis of DGGE banding pattern using simple matching also assists to determine diversity and phylogenetic affiliation of predominant bacterial consortia probably responsible for vegetable oil degradation. Figure 3.7 shows a schematic representation of the 16S rDNA gene neighbour-joining relationships tree depicting the phylogenetic relationship of the sequences of microbial assemblages in the respective sampling sites before and after biostimulation. From the 11 DGGE profiles analyzed, four were known cultured bacteria (that is., *Pseudomonas* sp S1007, *Pseudomonas* sp KBOS, *Saccharopolspora halophila* str. YIM 90500 and *Streptomycetaceae* bacterium), six unknown and one known (*Pseudonocardiaceae* bacterium) uncultured bacteria (Fig. 3.7). Unknown DGGE fragment (29) showed no significant nucleotide similarity on GenBank to known bacterial species, however still grouped with DGGE fragments 7, 8, 10 and 11. These microbes are grouped according to similarities in their genes, which reflect their evolutionary relationships (Muyzer, 1999).

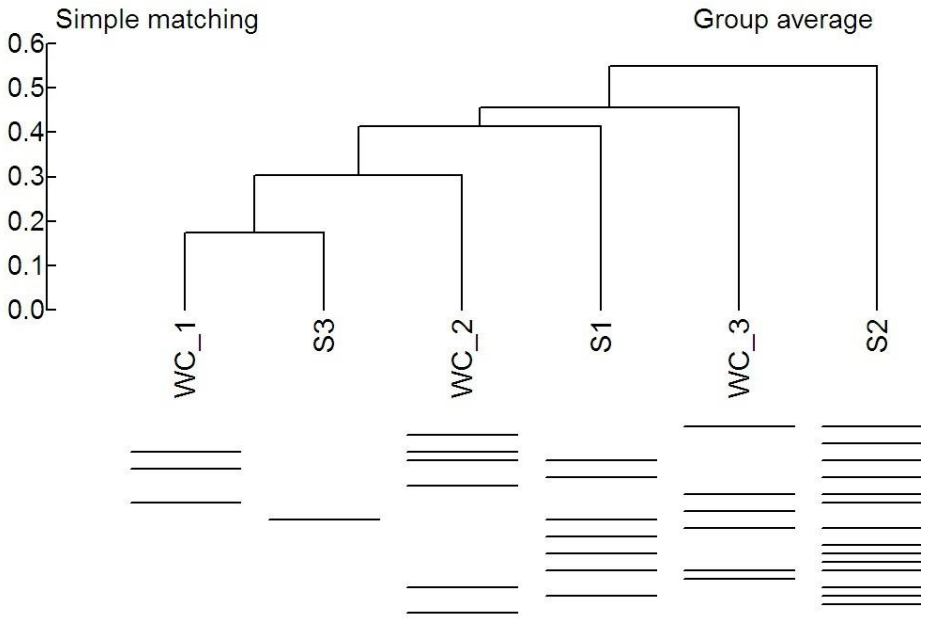

Figure 3.6: Species simple matching based on the DGGE band pattern similarity before and after biostimulation. Where WC1 (water column, site 1), WC2 (water column, site 2), WC3 (water column, site 3), S1 (sediment, site 1), S2 (sediment, site 2) and S3 (sediment, site 3).

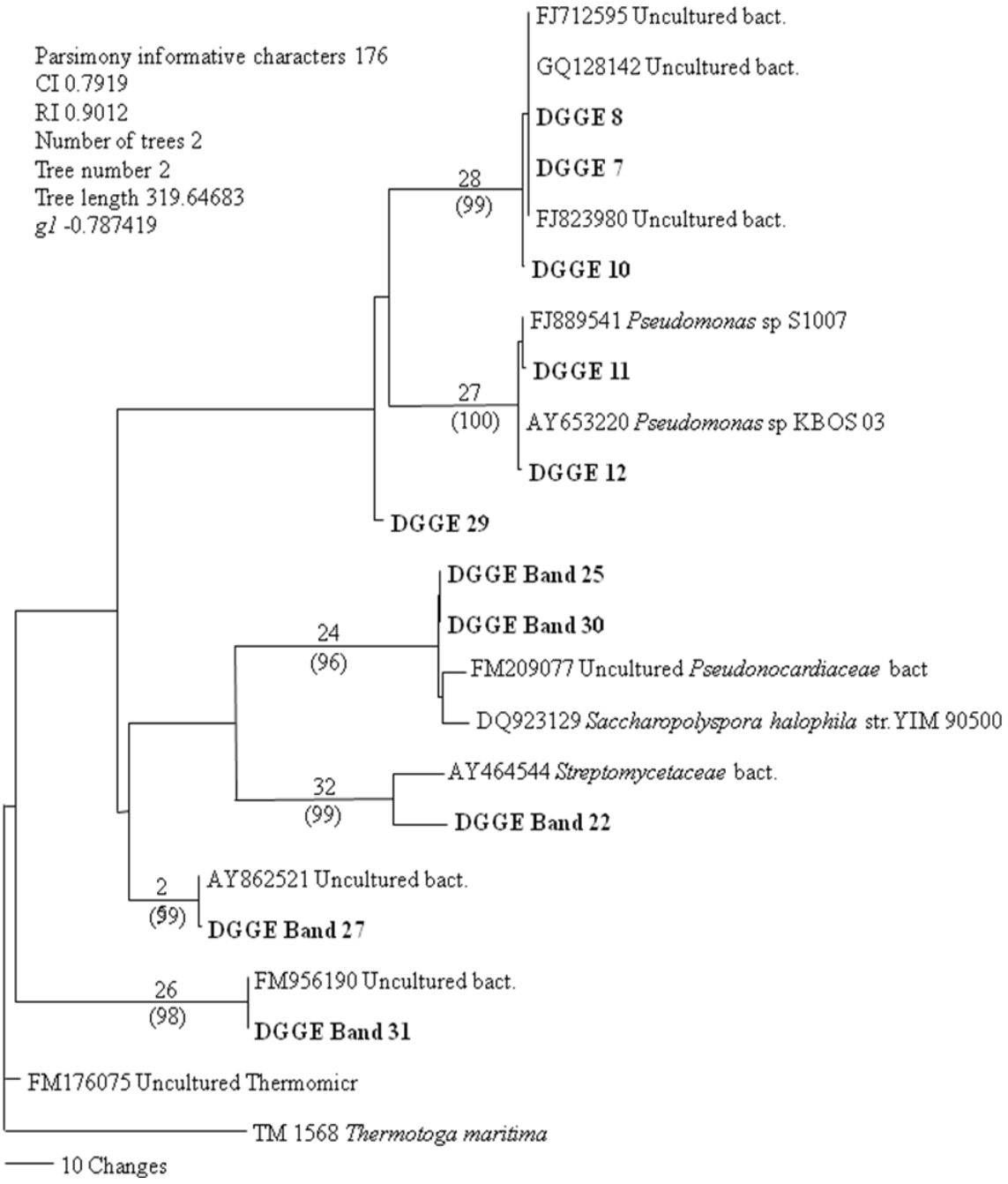

Figure 3.7: 16S rDNA gene neighbour–joining tree showing the phylogenetic relationship among the microbial amplicons (DGGE and amplicon number) from the three sampling sites obtained in the pilot study before and after biostimulation. The species names are preceded by GenBank accession numbers and sequence identity average value of ≥98 %. *Thermotoga maritime* in the main branch was used as supporting branch for the tree. The scale bar represents the changes per nucleotide position.

3.7. Discussion

Application of a nutrient rich fertilizer was found to significantly increase the biomass of microbes. Despite the observed increase in microbial activity at sites 1 and 2, a similar tendency was not observed at site 3. There may be two explanations for this, firstly, the degradation of the high vegetable oil concentrations within the sediment at sampling site 3 may have been hampered or retarded by the polymerized state of the vegetable oil as this site was severely contaminated (Table 3.1). In such instance, the vegetable oil was exposed to low energy and an oxygenated water environment that created an anoxic aquatic habitat and microbial activity (US EPA, 1997). Secondly, the application of fertilizer at a concentration of 800 g/m^2 exceeded the threshold value of phosphorous and nitrate for the biota and hampered their proliferation. The phytoplankton assemblages, protozoan and microorganisms were affected and showed little improvement at site 3 even after biostimulation with fertilizer concentration of 800 g/m^2 in comparison to sites 1 and 2, which showed great biological activity.

The addition of the organic fertilizer altered the physicochemical conditions within the water column and sediment (Table 3.1 and Fig. 3.5), for example the increase in TP and TN concentrations after stimulation, whilst pH levels remained relatively similar at all of the sites. US EPA (1997) reported that factors such as pH, temperature, dissolved oxygen, chemical contents, sediment characteristics, nutrient quantities and microbial consortia at the spill site profoundly influence the degradation of oil. Although sites 1 and 2 showed high content values of vegetable oil in both water column and sediment, they

have showed high microbial activity after the biostimulation. The higher BOD values at the selected sites after biostimulation were possibly due to vegetable oil biodegradation by the increase in microbial activity (Table 3.1 and Fig. 3.5).

The wetland already showed signs of eutrophication (Table 3.1) which implies that the addition of fertilizer could cause further depletion of the wetland ecosystem. The algae divisions Bacillariophyta, Chlorophyta, Cyanophyta and Euglenophyta were the main algal representative in the wetlands due to their ability to thrive under highly impacted environments with enrichment of primarily phosphorus and nitrate (Wahby and El-Moneim, 1979). An increase in the number of *Chlamydomonas* species at site 2, may be due to the light conditions caused by the water surface oil, limiting light penetration. The high numbers of the *Chlamydomonas* species also might have lead to an increased of phagotrophy (*i.e.*, species that depend more on ingested bacteria than on photosynthesis) (Oberholster *et al*., 2010). An increase in phytoplankton biomass as chl *a* after biostimulation at the different study sites might cause a species shift to noxious phytoplankton blooms (for example, blue green algae) that could be difficult to reverse.

Protozoa are mostly known as bacterivores (i.e., consumers of bacteria) (Kalff, 2002). The larger protozoa such as *Pseudomicrothorax agilis*, *Paramecium caudatum* and *Didinium nasutum,* which were present in the water column before biostimulation are likely to be bacterivores, and mostly consume algae or other protozoa (Fig. 3.3). According to the study of Fenchel (1988), each species of suspension-feeding ciliate tends to ingest a distinct size-spectrum of particles, related to the form and function of

their oral apparatus, and this spectrum generally shifts in larger ciliates towards larger particles, that is, algae and other protozoa. However, the low frequency of small ciliates at site 1 and 3 can also be indicators of the absence of bacteria at these sites after biostimulation (Fig. 3.6). In contrast, the increase in protozoans at site 2 may be an indication of the increase in bacteria after stimulation at this site.

At site 1, an increase in pollutant tolerant macroinvertebrates such as Chironomidae and Hirudinea (Fig. 3.4), were noticed prior to stimulation as compared to study by Camur-Elipek *et al*. (2010). This is because of the fact that early changes in macroinvertebrate community structures due to pollution are usually characterised by shifts from sensitive to less sensitive species (Norris *et al*., 1982; Clements, 1994). In the current study pollutant tolerant macroinvertebrates which generally indicate poor water quality mostly belong to the order Diptera namely, the family Chironomidae (Winner *et al*., 1980), as well as Culicidae, Muscidae, Psychodidae and Syrphidae. These organisms are then able to dominate the community in the absence of competitors. Hence, as a reduction in oil pollution was noticed at site 1 after biostimulation, an increase in the diversity of macroinvertebrates was noticed which resulted in a decrease in the dominance of the tolerant macroinvertebrates at this site. The increase in macroinvertebrate diversity at site 3, even though oil concentrations did not decrease in the sediment or water, could have been the consequence of the polymerization of the vegetable oil on the bottom sediment. In this state it may be less of a problem to the larger macroinvertebrates, as not all aquatic macroinvertebrates are fully aquatic throughout their entire lifecycles, and thus this site may be colonized externally by the dispersion of aquatic macroinvertebrates. The overall

macroinvertebrate diversity remained the same at site 2, which may be as a result of the decrease in phytoplankton noticed at the same site (Fig. 3.5), which may have resulted in a food limitation to these organisms and hence hampered the recruitment of these organisms. The abundance of macroinvertebrates is important due to the fact that low abundances of macroinvertebrates may have a long term adverse effect on fowl in the wetland, such as diving duck species that use macroinvertebrates as a major food source during protein demanding periods, e.g. during egg-laying.

Overall, the changes noticed in the biotic communities in relation to the water quality variables was in agreement with previous studies by Lee *et al*. (2002), who showed that the addition of organic fertilizers yield successful improvement in bioremediation oil contaminated environment.

The low abundance of microbial communities at sampling site 3 could be attributed to the presence of high vegetable oil content in the sediment, as well as the observed polymerization of vegetable oil residues. The improvement of sediment quality as measurement of oil content at sampling sites 1 and 2 after biostimulation (Table 3.1), corresponds with the observed bands on the DGGE gel (Figures 3.6, S2, Appendix). Sites with higher microbial activities showed higher number of bands on the DGGE gel, while site 3 with low microbial activity had fewer bands. The results from DGGE data has been reliably used for molecular community fingerprinting techniques of microbial assemblage diversity (Yu and Morrison, 2004). These results indicate that considerable diverse uncultured microbial assemblages exist in the wetland which is associated with vegetable

oil biodegradation. In the present study the dominant species was related to the genus *Pseudomonas* which agrees with the reported succession of microbial communities after biostimulation (Ogino *et al*., 2001). Although the use of DGGE for quantification of microbial assemblage is widely accepted, it should be noted that it does have a drawback in that the 16S rDNA specific primes also allows for cross reaction with members of other phylogenetic and physiological groupings in an environmental sample, especially when the sample contains a complex microbial gene pool (Rotthauwe *et al*. 1997). Furthermore, co-migration of short fragments with larger fragments in the DGGE can also yield undesired sequence data and the underestimation of microbial assemblage (Muyzer, 1999).

3.8. Conclusion

Biostimulation using organic fertilizer resulted in an increase in microbial consortia activity which promoted vegetable oil degradation at sampling sites 1 and 2; while site 3 showed less recovery due to the high amount of vegetable oil present, even after the addition of higher concentrations of fertilizer (800 g/m^2). Overall, the biostimulation of the wetland affected the different biotic communities studied to varying degrees either directly through the increase in TP and TN concentrations released from the fertilizer or indirectly through altering water quality (e.g. chlorophyll *a* and BOD) which affect the food web. This is as a result of the addition of high concentrations of fertilizer which may promote eutrophication in the wetland aquatic ecosystem and result in significant changes in phytoplankton biomass, this can have adverse effects by causing undesirable bloom formation during low flow periods in the winter months. Therefore, a pilot study is

recommended as a first step before full scale biostimulation with fertilizer may be conducted in the wetland environment. Due to the risk of eutrophication, we did not proceed with the whole wetland biostimulation, but rather monitor the wetland over a period of time to determine progressive changes that may occur in the aquatic ecosystem.

3.9. References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W. and Lipman, D. J., (1990). Basic local alignment search tool. *J. Mol. Biol*. **215**: 403-410.

Altschul S. F., Madden, T. L. and Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. and . Lipman, D. J., (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**: 3389-3402.

Aluyor, E. O., Obahiagbon, K. O. and Ori-jesu, M., (2009). Biodegradation of vegetable oils: A review. *Sci. Res. Essay* **4***:* 543-548.

American Public Health Association (APHA)., (1989). Standard methods for the examination of water and wastewater, 17th edition American Public Health Association, Washington, DC.

Balba, M. T., Al-Awadhi N. and Al-Daher, R., (1998). Bioremediation of oil-contaminated soil: microbiological methods for feasibility assessment and field evaluation. *J. Microbiol. Met.* **32**: 155-164.

Berger, W. H. and Parker, F. L., (1970). Diversity of planktonic *Foraminifera* in deep sea sediments. *Sci* **168**: 1345-1347.

Bucas, G. and Saliot, A., (2002). Sea transport of animal and vegetable oils and its environmental consequences. *Mar. Poll. Bull*. **44**: 1388-1396.

Campo, P., Zhao, Y., Suidan, M. T., Venosa, A. D. and Sorial, G. A., (2007). Biodegradation kinetics and toxicity of vegetable oil triacylglycerols under aerobic conditions. *Chemosphere* **68**: 2054-2062.

Camur-Elipek, B., Arslan, N., Kirgiz, T., Oterler, B., Guher, H. and Ozkan. N., (2010). Analysis of benthic macroinvertebrates in relation to environmental variables of Lake Gala, a National Park of Turkey. *Turk. J. Fish. Aquat. Sc.* **10**: 235-243.

Clarke, K. R. and Gorley, R. N., (2006). Primer v6: User Manual or Tutorial. PRIMER-E, Plymouth.

Clements, W. H., (1994). Benthic invertebrate community responses to heavy metals in the upper Arkansas River basin, Colorado. *J. N. Am. Benthol. Soc.* **13**: 30-44.

de Klerk, A. R. and Wepener, V., (2011). The influence of biotope and sampling method on the assessment of the invertebrate community structure in endorheic reed pans in South Africa. *Afr. J. Aquat. Sci.* **36**: 67-74.

Dutta, K., Sen S. and Veeranki, V. D., (2009). Production, characterization and applications of microbial cutinases. *Process Biochem.* **44**: 127-134.

Fenchel, T., (1988). Microfauna in pelagic food chains. In: Blackburn B, Sørensen J (Eds.). Nitrogen cycling in coastal marine, pp.59-65.

Fjellbirkeland, A., Torsvik, V. and Ovreas, L., (2001). Methanotrophic diversity in an agricultural soil as evaluated by denaturing gradient gel electrophoresis profiles of *pmoA*, *mxaF* and *16S rDNA* sequences. *Antonie van Leeuwenhoek* **79**: 209-217.

Groenewold, J. C., Pico, R. F. and Watson, K. S., (1982). Comparison of BOD relationships for typical edible and petroleum oils. *J. Water Poll. Control Fed.* **54**:398-405.

Hörnström, E., (2002). Phytoplankton in 63 lime lakes in comparison with the distribution in 500 untreated lakes with varying pH. *Hydrobiologia* **470**: 115-126.

Huelsenbeck, J. P. and Ronquist, F., (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754-755.

Kalff, J., (2002). Limnology: Inland water ecosystems. Prentice Hall, Upper Saddle River, New Jersey, USA: 1-535.

Larkin, M. A., Blackshields, G. and Brown, N. P., Chenna, R., McGettigan, P. A., McWilliam, H., Valentin, F., Wallace, I. M., Wilm, A., Lopez, R., Thompson, J. D., Gibson, T. J. and Higgins, D. G., (2007). Clustal W and Clustal X version 2.0. *Bioinformatics* **23**: 2947-2948.

Lee, L. E., Stassen, J., McDonald, A., Culshaw, C., Venosa, A. D. and Lee, K., (2002). Snails as biomonitors of oil-spill and bioremediation strategies. *Bioremediation J*. **6**: 373-386.

Li, Z., Lee, K., Cobanli, S. E., King, T., Wrenn, B. A., Doe, K. G., Jackman, P. N. and Venosa, A. D., (2006). Assessment of sediment toxicity during anaerobic biodegradation of vegetable oil using Microtox® and *Hyalella azteca* bioassays. *Environ. Toxicol.* **22**: 1-8.

Li, Z., Wrenn, B. A. and Venosa, A. D., (2005). Anaerobic biodegradation of vegetable oil and its metabolic intermediates in oil-riched freshwater sediments. *Biodegrad*. **16**: 341-352.

Merrit, R. W. and Cummins, K. W., (1996). An introduction to the aquatic insects of North America, 3rd edition Kendal/Hunt, Dubuque, Iowa, USA.

Minnesota Pollution and Control Agency (MPCA)., (2000). Macroinvertebrate Community Sampling Protocol for Depressional Wetland Monitoring Sites. Biological Monitoring Program. Standard Operating Procedures. MPCA, Minnesota.

Montagnes, D. J. S. and Lynn, D. H., (1987 a). A quantitative protorgal strain (QPS) for ciliates: a method description and test of its quantitative nature. *Mar. Microbiol. Food Webs* **2**: 83-93.

Montagnes D. J. S. and Lynn, D. H., (1987 b). Agar embedding on cellulose filters: an improved method of mounting protists for protargol and Chatton Lwoff staining. *Trans. Amer. Microsc. Soc*. **l06**: l83-l86.

Morant, P. D., (1983). Wetland classification: towards an approach for Southern Africa. J. *Limnol. Soc. South Afr*. **9**: 76-84.

Mudge, M. S., (1999). Shoreline treatment spilled vegetable oils. *Spill Sci. Technol. Bull.* **5**: 303-304.

Mudge, M. S., (1995). Deleterious effects from accidental spillage of vegetable oils. *Spill Sci. Technol. Bull*. **2**: 187-191.

Mueller, D. C., Bonner, J. S., McDonald, S. J., Autenrieth, R. L., Donnel, K. C., Lee, K., Doe, K. and Anderson, J., (2003). The use toxicity bioassays to monitor the recovery of oiled wetland sediments. *Environ. Toxicol. Chem*. **2**: 1945-1955.

Muyzer, G., (1999). DGGE/TGGE a method for identifying genes from natural ecosystems. *Curr. Opin. Microbiol.* **2**: 317-322.

Norris, R. H., Lake, P. and Swain, R., (1982). Ecological effects of mine effluents on the South Esk River, Tasmania: Benthic invertebrates. *Aust. J. Mar. Freshwater Res.* **33:** 789-809.

Oberholster, P. J., Blaise, C. and Botha, A.-M., (2010). Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland. *Ecotoxicol*. **19**: 1426-1439.

Oberholster, P. J., Botha, A.-M. and Cloete, T. E., (2005). Using a battery of bioassays, benthic phytoplankton and AUSRIVAS method to monitor long term coal tar

contaminated sediment in the Chatche la Poudre River, Colorado. *Water Res.* **39**: 4913-4924.

Ogino, A., Koshikawa, H., Nakahara, T. and Uchiyama, H., (2001). Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *J. Appl. Microbiol.* **91**: 625-635.

Pereira, M. G., Mudge, S. and Latchford, J., (2002). Consequences of linseed oil spills in salt marsh sediment. *Mar. Pollut. Bull.* **44**: 520-533.

Porra, R. J., Thompson, W. A. and Kriedemann, P. E., (1989). Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. *Biochimica Biophysica Acta* **975**: 384-394.

Poulton, B. C., Finger, S. E. and Humphrey, S. A., (1997). Effects of a crude oil spill on the benthic invertebrate community in the Gasconade River, Missouri. *Arch. Environ. Contam. Toxicol.* **33**: 268-276.

Ronquist, F. and Huelsenbeck, J. P., (2003). MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19**: 1572-1574.

Rotthauwe, J.-H, Witzel, K.-P. and Liesack, W., (1997). The ammonia monooxygenase structural gene amoA as a functional marker: Molecular fine-scale analysis of natural ammonia-oxidizing population. *Appl. Environ. Microbiol.* **63**: 4704-4712.

Shannon, C. E., (1948). A mathematical theory of communication. *Bell Syst. Tech. J.* 26:379-423, 623-656.

Shaw, P. J. A., (2003). Multivariate statistics for the environmental science. Arnold Publishers. London.

Shuhong, Y., Leichang, H., Li, Y. O., Ming, D., Yingying, H. and Dewen, D., (2006). Investigation on bioremediation of oil-polluted wetland at Liaodong Bay in northern China. *Appl. Microbiol. Biotechnol*. **71**: 543-548.

Skibbe, O., (1994). An improved quantitative protargol strain for cialiates and other planktonic protists. *Arch. Hydrobiol*. **130**: 339-347.

Surridge, A. K. J., (2007). Denaturing gradient gel electrophoresis characterization of microbial communities in polycyclic aromatic hydrocarbon and polychlorinated biphenyl contaminated soil. *PhD, University of Pretoria*, South Africa.

Swofford, D. L., (2002). PAUP. Phylogenetic analysis using parsimony (*and other methods). Version 4. Sinauer Associates, Sunderland, Massachusetts, USA.

Taylor, J. C., Harding, W. R. and Archibald, C. G. M., (2007). An illustrated guide to some common diatom species from South Africa. *WRC*, Report TT 282/07, Pretoria, pp. 1-178.

ter Braak, C. J. F. and Šmilauer, P., (2002). CANOCO reference manual and Canodraw for Windows User's Guide: Software for Canonical community ordination (version 4.5). Publishers, Microcomputer Power, Ithaca, New York, USA. pp. 500.

US EPA, (1997). Oil pollution prevention; non-transportation related onshore facilities. *Federal Register* **62**: 54508-54543.

Venosa, A. D., Suidan, M. T., Wrenn, B. A., Strohmeier, K. L., Haines, J. R., Eberhart, B. L., King, D. W. and Holder, E., (1996). Bioremediation of an experimental oil spill on the shoreline of Delawer Bay. *Environ. Sci. Technol.* **30**: 1764-1775.

Voeltz, N. J. and Ward, J. V., (1991). Biotic responses along the recovery gradient of a regulated stream. *Can J Fish Aquat Sci* **48**: 2477-2490.

van Vuuren, S., Taylor, J. C., Gerber, A. and van Ginkel, C., (2006). Easy identification of the most common freshwater algae. North-West University and Department of Water Affairs and Forestry, Pretoria, South Africa, pp. 1-200.

Wahby, S. D. and El-Moneim, A. M., (1979). The problem of phosphorus in the eutrophic lake Maryût. *Estuarine Coastal Mar. Sci.* **9**: 615-622.

Willen, E., (1976). Simplified method of phytoplankton counting. *British J. Phycol.* **11**: 265-278.

Winner, R. W., Boesel, M. W. and Farrell, M. P., (1980). Insect community structure as an index of heavy-metal pollutionin lotic ecosystem. *Can. J. Fish. Aquat. Sci*. **37**: 647-655.

Winter, T. C., (1988). Conceptual framework for assessing cumulative impact on the hydrology nontidal wetlands. *Environ. Manage.* **12**: 605-620.

Yu, Z. and Morrison, M., (2004). Comparisons of different hypervariable regions of *rrs* genes for using fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* **70**: 4800-4806.

CHAPTER 4

Assessing sunflower oil post spill recovery of the Con Joubert wetland using a battery of bioassays

4.1. Introduction

Toxicity is the potential of a substance to cause negative effects on living organisms, therefore toxicity tests are used to detect and predict the deleterious effects of chemicals on populations, communities and ecosystem level (Cairns, 1983). The standard values for a limited number of pollutants are used for the general risk assessment of water pollution. However, it is known that chemical and physical tests alone are not sufficient to assess the potential effects of complex chemical mixtures on aquatic biota, because existing analytical techniques and current knowledge of chemical toxicity and their interactions are limited (Hellawell, 1986).

According to Li *et al*. (2006) vegetable oils are harmful to sensitive aquatic organisms and ecosystems. The toxic effects of oils (petroleum and vegetable) are associated with their chemical composition and their interactions with organisms. Mudge (1995) and Campo *et al*. (2007) reported that the impact of vegetable oil on aquatic organisms were time dependent, and inhibits growth and eventually cause mortalities of different organisms. According to French-McCay (2002) and Wincele *et al*. (2004) only soluble intermediate constituents (e.g., free fatty acids and glycerol) of vegetable oil in the water are toxic to organisms. These chemical residues impact organisms by accumulating in the cellular membranes through disruption of cellular and tissues functionality. The insoluble oil constituents in freshwater became unavailable for aquatic organism interaction, and thus became less harmful to their survival (French-McCay, 2004).

The lack of complete characterization of complex chemical components present in environmental water resources requires the use of living organisms as indicators of pollution and recovery (Lee *et al*., 2002). According to Cairns *et al*. (1993) only biological specimens can determine the effects of chemical stressors in an ecosystem. These organisms respond differently to the physicochemical condition of the water source. A chain of various biological and physicochemical links could be applied to diversify the ecological spectrum using sensitive bioindicators species (Tripole *et al*., 2006).

Battery of bioassays are short term aquatic toxicity tests which are used to determine the adverse effects of pollutants in sediment and the water column (Clément *et al*., 2004; Oberholster *et al*., 2005). Van Dam *et al*. (1998) suggested that both toxicity bioassays and monitoring techniques should be applied in a wetland risk assessment program to determine degradation. Sauvant *et al*. (1995) acknowledged in their study that an ideal bioassay test should be able to predict the toxicity of any kind of chemical complexity in an aquatic system. These shorter term tests can be based on sensitivity end points e.g., reproductive inhibition, survival and growth, and the exposure times being less than seven days. Marsalek *et al*. (1999) reported that these short term tests deal with damaged to cells (cytotoxicity) or genetic material (genotoxicity).

The sensitivity of various plant and animal bioassay tests has been compared on numerous occasions and it was found to be chemical and species specific, which indicated that an array of different trophic levels must be applied in these tests (Lewis,

1995). The use of aquatic plants in water quality assessments is well recognized and a wide range of species have been used for many years as *in situ* bioindicators (Oberholster *et al*., 2005). According to Sortkjaer (1984) and Lewis (1995) aquatic macrophytes have been rarely used as early warning indicators of environmental impacts. The most commonly used macrophytes for bioassay purposes are floating duckweeds (van Dam *et al.*, 1998). Duckweeds (*Spirodela punctata*) are common to South Africa and are usually small to very small in size (< 1 cm), free-floating on the water surface and are not attached to the substrate. Their small size, ease to culture and rapid reproductive rate (doubling time 1-4 days) has led to their widespread use in batteries of bioassays in many countries to mimic trophic levels of macrophytes (Vujević *et al*., 2000). *Lemna minor* and *Lemna gibba* are the species that have been most commonly tested in numerous studies in Europe and North America (Lewis, 1995; Blinova, 2004).

Other commonly used species in bioassays also include algae, macroinvertebrates and vertebrate species. The unicellular green alga *Selenastrum capricornutum* Printz (*Raphidocelis subcapitata*) is the most commonly used species when algal toxicity tests are conducted. This test make used of phototoxicity in the aquatic environment by using chlorophyll *a* as an endpoint. The water flea (e.g. *Daphnia pulex*) is also commonly used in aquatic toxicity tests due to their sensitivity to contaminants in the aquatic environment, and tends to be the representative species of freshwater zooplankton (LeBlanc, 1980). *Physa acuta* is a common pulmonate snail that inhabits streams and dams throughout South Africa and has been used previously for toxicity tests (Musee *et*

al., 2010). All these tests were chosen in our study because they cover all trophic levels from producers to consumers.

In the present study a battery of bioassays were used to determine the impact of sunflower oil on the aquatic biota after a spill over a period of one year. For this we used survival, growth and histological change as endpoints at different trophic levels. The test organisms include *Raphidocelis subcapitata*, *Daphnia pulex*, *Physa acuta* and *Spirodela punctata* after exposure to sunflower oil contaminated of water from the Con Joubert Bird wetland. This study also compared chlorophyll data generated in the laboratory bioassay with chlorophyll measured during the field study.

4.2. Materials and methods

4.2.1. Study area description

This study was conducted on the Con Joubert Bird Sanctuary wetland. This Sanctuary is ±24.69 ha in size (26°11′ 20″S 27°41′03″E), and located in the Randfontein municipal borders (Fig. 4.1). The wetland acts as a habitat for 230 bird species. The 12.96 ha wetland has a maximum depth of 1.2 m during the raining season and the marshal zone is dominated by the following emergent macrophyte vegetation *Typha capensis*, *Schoenoplectus brachyceras*, *Phragmites australis*, *P. mauritianus* and *Persicaria lapathifolia*. In September 2007, a spill of 250 tons sunflower oil occurred at the Nola facility in the industrial area of Randfontein when a sunflower oil storage tank collapsed. The vegetable oil spilled inside the Nola facility and due to the volume of oil the multiple

trapping systems were overloaded and some of the oil followed the stormwater drains into the adjacent Con Joubert wetland area (Oberholster *et al*., 2010).

The Con Joubert Bird Sanctuary wetland can be classified as transitional open freshwater wetland type (Morant, 1983). The water budget of the wetland is governed by evaporation, precipitation and the inflow of stormwater inlets, which make the wetland a low-energy budget wetland with reduced flushing especially during the dry season (June to August).

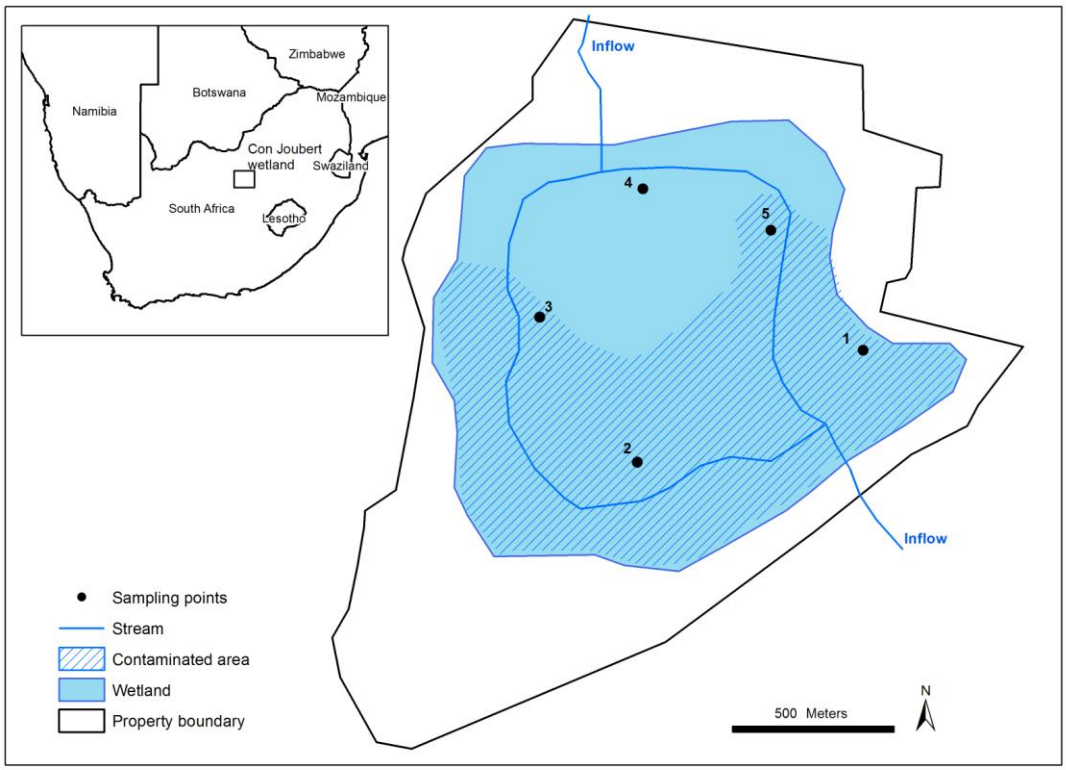

Figure 4.1: Study area (Con Joubert Bird Sanctaury wetland) and five sampling sites (Oberholster *et al*., 2010).

The study commenced after completion of a pilot study in October 2008, wherein biostimulation through addition of organic fertilizer at various sites selected in the wetland was done. The biostimulation was not performed in the whole wetland to avoid further eutrophication (Chapter 3 of this study). Due to the lack of a pre-spill control reference site (Oberholster *at al*., 2010), a toxicity profile had to be conducted using a battery of bioassays on five selected sites.

4.2.2. Sample collection and physicochemical determinations

The water column was sampled in duplicate at the surface and at the bottom using a grab bottle sampler (2 Liters). The grab bottle sampler was rinsed with alcohol after sample collection at each site to prevent oil cross contamination. Both the surface and bottom water samples from each site were pooled together to form one integrated. All the collected water samples were kept cold in a cooler box during transportation to the laboratory for analysis. Water samples were analysed within one week after collection. Various *in situ* water quality variables such as pH, electrical conductivity, temperature, dissolved oxygen (DO) and redox potential were measured in the field using a Hach™ sension 156 portable multiparameter (Loveland, CO, USA). Measurement of the other chemical variables in the water column and sediment (e.g. sunflower oil concentrations) were performed in the laboratory by using the spectrophotometric and US Environmental Protection Agency (EPA) partition-gravimetric method 413 (Code of Federal Regulations, part 136, US EPA, 1994).

4.2.3. Battery of bioassays

The battery of bioassays used in this study included three different trophic levels of organisms, which were included in the bioassays, with the following criteria in mind; low cost, organisms and suppliers locally available, sensitive to a wide-spectrum of organic compounds, reproducible and representative of more than two trophic levels (producers, grazers and filter feeders). All bioassays were performed on a two monthly bases after collection of integrated water samples from the selected sites, i.e. sites 1 to 5.

4.2.4. Algal phytotoxicity and growth inhibition test

In this study, we used a 72-hour growth standard freshwater algal toxicity test with the unicellular green alga *Raphidocelis subcapitata* (Pardos *et al.*, 1998). The green algae (*R. subcapitata*) culture was maintained axenically in 250 ml capacity Erlenmeyer flasks according to standard procedures (Slabbert, 2004). The algae were sub-cultured at weekly intervals to ensure that a constant supply of logarithmic growth phase cells was available for toxicity testing.

In this test we have used cell growth as the endpoint for the 72-hour bioassay. The tests were run in parallel in five replicate flasks, plus a control. *Raphidocelis subcapitata* (strain ATCC 22662, Canada) was cultured in standard AAP media under axenic conditions in 250 ml glass Erlenmeyer flasks (US EPA, 1985, 1989). The AAP medium had the following chemical composition, per liter: 25 mg/l $CaCl_2.2H_2O$; 0.78 µg/l $CoCl_2.6H_2O$; 0.009µg/l $CuCl_2.2H_2O$; 12.16 mg/l $MgCl_2.6H_2O$; 96 µg/l $FeCl_3.6H_2O$; 185.64 µg/l H_3BO_3; 175 mg/l K_2HPO_4; 75 mg/l $MgSO_4$; 264.27 µg/l $MnCl_2.4H_2O$; 7.26

µg/l $Na_2MoO_4.2H_2O$; 15 mg/l $NaHCO_3$; 250 mg/l $NaNO_3$; 32.7 µg/l $ZnCl_2$; and 333 µg/l $Na_2EDTA.2H_2O$. Culture flasks were shaken continuously at 100 rpm and incubated at 24 ºC ± 2 ºC under constant light (255 µmol photon $m^{-2}s^{-1}$). Algal inoculate were prepared for each water sample from fresh culture stocks sampled during the exponential growth phase, and the cells were concentrated by gentle centrifugation. The inoculums density was adjusted to 1 x 10^5 cells/ml (Ross *et al.*, 1988). Each of the replicate test flasks plus the control flask was inoculated with 1 ml stock culture that had been re-suspended in an appropriate volume (100 ml per flask) of nutrient-spiked (US EPA, 1989) filtered water sample of each sampling site to be tested. The sampled water of the five sampling sites was filtered three times through a 2.5 micron filter to prevent contamination by algae species occurring in the sampled water prior to the test. The following water quality parameters: pH, alkalinity, hardness and water temperature, were measured at the start and end of each test. Nutrient-spiked sterile (autoclaved) Milli-Q water inoculated with algae was used for control testing.

4.2.5. Daphnia pulex zooplankton toxicity test

The water flea, *Daphnia pulex*, is widely distributed in South Africa in ponds and dams and in slow-flowing streams and adult specimens have a maximum length of 3.5 mm. During most of the year, populations of *Daphnia* consist exclusively of females. The production of males is induced by high population densities and the subsequent accumulation of excretory products, and/or a decrease in food. The average life span of *Daphnia pulex* is about 50 days at 20 ºC. There are four distinct phases in the life cycle of Daphnia namely: the egg, juvenile, adolescent and adult. Minutes after moulding, a clutch

of eggs is released into the brood chamber. The eggs hatch in the brood chamber and the juveniles, which are similar in form to adults, are released in approximately 2 days when the female moults (casts off her exoskeleton). The organism used in the study was isolated from a pond in the Pretoria (South Africa) area in 1985 and has been maintained in laboratory culture since then (Slabbert, 2004).

Organisms aged 24-hours or less was used for the toxicity tests. To obtain the necessary number of young for a test, adult females bearing embryos in their brood pouches were removed from the stock cultures 24 hours prior to the initiation of the test, and placed in beakers containing moderately hard water (Table 4.1) and food suspension (trout chow, alfalfa and yeast).

Table 4.1: Moderately hard water made up for use in *Daphnia pulex* cultures.

KCl	$MgSO_4$	$NaHCO_3$	$CaSO_4.2H_20$		DO	Hardness	Alkalinity
mg/l				pH	mg/l	mg/l $CaCO_3$	
4	60	96	60	7.4-7.8	>6.8	80-100	60-70

[1] Prepared with Milli-Q®.
[2] Approximate equilibrium pH after aeration

Table 4.2 summarizes test conditions used in this study. Test specimens were transferred to a small intermediate holding beaker and transferred from there to the test beakers. The test was carried out using the water samples collected from the five sampling sites in the wetland and a control. A total of 10 specimens per sample were used in the test (2 sets of 5 beakers and 2 controls). Adverse effects were expressed as percentage lethality within 48 hours. Exposures were conducted in 250-ml glass beakers containing 50 ml test water.

The test was conducted as a screen (100 % concentration of wetland water). Mortality, defined as lack of movement after gentle prodding, was recorded at 24 and 48-h intervals.

Table 4.2: Summary of conditions for the *Daphnia pulex* toxicity test.

Test type	Static
Water temperature	20 ºC ± 2 ºC
Light quality	Ambient laboratory illumination
Photoperiod	10 hours dark: 14 hours light
Feeding	None during first 48 hours. Thereafter, suspension of trout pellets, alfalfa and yeast – prepared with Milli-Q® water.
Size of test container	250 ml
Volume of test sample	50 ml
Number of organisms/container	10
Number of replicate containers	18
Total number of organisms/container	10
Control and dilution water	Moderately hard water
Effects measured	1 and 2 day: Lethality (no movement on gentle prodding)

4.2.6. Physa acuta snail bioassay

The *Physa acuta* snails used in this bioassay were obtained from batch cultures of offspring collected from the Rietvlei nature reserve wetland area, South Africa, during 2004. The snails were maintained in standard snail water filled aquaria at 20 ºC and a 16:8-hour light:dark photoperiod. This culture water was also used for the controls in the experiment. The *P. acuta* snails were fed on commercial algae wafers.

The exposure of *Physa acuta* snails, to the sampled water to be tested (undiluted, *i.e.*, 100 %), was conducted as 96-hour static acute tests using juvenile snails (shell length, ≤ 5 mm) from the batch culture. Generally, in aquatic invertebrates, larvae, juvenile stages, and small individuals are reported to be more sensitive to toxicants than adult stages, and therefore juveniles were used (Aguirre-Sierra *et al.*, 2011). The water volume for the test vessels were 200 ml/beaker and a total of 5 test specimens per sample in triplicates were

used in the test. The control beakers contained five snails in triplicates filled to appropriate volume with snail cultured water. Adverse effects on tested organisms were expressed as percentage lethality as endpoint after 96 hours. The number of living and dead *Physa acuta* juvenile snails was recorded after 96 hours. Snails that did not respond to a gentle prodding with forceps were considered to be dead. Each bioassay was conducted at approximately 22-23 ºC in the laboratory with a 16:8-hour light:dark photoperiod.

4.2.7. Spirodela punctata biotest

Spirodela punctata, also known as duckweed, is a small vascular free floating aquatic macrophyte. *S. punctata* is a member of the family *Lemnaceae* and inhabits fresh water ponds, dams, stagnant waters and slow-flowing streams. The growth conditions include temperature ranges between 6-33 ºC and a wide pH range with optimal growth between pH 5.5 and 7.5. The fronts (represent leaves and stem systems) of *S. spirodela* occur singly or in clusters of two to nine. The fronts are flat and oval, 2 to 4 mm long, and are green to lime green in colour. Each thread-like bears one or more thread-like roots. Vegetative growth is by bilateral branching and rapid compared with other vascular plants. The test species (*S. punctata*) used in this study was collected from the Rietvlei dam wetland, Pretoria (South Africa) and prepared according to standard procedures for laboratory used (Bowker *et al*., 1980).

All experiments were carried out using axenic plants of the duckweed *S. punctata*. The aquatic plants were cultivated for several months in modified Provasoli's medium

(Pflugmacher and Steinberg, 1997). Plants were then pre-cultivated for two weeks in 100 ml Erlenmeyer flasks under continuous illumination at approximately 60 µmol photons.$m^{-2}.s^{-1}$ at 22 - 24 ºC before exposure to water from the different sampling sites. Five plants of *S. punctata* with two fronds each were then transferred from the preliminary culture into sterile Erlenmeyer flasks (100 ml) with constant stirring. The test was conducted in triplicate, and the plants were exposed to the water samples from different sampling sites (1-5). Culture water was used as control. Incubation took place for 5 days under the same culture conditions as the preliminary cultures in the growth chambers.

Growth end points as indicators of toxicity

The end point assessed included frond numbers and root length. The number of the fronds of the *S. punctata* plants was counted every day and growth rate was calculated on the basis of the increase in fronds over a period of 5 days according to an exponential growth model.

Growth rate was calculated as:

$$t^{-1} \cdot \log (F_t / F_0) \cdot (\log 2)^{-1} \qquad \text{Equation 1.}$$

Where t is the duration of the test in days, F_0 is the initial number of fronds, and F_t is the number of fronds at the end of the test. Root elongation (mm) was also measured on a daily basis.

4.2.8. Suspended chlorophyll analysis

A 100 ml-subsample of the integrated sampled water collected for planktonic algae enumeration was used to determine suspended chlorophyll *a* in the water column at each site. One liter sample from each sampling site were filtered using glass microfibre filter (GF/C) papers to concentrate planktonic phytoplankton biomass. These GF/C filter papers were dissolved in 80 % acetone and incubated overnight in the dark at room temperature. Chlorophyll *a* and *b* contents were determined spectrophotometrically at 664 nm and 647 nm, respectively according to Porra *et al*. (1989). A PerkinElmer™ Lambda 25 spectrophotometer was used to determine absorbance.

4.2.9. Data analyses of biotest endpoints

The test results were recorded on standard Excel spreadsheets for data processing, and statistical analysis was performed using the SYSTAT® 7.0.1 software package (SYSTAT, 1997). The responses of bioassay samples were subjected to an analysis of variance ($p<0.05$) using SYSTAT ® 7.0.1 (1997) to examine the endpoint variability of each test.

4.3. Results

Key physicochemical parameters of water quality analyzed during the study are summarized in table 4.3. The pH (7.3) of the wetland water was stable around neutral at 4 sites with the exception of site 1, where the pH was slightly higher (8.1) as compared with the other sites. The electrical conductivity (EC) had shown fluctuation amongst sampling sites ranging between 369 and 982 µS/cm. Site 1 also displayed the highest

sunflower oil concentration (25.73 g/kg) when compared to the other sites. Generally, the wetland water column showed low dissolved oxygen (DO) saturation which ranges between 1.96 mg/l and 5.0 mg/l. Chlorophyll *a* contents for sites 3 to 5 were 14, 33.25 and 23.3 µg/l, respectively which were high as compared with sites 1 and 2 (4.2±1 and 4.78±2 µg/l). Redox potential in the sediments remained negative for all sampling sites during the study period. Average of salinity remained stable at 0.38 ‰. The average temperatures were at 25±2 ^{o}C in summer and 16 ^{o}C in winter, respectively.

Table 4.3: Physicochemical analyses of the wetland water over the one year period at five sampling sites in the Con Joubert Sanctuary wetland (n=12).

	Site 1	Site 2	Site 3	Site 4	Site 5
pH	8.0 ± 0.3	7.4 ± 1	7.6 ± 2	7.2 ±	7.2 ± 0.15
TDS mg/l)	546±140	356±86	199.2±18	348±12	384±27
EC(µS/cm)	982±210	587±48	369±44	645±87	749±141
Salinity (‰)	0.5±0.1	0.4±0.1	0.2±0.1	0.3±0.1	0.5±0.2
DO (mg/l)	3.9±1.2	2.4±0.25	5.0±0.27	4.5±0.4	1.96±0.04
Temperature (^{o}C)	25.5±2	15.8±2	26±4	16±2	24±1.6
Chl *a* (µg/l)	4.2 ± 1	4.78 ±2	14 ± 4	33.25 ± 1	23.3 ±0.1
Redox (sediment)	-365±40	-257±30	-155.7±27	-164.3±11	-277.3± 21
Sediment oil (g/kg)	25.73±4	2.18±0.10	5.55±2.3	5.11±1.9	1.6±0.4
Oil in water (g/kg)	3.75±0.25	2.1±0.48	2.51±0.18	2.54±0.74	1.04±31

4.3.1. R. subcapitata test and chlorophyll concentrations

The highest average chlorophyll *a* concentrations were above 80 % at sites 3, 4 and 5, while sites 1 to 2 were just above 60 % (Fig. 4.2). Chlorophyll *a* concentrations measured in the field samples were higher in comparison to the laboratory studies throughout the study period.

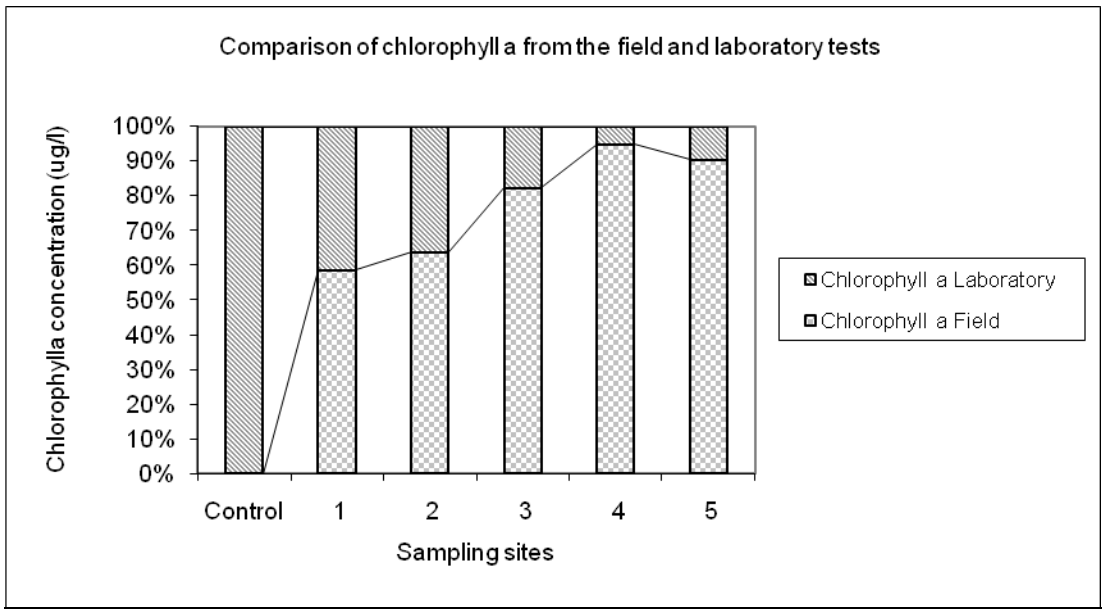

Figure 4.2: Comparison between field and laboratory chlorophyll *a* concentrations during the study period of one year.

Inhibitory or stimulatory mechanisms on the *R. subcapitata* biotest have shown that growth was affected by the sunflower oil in the water (Fig. 4.2). All sampling sites (1-5) showed a decrease in chlorophyll *a* and *b* concentration (3.01-1.75 and 2.21-1.03 µg/l, respectively) as compared with the average of the control sample (chl *a* =4.78 µg/l and chl *b* =1.77 µg/l). The *R. subcapitata* biotest showed toxicity responses due to an observed reduction in the chlorophyll *a* and *b* of the field water samples (Fig. 4.2). Chlorophyll *a* and *b* concentrations determined in the field water samples were also compared amongst the five sampling sites during the study period (Fig. 4.3). In the winter period, the reduction of chlorophyll *a* was very high when compared with the other seasons (summer, autumn and spring), which showed an average decrease of chlorophyll *a* as compared to the control (Fig. 4.3).

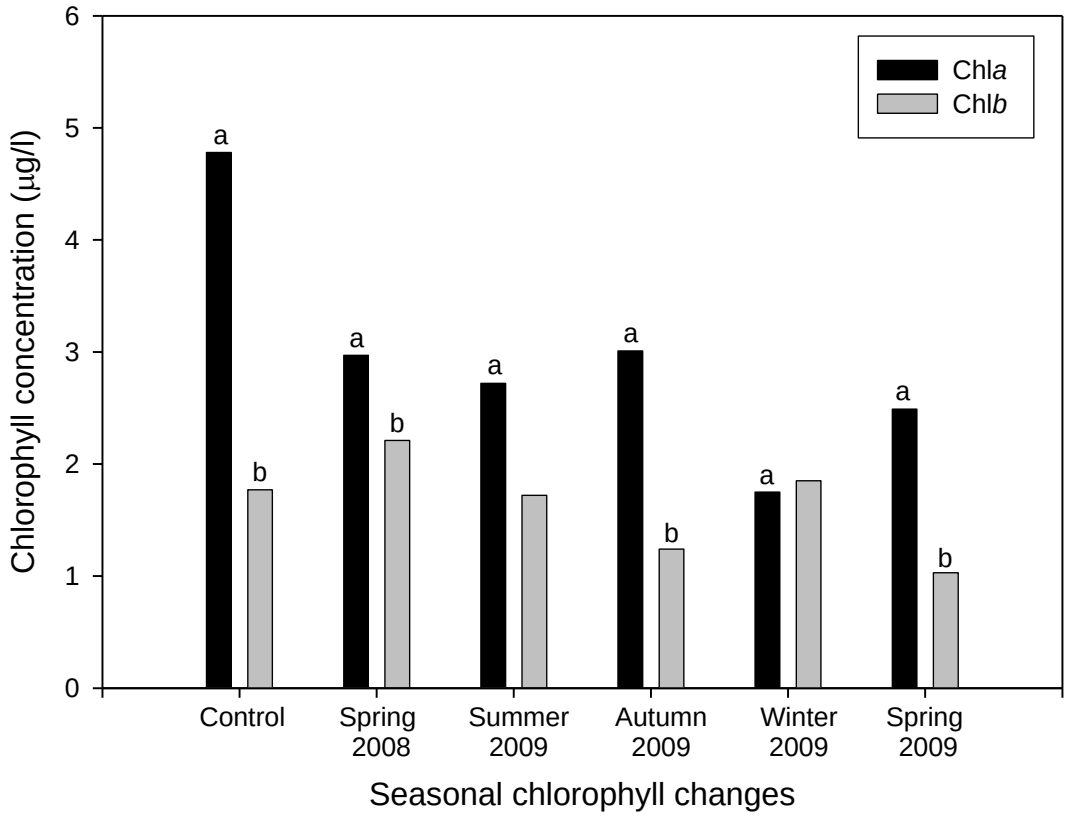

Figure 4.3: Chlorophyll contents from *R. subcapitata* biotest in response to sunflower oil contamination. Seasonal variation in chlorophyll concentrations: 1) Control; 2) spring (October 2008); 3) summer (January 2009); 4) autumn (April 2009); 5) winter (June 2009) and 6) spring (August and October 2009). Alphabetical letters "a" and "b" indicate significant differences to the control test ($P<0.05$).

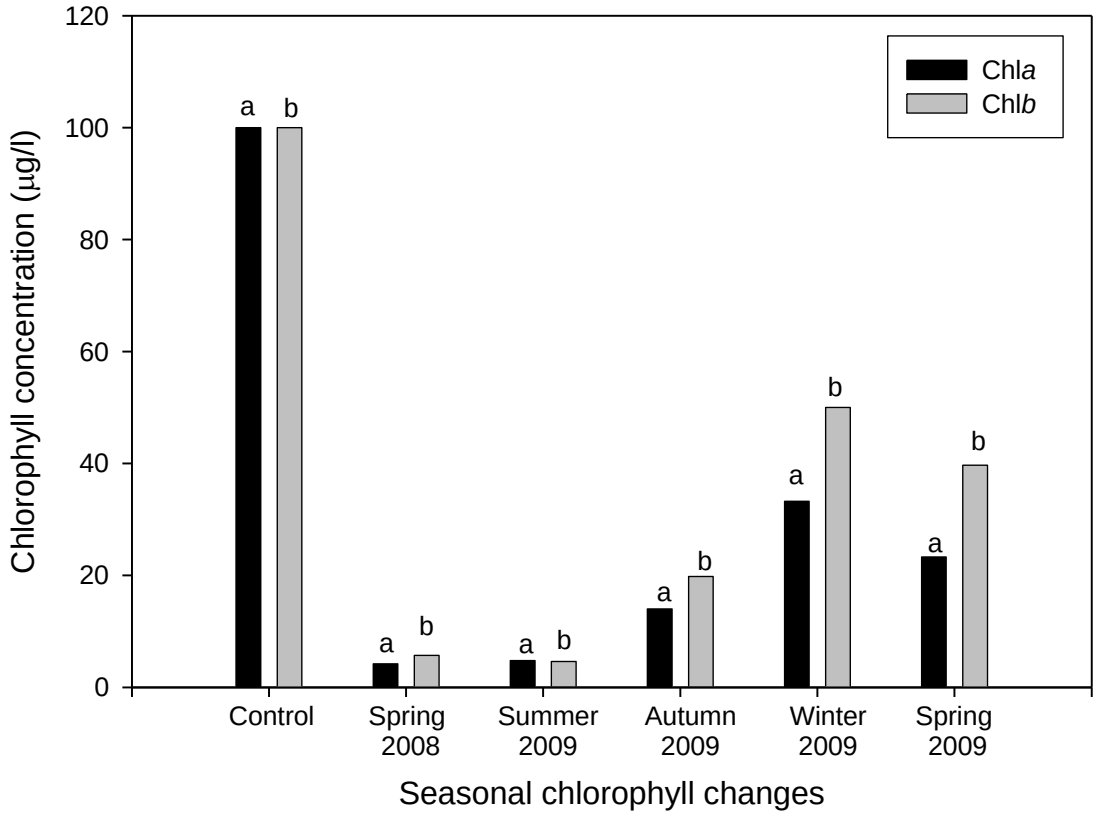

Figure 4.4: Field chlorophyll contents from planktonic phytoplankton biomass in response to sunflower oil contamination. Seasonal variation in chlorophyll concentrations: 1) Control; 2) spring (October 2008); 3) summer (January 2009); 4) autumn (April 2009); 5) winter (June 2009) and 6) spring (August and October 2009). Alphabetical letters, "a" and "b" indicates significant differences to the control test ($p<0.05$).

4.3.2. Toxicity variations among bioassays: P. acuta and D. pulex

Daphnid mortality was noticeable in the spring 2008 and summer 2009 season samples. The recorded daphnid survival rates were ±85 % (summer) and ±70 % (spring) after the 48 hours treatment in comparison to 100 % survival in the control test (Fig. 4.5). However, in the succeeding seasons (months April 2008 to October 2009) no effects were observed on the biotests species as compared to control test species. This was not

surprising since daphnid species (approximately ±500 individuals/l) were observed at sampling sites 3 to 5 in summer (January 2009), which indicate an improvement of wetland water quality especially at these particular sites.

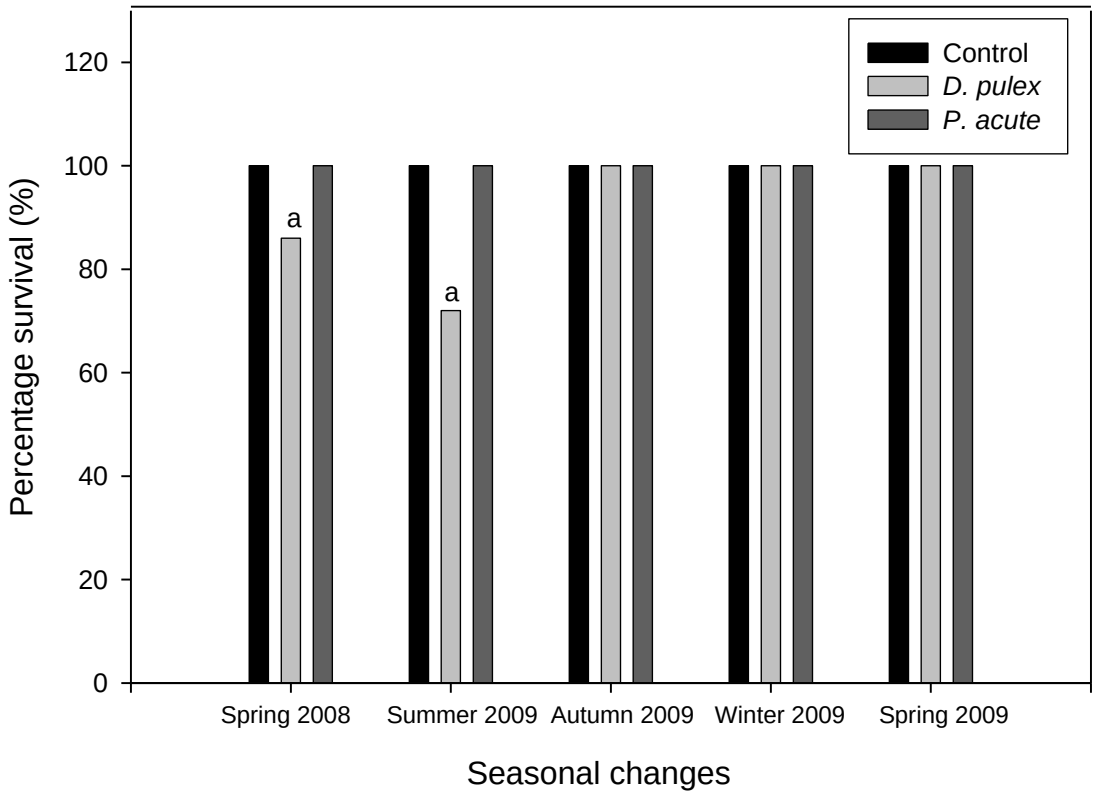

Figure 4.5: Toxicity response among multispecies biotests during four seasons of the study. Samplings dates: spring (October 2008), summer (January 2009), autumn (April 2009), winter (June 2009), spring (August and October 2009). Alphabetical letter, "a" indicates significant difference with the control test ($P<0.05$).

The survival rates of *P. acuta* in the water samples collected at sites 1 to 5 showed no effect on the specimens in comparison to the control test during all seasons, as no mortality or immobility of *P. acuta* was found in all test and control containers during the exposure time (100 % survival) (Fig. 4.5).

Figure 4.6 shows the response of the biotest species (*S. punctata*) to the sunflower oil contaminated water samples. There was no reduction in fronds growth observed over the 72-hour incubation period in *S. punctata* compared to control plants. Generally, the sensitivity rates for wetland water of the sampling sites were in the following order: *R. subcapitata* > *D. pulex* > *P. acuta* > *S. punctata*, with no difference between the last two test species.

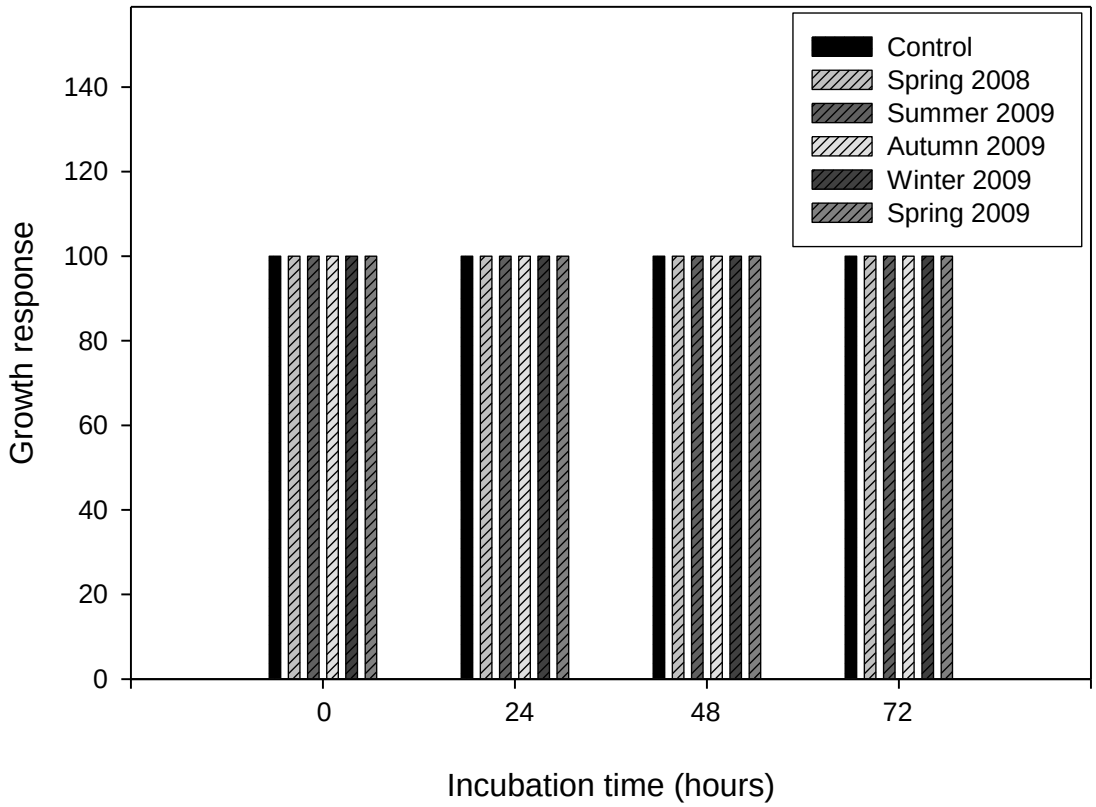

Figure 4.6: Toxicity response of *S. punctata* after 24, 48 and 72 hours of incubation with undiluted wetland water (100 % concentration of oil contaminated water) of the five sampling sites. No significant differences with control test were observed (P>0).

4.4. Discussion

Overall, the physicochemical quality of the water column samples collected at five sampling sites in the Con Joubert Bird Sanctuary wetland showed different responses to the spilled sunflower oil. The only noticeable exception was from the high sunflower oil concentration at sites 1 and 2 as compared with other sites (3-5). The exposures of bioindicators to water samples contaminated with sunflower oil have focused on evaluating individual species under laboratory conditions mimicking environmental conditions. The bioassays indicated that the wetland sampled water did have adverse effects on different trophic levels and was therefore species specific.

4.4.1. R. subcapitata biotest

The species *R. subcapitata* showed significant different toxicity effects after exposure over a period of 72hr to the water samples from the wetland as measured in chlorophyll *a* and *b* concentrations in comparison to the control (Fig. 4.3). According to Baun *et al*. (2000) the use of this species is regarded as the most sensitive bioassay test of pollutants followed by the daphnids and duckweeds tests. The mechanisms causing the biotest's sensitivity are strongly related to processes of adaptation of *R. subcapitata* to the experimental water samples (Masojídek *et al*., 2011). The different seasonality affected the quality of the water *e.g.* during winter period the level of chlorophyll *a* was lower than chlorophyll *b* concentrations (Fig. 4.3). The latter can possibly be related to the higher concentration of salts (EC) (Fig. 6.2, Chapter 6) in the winter dry season.

The planktonic algae species in the field showed more tolerance to the adverse effects of the sunflower oil relative to the specific species that was used in the bioassay (Fig 4.2) as reflected by the chlorophyll *a* and *b* concentrations. Since chlorophyll *a* is a field metric for phytoplankton biomass, its variability in the laboratory can be strongly linked to physiological shifts in intracellular pigmentation in response to the changing growth conditions such as light, nutrients and temperature. From the field sampling it was evident that the sunflower oil concentrations (e.g. October 2008, January 2009 and April 2009) caused major reductions in chlorophyll *a* in the first three consecutive sampling trips (Fig. 4.2).

There were also significant differences amongst the seasons (October 2008, January 2009, April 2009, June 2009, August 2009 and October 2009) in chlorophyll *a* concentrations as compared to the control sample (Fig. 4.3). From the results it is apparent that toxicity was also occurred on cellular level (i.e., affected the photosynthetic pathway) as reflected by its effect on chlorophyll *a* and *b* (Baryla *et al*., 2001). It also showed that toxicity was below threshold values and that it adversely affected growth of *R. subcapitata*. The obtained results supported those by Oberholster *et al*. (2010) in their previous studies with respect to the effects of sunflower oil on algae.

4.4.2. Daphnia pulex biotest

The test specimen *D. pulex* was sensitive to the sunflower oil contaminated freshwater from two sampling sites (1 to 2) while at sites 3 to 5 no toxicity was observed during the entire study period (Fig. 4.5). This may indicate that the toxicity of the sunflower oil

concentrations at sites 1 to 2 were above threshold level in comparison to the control. These results agree with the findings from a previous study by Baun *et al*. (2000) that the mobility of daphnids after a 48 hour incubation represent detectable levels below toxicity. Baun *et al*. (2000) found that the test species *D. magna* were insensitive to the organic compounds in the leachate-polluted water samples in their range of test concentrations. Toxic compounds are often adsorbed or chemically bound to suspended particles in the water column, which depends upon the physicochemical conditions of water. Since daphnids are filter feeders and they can gradually thrive in slightly cloudy water in the wetland injesting bacteria, fungi and algae (Weltens *et al*., 2000). These species filtrates large amounts of surrounding water, redraw particles as a food source, which means contaminated particles threaten the health of these particle-feeding organisms (Weltens *et al*., 2000). The obtained results therefore implies that the water at sampling sites 3 to 5 of the wetland was less toxic to the *Daphnia* specimens, while higher concentrations of sunflower oil pollutions were still present in the water from sites 1 and 2 and therefore more toxic to the *Daphnia* specimens.

4.4.3. Physa acuta biotest

Mollusca species are abundant and widely distributed in various environmental waters, and have been used in water biomonitoring due to their susceptibility to slight pollution (Lee *et al*., 2002). *Physa acuta* (snail) biotest showed no susceptibility or stress and mortality to the sunflower oil impacted water samples during the incubation period at all sampling sites (1-5) when compared to the control sample (Fig. 4.5). Greer *et al*. (2001) reported in their study that the snail growth in oil impacted environments could be related

to the reduction of petrochemical toxicity and an increase in microorganism's growth following nutrient amendment. In the case of sunflower oil pollution, there is no previous published information focusing on the assessment of its toxicity to different life stages of aquatic snails.

4.4.4. Battery of biotests

In conclusion, the tests performed on these organisms were the basis for the comparison of the relative acute toxicities of chemicals to aquatic plants and animals reported in the previous studies (Kenaga and Moolenaar, 1979). Chemical monitoring generally assist in identifying the toxicity of components in the water body associated with sunflower oil contamination. The comparison between outcomes of the bioassay assessment and analyses of physicochemical indicators showed no early warning with regards to sunflower oil contamination (Fig. 4.5). Blaise *et al*. (2004) and Oberholster *et al*. (2005) reported that biotests are highly rated in oil spill remediation programs to provide operational guidance, because it can identify potential detrimental effects from applications and provide a means of quantifying treatment success. It was evident from these results that the multispecies bioassays detected variations in toxicity effects.

Since various forms of biota differ in their sensitivity to toxicants, it is highly recommended to use test battery bioassays comprising of species from different trophic levels during environmental assessments to ensure ecological relevance (Zhu *et al*., 2004). These results confirmed no impacts of sunflower oil on *P. acuta* and *S. punctata* in the wetland, while *D. pulex* and *R. subcapitata* showed response under similar conditions.

Differences in sensitivity between organisms may be related to the mode of action of toxicants but results were hampered by the scarce information on the impact of sunflower oil on different life stages of organisms. It is therefore necessary to conduct studies on the physiological responses of biological variants.

4.5. References

Aguirre-Sierra, A., Alonso, A. and Camargo, J. A., (2011). Contrasting sensitivities to fluoride toxicity between juveniles and adults of the aquatic snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca). *Bull. Environ. Contam. Toxicol.* **86**: 476-479.

Baryla, A., Carrier, P., Franck, F., Coulomb, C., Sahut, C. and Havaux, M., (2001). Leaf chlorosis in oilseed rape plants (*Brassica napus*) grown on cadmium-polluted soil: causes and consequences for photosynthesis and growth. *Planta* **212**: 696-709.

Baun, A., Jensen, S. D., Bjerg, P. L., Christensen, T. H. and Nyholm, N., (2000). Toxicity of organic chemical pollution in groundwater downgradient of a landfill (Grindsted, Denmark). *Environ. Sci. Technol.* **34**: 1647-1652.

Blaise, C., Gagné, F., Chévre, N., Harwood, M., Lee, K., Lappalainen, J., Chial, B., Persoone, G. and Doe, K., (2004). Toxicity assessment of oil-contaminated freshwater sediments. *Environ. Toxicol.* **19:** 267-273.

Blinova, I., (2004). Use of freshwater and algae duckweeds for phytotoxicity testing. *Environ. Toxicol.* **19**: 425-428.

Bowker, D. W., Duffield, A. N. and Denny, P., (1980). Methods for the isolation, sterilization and cultivation of Lemnacea. *Freshw. Biol*. **10**: 385-388.

Cairns, J. Jr., (1983). Are single species toxicity tests alone adequate for estimating environmental hazard? *Hydrobiologia* **100**: 47-57.

Cairns, J., McCormick, P. V. and Niederlehner, B. R., (1993). A proposed framework for developing indicators of ecosystem health. *Hydrobiologia* **263**: 1-44.

Campo, P., Zhao, Y., Suidan, M. T., Venosa, A. D. and Sorial, G. A., (2007). Biodegradation kinetics and toxicity of vegetable oil triacylglycerols under aerobic conditions. *Chemosphere* **68**: 2054-2062.

Clément, B., Devaux A, Perrodin Y., Danjean, M. and Ghidini-Fatus, M., (2004). Assessment of sediment ecotoxicity and genotoxicity in freshwater laboratory microcosms. *Ecotoxicol.* **12**: 323-333.

French-McCay, D. P., (2004). Oil spill impact modelling: Development and validation. *Environ. Toxicol. Chem.* **23**: 2441-2456.

French-McCay, D. P., (2002). Development and application of an oil toxicity and exposure model, OilToxEx. *Environ. Toxicol. Chem.* **21**: 2080-2094.

Greer, C. W., Fortin, N., Roy, R., Whyte, L. G. and Lee, K., (2001). Microbial population dynamics and degradation activity in response to a controlled oil spill on a freshwater wetland. *Biorem. J.* **6**: 2002.

Hellawell, J. M., (1986). Biological indicators of freshwater pollution and environmental management. *Appl. Sci.*, pp. 546.

Kenaga, E. E. and Moolenaar, R. J., (1979). Fish and *Daphnia* toxicity as surrogates for aquatic vascular plants and algae. *Environ. Sci. Technol.* **13**: 1479-1480.

Lee, L. E. J., Stassen, J., McDonald, A., Culshaw, C., Venosa, A. D. and Lee, K., (2002). Snails as biomoitors of oil-spill and bioremediation strategies. *Bioremediation J.* **6**: 373-386.

LeBlanc, G. A., (1980). Acute toxicity of priority pollutants to water flea (*Daphnia magna*). *Bull. Environ. Contam. Toxicol.* **24**: 684-691.

Lewis, M. A., (1995). Use of freshwater plants for phytotoxicity testing: a review. *Environ. Poll*. **87**: 319-336.

Li, Z., Lee, K., Cobanli, S. E., King, T., Wrenn, B. A., Doe, K. G., Jackman, P. N. and Venosa, A. D (2006). Assessment of sediment toxicity during anaerobic biodegradation of vegetable oil using Microtox® and *Hyalella azteca* bioassays. *Environ. Toxicol.* 1-8.

Marsalek, J., Rochfort, Q., Brownlee, B., Mayer T., Servos, M. and Dutka, B., (1999). Toxicity testing for controlling urban wet-weather pollution: advantages and limitations. *Urban Water* **1**: 91-103.

Masojídek, J., Souček, P., Máchová, J., Frolík, J., Klem, J. and Malý, J., (2011). Detection of photosynthetic herbicides: Algal growth inhibition test vs. electrochemical photosystem Π biosensor. *Ecotoxicol. Environ. Safety* **74**: 117-122.

Morant, P. D., (1983). Wetland classification: towards an approach for Southern Africa. *Limnol. Soc. South Afr.* **9**: 76-84.

Mudge, S. M., (1995). Deleterious effects from accidental spillage of vegetable oils. *Spill Sci. Technol. Bull.* **2**: 187-191.

Musee, N., Oberholster, P. J., Sikhwivhilu, L. and Botha, A.-M., (2010). The effects of engineered nanoparticles on survival, reproduction, and behaviour of freshwater snail, *Physa acuta* (Draparnaud, 1805). *Chemosphere* **81**: 1196-1203.

Oberholster, P. J., Blaise, C. and Botha, A.-M., (2010). Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland. *Ecotoxicol*. **19**: 1426-1439.

Oberholster, P. J., Botha, A.-M. and Cloete, T. E., (2005). Using a battery of bioassays, bethic phytoplankton and contaminated sediment in the Cache la Poudre River, Colorado. *Water Res.* **39**: 4913-4924.

Pardos, F., Higgins, R. P. and Benito J., (1998). Two new Echinoderes (Kinorhyncha, Cyclorhagida) from Spain, including a reevaluation of Kinorhynch taxonomic characters. *Zool. Anz*. **237**: 195-208.

Pflugmacher, S. and Steinberg, C. E. W., (1997). Activity of phase I and phase II detoxication enzymes in aquatic macrophytes. *J. Appl. Bot*. **71**: 144-146.

Porra, R. J., Thompson, W. A. and Kriedemann, P. E., (1989). Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. *Biochem. Biophys. Acta* **975**: 384-394.

Ross, P. J. V. and Sloterdijik, H., (1988). A rapid bioassay using the green algal *Selenastrum capricornutum* to screen for toxicity in St. Lawrence River sediment

elutriates. In "Cairns J, Pratt J. R (eds) Functional testing of aquatic biota fro estimating hazards of chemicals. ASTM STP 988. *ASTM, Philadelphia, USA*, pp 68-73.

Sauvant, M. P., Pépin, D., Bohatier, J. and Groliére, C. A., (1995). Comparison of six bioassays for assessing in vitro acute toxicity and structure-activity relationships for vinyl chloride monomer, its main metabolites and derivates. *Sci. Total Environ.* **172**: 79-92.

Slabbert L. (2004). Methods for direct estimation of ecological effect potential (DEEEP). WRC report 1313/01/04, *WRC Pretoria*. pp 1-100.

Sortkjaer, O., (1984). Macrophytes and macrophyte communities as test systems in ecotoxicological studies of aquatic systems. *Ecol. Bull* **36**: 75-80.

Tripole, S., Conzalez, P., Vallania, A., Garbagnati, M. and Mallea, M., (2006). Evaluation of impact of acid mine drainage on the chestry and the macrobenthos in the Carolina stream (San Luis-Argentina). *Environ. Monit. Assess.* **114**: 377-389.

US EPA, (1994). Oil pollution prevention; non-transportation-related onshore facilities; final rule. *Federation Registration*, pp. 59.

U.S. EPA,. (1989). Short term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms, 2nd ed. EPA 600/4-89/001. *Technical Report*. Cincinnati, OH.

US EPA., (1985). Short term methods for estimating the chronic toxicity of effluents and receiving waters to freshwater organisms. EPA 600/485/014. *Technical Report*. Cincinnati, OH.

Weltens, R., Goossens, R. and Van Puymbroeck, S., (2000). Ecotoxicity of contaminated suspended solids for filter feeders (*Daphnia magna*). *Arch. Environ. Contam. Toxicol.* **39**: 315–323.

Wincele, D. E., Wrenn, B. A. and Venosa, A. D., (2004). Sedimentation of oil-mineral aggregates for remediation of vegetable oil spills. *J. Environ. Eng*. **130**: 50-58.

van Dam, R. A., Camilleri, C and Finlayson, C. M., (1998). The potential of rapid assessment techniques as early warning indicators of wetland degradation: A review. *Environ. Toxicol. Water Quality* **13**: 297-312.

Vujević, M., Vidaković-Cifrek, Ž., Tkalec, M., Tomić, M. and Regula, I., (2000). Calcium chloride and calcium bromide aqueous solutions of technical and analytical grade in *Lemna* bioassay. *Chemosphere* **41**: 1535-1542.

Zhu, X., Venosa, A. D., Suidan, M. T. and Lee, K., (2004). Guidelines for the bioremediation of oil-contaminated salt marshes. *US EPA. Cincinnati, OH* **45268**, pp. 1-61.

CHAPTER 5

Succession of planktonic phytoplankton in a freshwater wetland, one year after a vegetable oil spill

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5.1. Introduction

Petroleum and vegetable oils share physical properties and exhibit similar characteristics when spilled in aquatic environments. These oils have the ability to either float on the water surface and become solubilised or emulsify in the water column or settle on the bottom substrate (Crump-Wiesner and Jennings, 1975; Frink, 1994). The main toxic constituent of vegetable oils is free fatty acids produced during environmental exposure (USEPA, 1997). The lack of adequate monitoring carried out prior to a spill and as such the lack of sufficient historical data are major limitations for the evaluation of impacts of oil spills in both marine and freshwater ecosystems (Varela *et al.*, 2006; Oberholster *et al.*, 2010). The response of phytoplankton to an oil spill is an excellent indicator to examine the adverse effects of an oil spill in the aquatic ecosystem (Miller *et al*., 1978). Evidence from previous studies supports the fact that the elimination of grazers in the respective aquatic ecosystem is the principal cause of altered phytoplankton compositions and increased biomass in wetlands (McComb and Davies, 1993).

According to Wehr and Sheath (2003), phytoplankton represents between 30 and 50 % of primary producer biomass in wetland systems, and their activity is noticeable in the large diurnal changes in dissolved oxygen and carbon dioxide. The presence of phytoplankton biomass in the wetland reflects changes in the water quality and was shown to be a sensitive indicator of biological integrity and physicochemical conditions of the respective wetland system (Korneva and Mineeva, 1996; Willén, 2001).

Oberholster *et al*. (2010) reported that shifts in the functional phytoplankton groups, such as different growth forms and divisions of phytoplankton, can also be indicative of important changes in food quality and habitat structure for invertebrates. Phytoplankton is regarded as the primary energy producers in nutrient cycling and the food web that drives aquatic ecosystems (Walter, 2006; Kane *et al*., 2009). Phytoplankton also serves as energy source for zooplankton in freshwater wetlands. The growth responses of phytoplankton are predictive and sensitive to environmental factors, including pH, temperature, electrical conductivity and nutrient concentrations (*e.g.*, nitrogen, phosphorus and silica load) in various water environments (Adrian *et al*., 1999; Oberholster, 2011).

The objective of this study was to determine the response of planktonic phytoplankton assemblages over a period of one year after a vegetable oil spill in the Con Joubert Bird Sanctuary wetland in relation to water quality variables. Through this study we wish to establish a benchmark of some of the most important changes in a planktonic phytoplankton community of a freshwater wetland recovering from a vegetable oil spill.

5.2. Materials and methods

5.2.1. Study area and site selection

The Con Joubert Bird Sanctuary wetland studied is a lake freshwater wetland (26°11′ 20″S 27°41′03″E), approximately 25 ha in size. According to Oberholster *et al*. (2010), 250 tons of sunflower oil was spilled at a vegetable oil storage facility in Randfontein (South Africa) when a sunflower oil storage tank collapsed in September 2007. The oil

entered the Con Joubert Bird Sanctuary wetland via storm water outlets that drain into the wetland. According to the authors, this was the largest global sunflower oil spill in a freshwater wetland.

This wetland is a sanctuary for approximately 250 bird species and contains large stands of reed beds, while patches of short emergent vegetation and mudflats are visible in the dry season. The wetland is surrounded to the south and east by an industrial area and subjected to stormwater inflow during rainy seasons. Five sampling sites were selected (Fig. 5.1). Sites 1 and 2 were in close proximity to the stormwater inlet from where the spilled vegetable oil entered the wetland. Sites 3 and 4 were located on the eastern side of the wetland where it borders a residential area. Site 5 was located on the northern side of the wetland. These sites were chosen to be representative of the entire wetland system. The maximum water depth of the wetland is approximately 1.2 m during the wet season.

Figure 5.1: An aerial photo of the wetland studied, indicating the five selected sampling sites. The red arrow indicates the source of the vegetable oil contamination.

5.2.2. Water quality

Post spill samples were collected every second month over a period of one year (from October 2008 to October 2009). Water samples were collected to determine the concentrations of the oil, as well as other water quality variables during this period. Various *in situ* water quality parameters, including dissolved oxygen, electrical conductivity, pH, redox potential and temperature were measured in the field using a Hach™ sension 156 portable multiparameter meter.

The water samples were collected in triplicate at the surface and the bottom using a grab bottle sampler (two liters). Both the surface and bottom grab samples were pooled together to form one integrated sample. The collection bottle was filled to the brim and

kept cold and in the dark until analyses could be performed to prevent additional chemical reactions. The grab bottle sampler was cleaned with alcohol after collection at each site to prevent possible cross contamination of oil. The water samples were analysed for silica, total phosphate and total nitrogen, which constitutes of nitrate, nitrite and total kjeldahl nitrogen, to determine changes in the water quality at each sampling site. The phosphate and nitrogen concentrations were measured using appropriate spectrophotometric methods (APHA, AWWA and WPCF, 1980). Sampling of bottom sediment to determine concentrations vegetable oil was done according to Oberholster *et al*. (2005). Concentrations of vegetable oil in the sediment and in the water column were measured according to a protocol by the United States Environmental Protection Agency (USEPA, 1994).

A subsample of the pooled water sample from each site was used to determine the chlorophyll *a* and *b* concentrations in the water column at each site. This sample was filtered through a glass microfibre filter (GF/C) to concentrate the algal sample. These GF/C filter papers were dissolved in 80 % acetone and incubated overnight in the dark at room temperature. The chlorophyll *a* and *b* concentrations were determined spectrophotometrically at 664 nm and 647 nm wavelengths, respectively, according to Porra *et al*. (1989) using a PerkinElmer™ Lambda 25 spectrophotometer.

5.2.3. Planktonic phytoplankton

A subsample (100 ml) of the integrated samples collected at each site was preserved with formaldehyde to a final concentration of 1 %. These sub-samples were concentrated 10-

fold by centrifugation at 400 rpm and used for planktonic phytoplankton identification and enumeration. The phytoplankton cells were counted at a magnification of 1250 times, using an Olympus inverted binocular compound microscope with phase contrast, using the strip-count method (American Public Health Association, 1989). Planktonic phytoplankton genera were identified according to Wehr and Sheath (2003) and van Vuuren *et al*. (2006). Before diatoms were identified, they were cleared of organic matter using a potassium dichromate and sulphuric acid solution. The cleared sample was then diluted and mounted in Pleurax medium for microscopic examination. Diatom genera were identified and counted at a magnification of 1250 times. Genera identification was performed according to Patrick and Reimer (1975), Wehr and Sheath (2003) and Taylor *et al*. (2007). The total number of planktonic phytoplankton was recorded after careful examination for at least 15 minutes until no additional taxa were found thereafter. The total number of dominant planktonic taxa and their abundance during the study were categorized according to Hörnström (2002).

5.2.4. Statistical analyses

All the recorded data were subjected to the most appropriate univariate and multivariate statistical analysis to determine correlations between changes in the planktonic phytoplankton community structures and water quality variables (Ter Braak and Šmilauer, 2002). Planktonic phytoplankton diversity was calculated using the Shannon diversity index (Shannon and Weaver, 1949), whilst the Margalef's index (d) was used to measure phytoplankton genus richness (Margalef, 1951). A redundancy analysis plot was used to determine any similarity or dissimilarity between the different sites with regard to

the changes in the planktonic phytoplankton community and it was overlain with the prevailing water quality variables within the wetland.

5.3 Results

5.3.1. Water quality

The water quality results from the different sites within the wetland are summarized in Table 5.1. At sites 1 and 2 the chlorophyll *a* and *b* concentrations were found to be the lowest. The dissolved oxygen concentration of ~2.4 mg/l at site 1 and ~2.7 mg/l at site 4 were low in comparison to the dissolved oxygen concentrations at the remaining three sites. The electrical conductivity at site 1 (~729 mS/cm) was relatively similar in comparison to the other four sampling sites where it varied between ~637 and ~699 mS/cm. The pH values and silicate concentrations were relatively similar between the different sites. pH ranged between ~7.4 and ~9.3 and the silicate concentrations were between ~3.30 and ~5.32 mg/l. At sites 1 and 2 the total nitrogen and total phosphate concentrations were found to be higher than those recorded at the rest of the sites. The average oil concentrations in the water were relatively similar at all the selected sites, with the highest concentration at site 1 (~3.75 mg/l), whilst the average oil concentration found in the sediment was high at sites 1 and 2 (~25.73 g/kg and ~21.88 g/kg, respectively) in comparison to sites 3, 4 and 5, where the oil concentration remained below 6 g/kg.

Table 5.1

Water quality variables analysed at the five selected sampling sites over a period of one year. Average values and standard deviations are reported.

Parameters	Site 1	Site 2	Site 3	Site 4	Site 5
Chlorophyll *a* (µg/l)	4.2 ± 1.9	4.7 8 ± 3.28	14.21 ± 1.6	33.22 ± 21	23.31 ± 14.2
Chlorophyll *b* (µg/l)	3.44 ± 1.7	3.01 ± 0.02	10.48 ± 0.7	20.4 ± 8	17.26 ± 3
Dissolved oxygen (mg/l)	2.4 ± 0.1	3.8 ± 1.3	4.2 ± 0.3	2.7 ± 0.1	3.7 ± 0.7
Electrical conductivity (mS/cm)	729 ± 115	683 ± 279	699 ± 101	685 ± 49	637 ± 152
pH	7.4 ± 1.0	8.1 ± 1.7	8.5 ± 0.8	8.4 ± 1.1	9.3 ± 1.6
Redox potential (mV)	-125.35 ± 13	-136.05 ± 11	-120.67 ± 3.6	-122.31 ± 4.1	-188.8 ± 10.1
Silicate (mg/l)	5.32 ± 1.9	4.81 ± 2.0	3.66 ± 1.5	3.3 ± 0.6	4.31 ± 1.7
Temperature (^{o}C)	11.7 ± 1.8	12.2 ± 0.6	11.4 ± 1.2	12.1 ± 0.6	11.5 ± 2.0
Total nitrogen (mg/l)	22.06 ± 3.5	49.60 ± 2	10.45 ± 0.27	16.50 ± 0.1	9.70 ± 0.25
Total phosphate (mg/l)	3. 87 ± 0.20	5. 92 ± 0.36	0.91 ± 0.18	1. 44 ± 0.12	0.67.6 ±0.07
Oil in water (mg/l)	3.75 ± 0.3	2.1 ± 0.01	2.51 ± 0.3	2.54 ± 1.3	1.04 ± 0.01
Oil in sediment (g/kg)	25.73 ± 11	21.88 ± 1.8	5.55 ± 0.9	5.11 ± 0.7	1.6 ± 03

5.3.2. Planktonic phytoplankton

Throughout the sampling period a total of 20 genera were recorded at all five sites (Table 5.2). Chlorophyta was the most dominant phytoplankton group found during this study with *Chlamydomonas* sp., *Scenedesmus* sp. and *Spirogyra* sp. being the most dominant genera.

Table 5.2

Planktonic phytoplankton assemblages at the five selected sites over a period of one year. The planktonic phytoplankton abundances during the study were categorized according to Hörnström (1999), where 0 = 0; 1 = ≤ 500; 2 = 501 – 5000; 3 = 5001 – 25000; 4 = 25001 – 100000 cells/mℓ. A = October 2008; B = January 2009; C = April 2009; D = June 2009; E = August 2009; and F = October 2009.

Division	Genus	Site 1						Site 2						Site 3						Site 4						Site 5					
		A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F	A	B	C	D	E	F
Bacillariophyta	*Cyclotella* sp.	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Gomphonema sp.	0	0	2	0	0	0	0	0	1	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	1	0	0	0
	Melosira sp.	0	0	1	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0
	Navicula sp.	0	0	3	2	0	2	0	0	3	0	0	0	0	0	3	3	3	3	0	0	2	3	3	0	0	0	0	0	0	2
	Pinnularia sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	0	0	0	0	0	1
	Rhopalodia sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	1	0	0	0	0	0	0	0
	Synedra sp.	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0
Chlorophyta	*Chlamydomonas* sp.	3	4	4	3	4	4	4	2	4	3	4	0	4	0	4	4	4	4	3	4	4	4	4	4	0	0	4	4	4	4
	Chlorogonium sp.	1	1	1	0	0	0	0	0	0	0	4	0	0	0	0	0	3	0	0	0	0	0	1	1	0	0	0	0	0	0
	Oedogonium sp.	0	0	0	0	0	0	2	4	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Pediastrum sp.	0	0	0	0	0	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Scenedesmus sp.	0	0	4	4	4	4	0	0	4	4	4	3	0	0	4	4	4	4	0	0	4	4	4	4	0	0	0	0	0	4
	Spirogyra sp.	4	4	4	3	4	4	4	4	0	3	4	4	0	4	4	2	4	4	4	4	4	3	4	4	4	4	4	4	4	0
	Stigeoclonium sp.	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Cyanophyta	*Nostoc* sp.	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Planktothrix sp.	1	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	Oscillatoria sp.	1	2	0	0	2	2	0	0	2	2	2	0	0	0	2	2	0	0	0	0	0	0	0	2	0	0	1	0	2	2
Dinophyta	*Ceratium* sp.	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Euglenophyta	*Euglena* sp.	1	1	0	0	0	1	1	2	0	0	0	2	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0
	Phacus sp.	0	0	0	3	0	3	0	0	3	0	1	0	0	0	3	2	0	0	0	0	3	3	2	0	0	0	0	0	0	0

Figure 5.2 is a schematic representation of the Shannon Diversity Index (H') and Margalef Species Richness (d) results. In general, the Shannon diversity and Margalef richness index showed a low species diversity and richness at sites 1, 2 and 3 during the initial two sampling surveys when compared to the remaining sites. However, the diversity and richness subsequently increased from the third sampling survey.

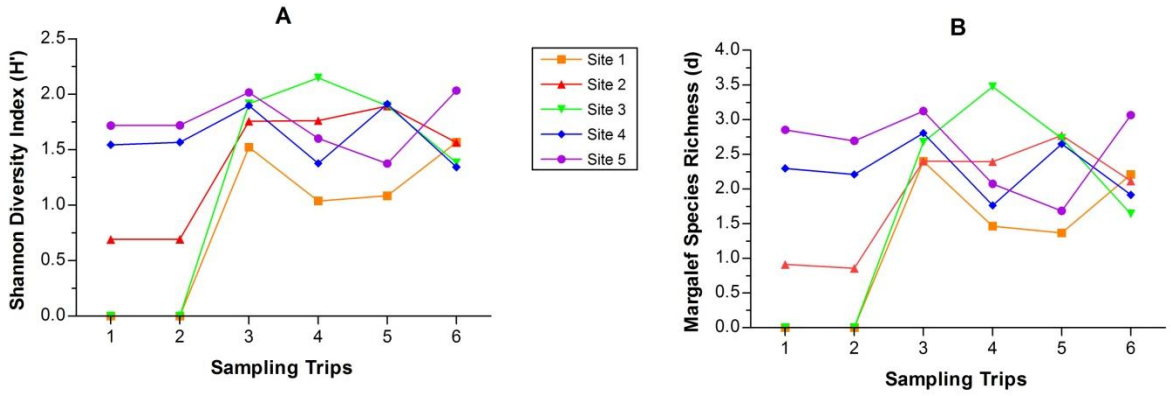

Figure 5.2: The Shannon diversity index (A) and Margalef species richness index (B) of the planktonic phytoplankton assemblages present at the five selected sites during the six sampling trips.

5.3.3. The interrelationship between the planktonic phytoplankton assemblages and water quality variables

Figure 5.3 represents an ordination plot of the planktonic phytoplankton assemblages with the respective water quality variables overlain. *Oedogonium* sp. and *Cyclotella* sp. (and to a lesser extent *Spirogyra* sp. and *Planktothrix* sp.) showed a close relationship with total phosphate, total nitrogen and oil concentrations in the sediment during the first two sampling trips at both sites 1 and 2. An increase in these genera was thus observed at

sites 1 and 2 during these two sampling surveys. It can also be noted that the increase in oil pollution was accompanied by an increase in phosphate and nitrogen.

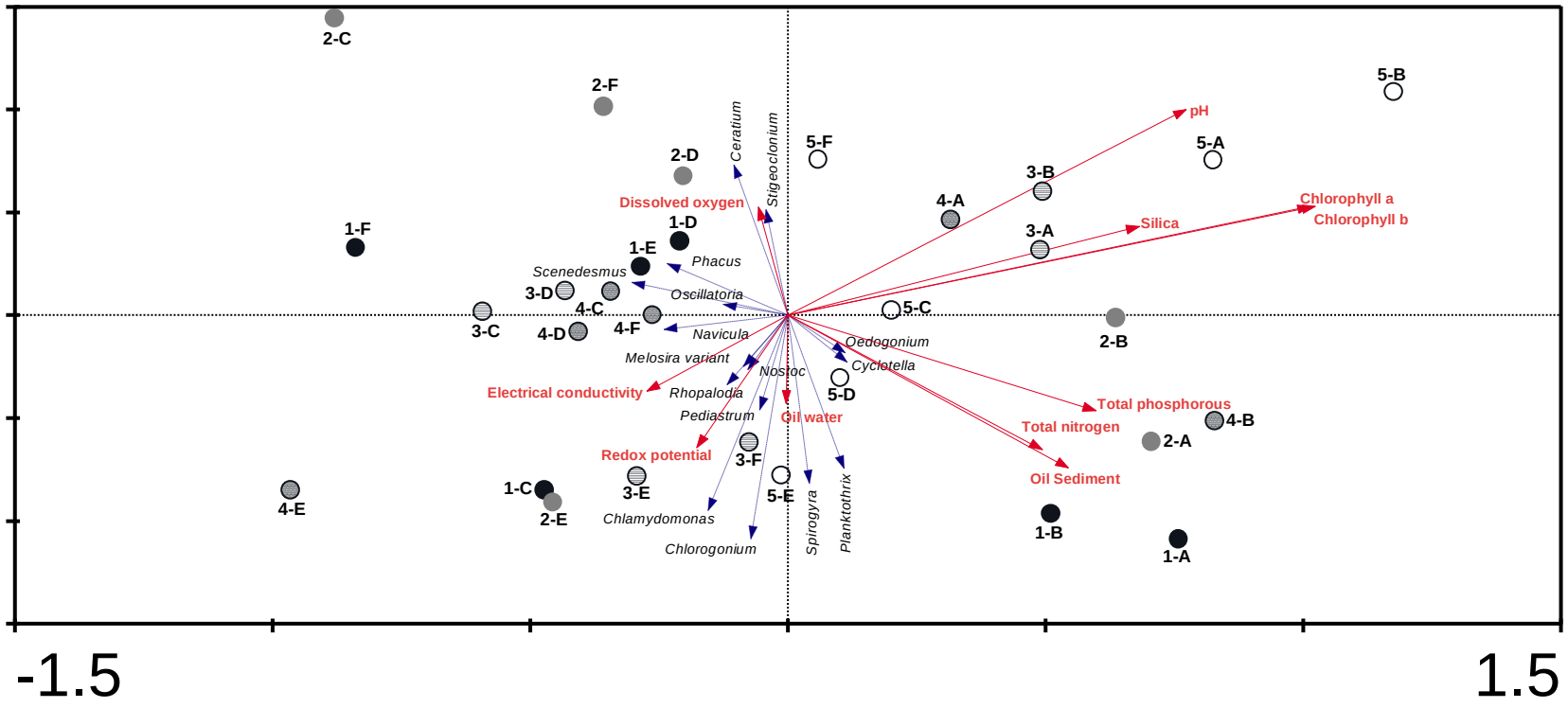

Figure 5.3: Redundancy analysis plot to determine interrelationships between planktonic phytoplankton assemblages and physico-chemical variables during the six sampling trips: A (October 2008), B (January 2009), C (April 2009), D (June 2009), E (August 2009) and F (October 2009). Percentage variance explained on the first axis is 34.9 % and on the second axis is 20.4 %.

5.4. Discussion

Because there was insufficient data on the phytoplankton assemblages for the wetland studied prior to the sunflower oil spill, variations in planktonic phytoplankton assemblages were used as indicators of wetland recovery during post spill conditions. Changes in the diversity of phytoplankton have been shown to be a useful indicator of water quality and can be used to determine recovery of biotic communities after anthropogenic impacts (De Lange, 1994; Ariyadej *et al*., 2004). According to Ortiz and Sáez (1997), nutrients influence phytoplankton biomass with phosphate being regarded as the limiting nutrient for phytoplankton growth, irrespective of whether other nutrients are available or not.

The average total phosphate concentrations obtained at all five sampling sites were below excessive pollution threshold of approximately ± 200 mg/l (Friedrich, 1996). From the results it is clear that this nutrient, together with total nitrogen increased in close relation with oil concentrations in sediment. The oil may thus be able to retain nutrients, while at the same time deplete the system from oxygen, because of the oil layer that forms on the surface water and thus prevent the input of atmospheric oxygen to the system. Ortiz and Sáenz (1997) reported that chlorophyll *a* concentrations are influenced by continuous contributions of phosphate. The ratio of planktonic phytoplankton fresh weight to chlorophyll *a* is accepted as an indirect measure of phytoplankton biomass (Voros and Padisak, 1991). This allows for the estimation of the trophic state of the water based on phytoplankton productivity parameters (Korneva and Mineeva, 1996). In this study the chlorophyll *a* concentration increased at the sites further away from where the oil was

spilled. This can be seen in Figure 5.2, where it is shown that the diversity and species richness increased at sites 3, 4 and 5. Variations in pH were reported six months after a vegetable oil spill in a salt marsh (Pereira *et al*. (2003) and three years after a petroleum oil spill in a reedbed wetland (Ji *et al*., 2007). However, the relatively similar pH levels measured throughout the study at the five samplings sites contradicted the data reported by Pereira *et al*. (2003) and Ji *et al*. (2007).

The close relationship between total phosphate, total nitrogen and vegetable oil pollution to the genera *Oedogonium* sp., *Cyclotella* sp., *Spirogyra* sp. and *Planktothrix* sp. was noted at sites 1 to 2 during the first two post oil spill surveys. Thus, it can be deduced that these species showed higher level of tolerance to the adverse effects of the oil spill and lower oxygen conditions when compared to the other species found in the wetland. Previous studies have indicated that *Oedogonium* sp. and *Cyclotella* sp. abundances increase with elevated nutrient concentrations and can be used as indicator of eutrophic conditions, whilst *Spirogyra* sp. is widespread in all freshwater ecosystems (van Vuuren *et al*., 2006). This is confirmed by the present study. Sites 3, 4 and 5 were similar throughout the study period and showed less variation in planktonic phytoplankton biomass diversity and richness as compared to sites 1 and 2. The main differences between these two groups of sites (or sections of the wetland) are due to the inlet located near sites 1 and 2 from where the oil flowed into the wetland. This oil spill, which resulted in an increase in total phosphate and total nitrogen concentrations, consequently affected the phytoplankton assemblages at these two sites. This was most noticeable during the first two post spill surveys when the planktonic phytoplankton diversity and

richness were low in comparison to the later sampling surveys. Subsequent surveys showed a gradual increase in planktonic phytoplankton diversity and richness, although it was still low. In a previous study conducted by Oberholster *et al*. (2010), the authors showed that some species (*e.g.*, *Chlamydomonas africana*) have a competitive edge over susceptible genera, and can thus become dominant in polluted water. In the present study, this genus, together with *Chlorogonium* sp., was in close relation with oil pollution in the water, as well as electrical conductivity and was thus also shown to be tolerant of these pollution effects. During this study, the filamentous cyanobacterium *Oscillatoria* sp. became more abundant after the second sampling survey. McComb and Davies (1993) reported in their study that the blooms of *Oscillatoria* sp. occur irregularly in polluted waters in summer and are thought to be characteristic of nutrient-poor waters and not necessarily in a response to eutrophication. This was another indication of the recovery of the wetland post spill, where the decrease in nutrients resulted in an increase in *Oscillatoria* sp. Furthermore, species that became more abundant after the second sampling survey was the cyanobacteria, *Nostoc* sp. This confirms previous studies by Fernandez-Pinaz *et al*. (1991) and Douterelo *et al*. (2004), who found this genus to be positively correlated with low nutrient concentrations.

5.5. Conclusions

An increase in planktonic phytoplankton diversity was observed during subsequent surveys of the wetland after the oil spill. This was an indication of the wetland recovering over time. Although there is no information available on the phytoplankton species composition before the oil spill, we found that this freshwater wetland was able to

increase its phytoplankton diversity and abundance and to start to recover within one year after such a spill. It is recommended that this wetland should be monitored on a continuous basis to establish a water quality benchmark and phytoplankton composition of the wetland on the long term after the oil spill. This will provide data that can be used for future management purposes in the event of a recurring oil spill, as well as to provide valuable data on changes within a freshwater wetland after a vegetable oil spill.

5.6 References

Adrian, R., Waltz, N., Hintze, T., Hoeg, S. and Rusche, R., (1999). Effects of ice duration on plankton succession during spring in a shallow polymictic lake. *Freshw. Biol.* **41**: 621-632.

American Public Association (APHA)., (1989). Standard methods for the examination of water and wastewater. (17th edition.-American Public Health Association), Washington DC.

APHA, AWWA. and WPCF, (1980). Standard methods for the examination of water and waste water , 15th ed. American Publica Health Association, Washington DC.

Ariyadej, C., Tansakul, R., Tansakul, P. and Angsupanich, S., (2004). Phytoplankton diversity and its relationship to the physiochemical environment in the Banglang Reservoir, Yala Province. *J. Sci. Technol*. **26**: 595-607.

Crump-Wiesner, H. J. and Jennings, A. L., (1975). Properties and effects of nonpetroleum oils. In "Proceedings of 1975 conference on prevention and control of pollution". American Petroleum Institute, Washington, DC, pp. 29-32.

De Lange, E. (1994). Manual for simple water quality analysis. International Water Tribunal (IWT) Foundation: Amsterdam.

Douterelo, I., Perona, E. and Mateo, P., (2004). Use of cyanobacteria to assess water quality in running waters. *Environ. Pollut.* **127**: 377-384.

Fernández-Piñas, F., Leganés, F, Mateo, P., Bonilla, I., (1991). Blue-green algae (cyanobacteria) as indicators of water quality in two Spanish rivers. In "Whitton, B. A., Rott, E. and Friedrich, G. (eds), Use of Algae for Monitoring Rivers". *Institut für Botanik, Innsbruck*, Austria: pp, 151–156.

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Friedrich, G., Chapman, D. and Beim, A., (1996) The Use of Biological Material *In*: Chapman, D (ed.) Water Quality Assessments: A Quide to Use of Biota, Sediments and Water in Environmental Monitoring, 2nd edition.

Frink, L., (1994). Statement on regulatory standards for the transportation of edible oil. *Tri-State Bird Rescue and Res.* Inc., January 30.

Hörnström, E., (2002). Phytoplankton in 63 limed lakes in comparison with the distribution in 500 untreated lakes with varying pH. *Hydrobiologia* **470**: 115-126.

Ji, G., Sun, T. and Ni, J., (2007). Impact of heavy oil-polluted soils on reed wetlands. *Ecol. Eng.* **29**: 272-279.

Kane, D. D., Gordon, S. I., Munawar, M., Charlton, M. N. and Culver, D. A., (2009). The planktonic index of biotic integrity (P-IBI): An approach for assessing lake ecosystem health. *Ecol. Indicators* **9**: 1234-1247.

Korneva, L. G. and Mineeva, N. M., (1996). Phytoplankton composition and pigment concentrations as indicators of water quality in the Rybinsky reservoir. *Hydobiologia* **322**: 255-259.

Margalef, R., (1951). Diversidad de species en las comunidades natuales. *Publicaciones del Instituto Aplicda de., Barcelona* **6**: 59-72.

McComb, A. J. and Davies, J. A., (1993). Eutrophic waters of southwestern Australia. *Fertilizer Res.* **36**: 105-114.

Miller, M. C., Alexander, V. and Barsdate, R J., (1978). The effects of oil spills on phytoplankton in an Arctic Lake and ponds. *Arctic* **31**: 192-218.

Oberholster, P. J., (2011). Using epilithic filamentous green algae communities as indicators of water quality in the headwaters of three South African rivers systems during high and medium flow periods. In "Zooplankton and phytoplankton". Kattel, G., (ed). *Chapter 5, Nova Science Publishers*, Inc, USA, pp, 121-125.

Oberholster, P. J., Blaise, C. and Botha, A.-M., (2010). Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland. *Ecotoxicol*. **19**: 1426-39.

Oberholster, P. J., Botha, A.-M. and Cloete, T. E., (2005). Using a battery of bioassays, benthic phytoplankton and the AUSRIVAS method to monitor long term coal tar contaminated sediment in the Cachela Poudre River, Colorado. *Water Res*. **39**: 4913-4924.

Ortiz, M. C. and Sáenz, J. R., (1997). Detergents and orthorphoshates inputs from urban discharges to Chetumal Bay, Quintana Roo, Mexico. *Bull. Environ. Contam. Toxicol*. **59**: 486-491.

Patrick, R. and Reimer, C. W., (1975). The diatoms of the United State Exclusive of Alaska and Hawaii, vol 2, Part 1. Monograph 13, Academy of National Sciences, Philadelphia, PA.

Pereira, M. G., Mudge, S. and Latchford, J., (2003). Polymerisation versus degradation of sunflower oil spilled in the marine environments. *Mar. Poll. Bull.* **46**: 1078-1081.

Porra, R. J., Thompson, W. A. and Kriedemann, P. E., (1989). Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. *Biochem. Biophys. Acta.* **975**: 384-394.

Shannon, C. E. and Weaver, W., (1949). The mathematical theory of communications. *University of Illinois Press, Urbana*, pp. 125.

Taylor, J. C., Harding, W. R. and Archibald, C. G. M., (2007). An illustrated guide to some common diatom species from South Africa. *WRC*, Report TT 282/07, Pretoria, pp. 1-178.

ter Braak, C. J. F. and Šmilauer, P., (2002). CANOCO reference manual and Canodraw for Windows User's Guide: Software for Canonical community ordination (version 4.5). Publishers, Microcomputer Power, Ithaca, New York, USA. pp. 500.

US EPA., (1997). Oil pollution prevention; non-transportation related onshore facilities. *Federal Register* **62**: 54508-54543.

US EPA, (1994). Oil pollution prevention; non-transportation-related onshore facilities; final rule. *Federation Registration*, pp. 59.

Van Vuuren, S., Taylor, J. C., Gerber, A. and van Ginkel, C., (2006). Easy identification of the most common freshwater algae. North-West University and Department of Water Affairs and Forestry, Pretoria, South Africa, pp. 1-200.

Varela, M., Bode, A., Lorenzo, J., Álvarez-Ossorio, M. T., Miranda, A., Patrocinio, T., Anadón, R., Viesca, L., Rodríguez, N., Valdés L., Cabal, J, Urrutia, Á, García-Soto, C., Rodríguez, M., Alvarez-Salgado, X. A. and Groom, S., (2006). The effect of the ‘‘Prestige’’ oil spill on the plankton of the N–NW Spanish coast. *Mar. Poll. Bull.* **53**: 272–286.

Voros, L. and Padisak, J., (1991). Phytoplankton biomass and chlorophyll-a in some shallow lakes in central Europe. *Hydrobiologia* **215**: 111-119.

Walter, K. D., (2006). Eutrophication and trophic state in rivers and streams. *Limnol.Oceanogr*. **51**: 671-680.

Wehr, J. D. and Sheath, R. G., (2003). Freshwater of North America: ecology and classification. *Academic Press*, Massachusetts, USA, pp. 1-834.

Willén, E., (2001). Phytoplankton and water quality characterization: Experiences from the Swedish large lakes Mälaren, Hjälmaren, Vätten and Vänern. *Ambio* **30**: 529-537.

CHAPTER 6

Effects of vegetable oil pollution on aquatic macroinverterbrate assemblage in a freshwater wetland and its use as a remediation tool

6.1. Introduction

Aquatic macroinvertebrates can be used to monitor aquatic environments because they are in constant contact with the surrounding aquatic environment (Chutter, 1998; De la Rey *et al*., 2004). They are useful for such tasks due to their visibility to the naked eye, ease of identification, rapid life cycle and their position in the food chain (Dickens and Graham, 2002). Generally, aquatic macroinvertebrates such as Dytiscidae, Elmidae, Hirudinae, Belostomatidae, Oligochaeta and Chironomidae are responsive to quick changes in water quality with respect to anthropogenic pollution (Mousavi *et al*., 2003; Rainio and Niemela, 2003; Arimoro *et al*., 2007; Oberholster *et al*., 2009). Aquatic macroinvertebrates have relatively short life cycles (Wu *et al*., 2004; Takahashi *et al*., 2008), and changes in the composition and structure of their communities are dependent on water quality (Marques and Barbosa, 2001; Nazarova *et al*., 2008). Most importantly, it is known that aquatic macroinvertebrates are sensitive to oil spills and the effect depends on the type of hydrocarbon-sensitive species present in the aquatic system (Gesteira *et al*., 2003; Oberholster *et al*., 2010).

When oil is spilled in an aquatic environment it can be transformed through various biological, chemical and physical weathering processes. These processes can then change the behaviour, composition, exposure routes and toxicity of the oil (USDOC/NOAA, 1996). The biodegradation of oil is affected by dissolved oxygen, pH, occurrences of nutrients in the proper proportions, soil types, the dispersal of oil, the type of oil, as well

as the concentration of undissociated fatty acids in the water (Cornish *et al.*, 1993; Rigger, 1997).

Although there are a lot of information available on the effects of an oil spill on marine, river and coastal marsh environments (*e.g.*, Zoun *et al.*, 1991; Mudge *et al.*, 1993; Mudge, 1995), not much is known about the effect of such a spill on a freshwater wetland. In addition, the effect of oil contamination within an aquatic environment is difficult to evaluate because of the unavailability of data prior to a spillage (Bury, 1972; Oberholster *et al.*, 2010), but it is known that an oil spill can cause severe damage to sensitive aquatic organisms and ecosystems (Mudge, 1995). There is also a known delay between exposure during the oil spill and the expression of adverse effects. This is mainly depended on the toxic constituents within the oil in question or due to the formation of toxic by-products during degradation within the environment (USEPA, 1997). According to Poulton *et al*. (1997), oil spills in rivers are known to be associated with an increase in tolerant aquatic macroinvertebrates (*e.g.*, Chironomidae and Oligochaeta). The reduction of sensitive taxa such as Trichoptera and Plecoptera in response to such a spill has also been observed and is likely to persist for a considerable period of time (Lytle and Peckarsky, 2001). Different types of vegetable oils and fats, namely soyabean, rapeseed, palms and coconut, show similar characteristics in aquatic environments (Frink, 1994). When these oils enter an aquatic environment their fatty acid constituent enables it, depending on the prevailing physicochemical properties, or either float on top of the water surface and become solubilised, emulsify in the water column or settle on the sediment (USEPA, 1997).

Currently, information relating to the effects of vegetable oil spills on freshwater wetlands including their associated biota is very limited. Thus, the aim of this study is to determine the temporal and spatial changes of aquatic macroinvertebrates and selected abiotic variables at various sites within a wetland at the Con Joubert Bird Sanctuary after a sunflower oil spill. Through the information generated by this study, the authors hope to improve the current knowledge base on the aquatic environmental effects of vegetable oil spills in freshwater wetlands.

6.2. Materials and methods

6.2.1. Study area

This study focused on a wetland, approximately 25 ha in size, in the Con Joubert Bird Sanctuary (26°11′ 20″S 27°41′03″E) within the Randfontein municipality, South Africa. In the beginning of September 2007 a sunflower oil storage tank collapsed, causing a spill of 250 ton of sunflower oil. The spilled oil flowed into the wetland via storm water drains. Five sampling sites were chosen to be representative of the entire wetland. The location of the study area as well as the respective sampling sites is presented in Figure. 6.1. Site 1 was located on the eastern side of the wetland, close to a stormwater inflow from where the sunflower oil spillage occurred. The marginal vegetation at this site consists mainly of *Typha capensis*, *Phragmites australis* and *Lemna* sp. Polymerized oil was observed floating on the water surface at this site. Site 2 was located in the southern part of the wetland and is mainly characterized by *Juncus effuses*, *Persicaria decipiens*, *Schoenoplectus brachyceras*, *Azolla pinnata* and *Lemna gibba*. Sites 3 to 5 were located

on the western side of the wetland and consisted mainly of *Azolla pinnata*, *Juncus effuses*, *Typha capensis*, *Lemna gibba* and *Persicaria decipiens*. The substrate of all five sampling sites was mainly made up of clay and organic material. The average water depth over the study period at all of the sites was about 20 cm. Sampling was conducted over a period of one year that includes the following months: October 2008, and January, April, June, August and October 2009.

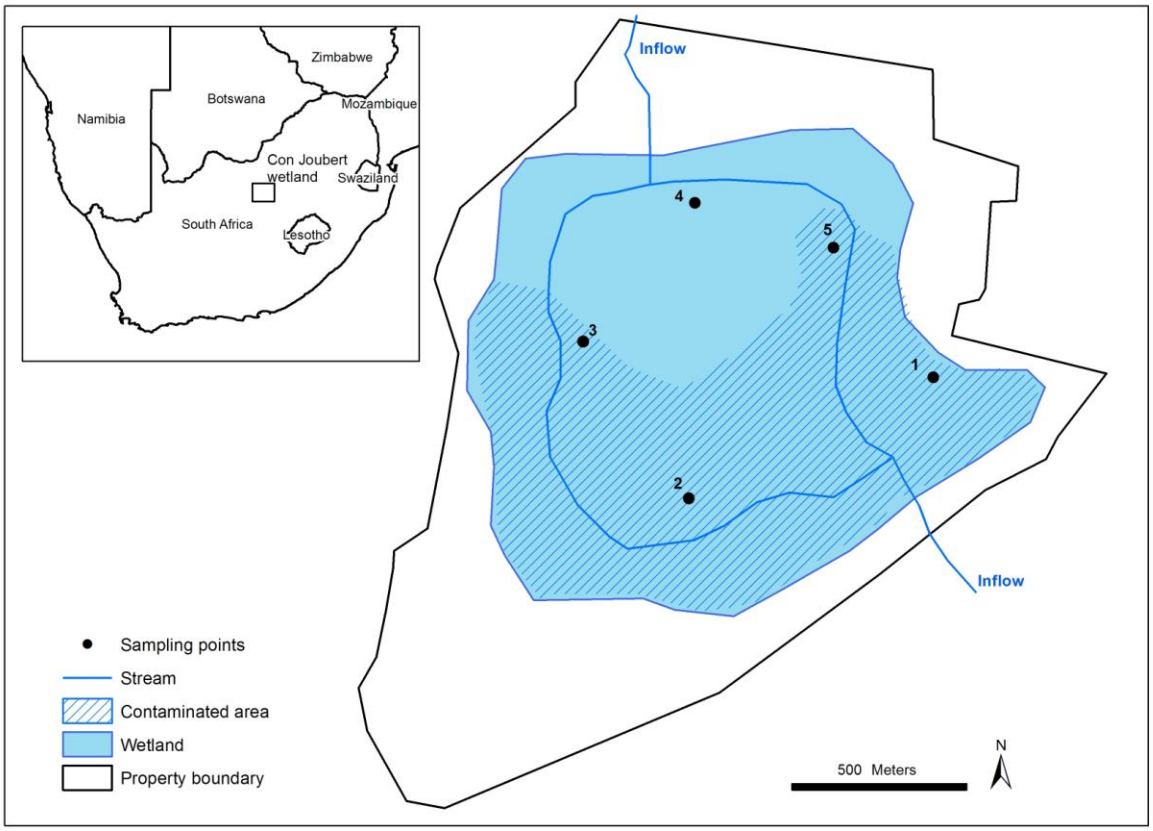

Figure 6.1: Map of the Con Joubert Bird Sanctuary wetland showing the location of the five sampling sites and the position of the inflowing strormwater inlets. Inset shows the location of the map area in South Africa. The visually observed extent of the oil pollution after the spillage occurred is also indicated

6.2.2. Water and sediment

Various *in situ* measurements such as pH, electrical conductivity and dissolved oxygen were measured in the field using a Hach™ sension 156 portable multiparameter. Duplicate water samples were collected at the surface and at the bottom using a grab bottle sampler. The grab bottle sampler was cleaned with alcohol after sample collection at each site to prevent possible cross contamination of oil. Both the surface and bottom grab bottle samples were pooled together to form one integrated sample and used to fill three pre-cleaned containers. These containers were kept in a cold and dark environment to minimize risks of chemical and biological processes occurring in the bottles whilst in transit to the laboratory. Various chemical parameters, such as total phosphate (expressed as PO_4^{3-}), total nitrogen (expressed as the sum of NH_4^+, NO_3^- and NO_2^-) and silica concentrations were determined in each water sample. Total nitrogen and total phosphate concentrations were measured using the most appropriate spectrophotometric methods (APHA, AWWA and WPCF, 1992).

Chlorophyll *a* and *b* were measured using sub-samples of the integrated water samples collected. Chlorophyll was extracted using 80 % acetone after an overnight incubation. The chlorophyll *a* and *b* contents were determined spectrophotometrically at 664 nm and 647 nm wavelengths, respectively, according to Porra *et al*. (1989).

Triplicate sediment samples (upper 5 cm) were taken randomly at each sampling site using a perspex sediment corer (5 cm in diameter). This was done in order to determine the spatial extent of oil contamination within the sediment profile (Oberholster *et al*.,

2005). The sediment corer was cleaned with alcohol after each site was sampled to prevent any oil cross contamination. Sunflower oil in the sediment and water column was determined according to USEPA (1994).

6.2.3. Aquatic macroinvertebrates

Aquatic macroinvertebrates were collected using a sweep net (300 x 300 mm frame, 1000 µm mesh), which included sampling all of the available biotopes (MPCA, 2007; de Klerk and Wepener, 2011). The macroinvertebrate samples were immediately preserved in 70 % ethanol and later sorted using a 75 µm mesh sieve. The macroinvertebrates were identified to family level according to Merritt and Cummins (1996) and Thorp and Covich (2001) by randomly sub-sampling one-tenth of the total sample under a dissection microscope at 20 times magnification. These samples were then enumerated. The macroinvertebrates collected within a sub-sample of the 5 cm diameter sediment core sample were also removed, identified and enumerated.

6.2.4. Statistical analyses

Univariate statistics such as Shannon's diversity index (Shannon, 1948) was used to determine the aquatic macroinvertebrate diversity at each site with the software program PRIMER version 6.0 (Clarke and Gorley, 2006). Both species richness and equitability components are incorporated in the Shannon diversity index. Thus, to obtain a measure of species richness which is based on a presumed linear relation between the numbers of species and logarithm of the number of individuals, Margalef's index (d) was used (Margalef, 1951). Multivariate statistics, such as a redundancy analysis (RDA) plot was

constructed to determine the relationship between the macroinvertebrate community structures identified at each site and the respective environmental variables measured. These plots were expressed two-dimensionally where distances among them reflect their relative similarity or dissimilarity. In an ordination plot the arrows may be used to indicate the direction of the steepest increase, as well as if any correlation between variables exist depending on whether the angles between them are acute or not. Significant spatial differences in the concentrations of the selected abiotic variables between different sites were determined using a two-way analysis of variance (ANOVA). Significance was assumed as a probability level of $p \leq 0.05$. The temporal data was also subjected to Kendall correlation analyses to determine significantly ($p \leq 0.05$) positive (Tau value is positive) and negative (Tau value is negative) trends.

6.3. Results

The oil concentrations in the water and sediment samples are presented in Table 6.1. The oil concentrations found within the sediment remained relatively consistent at the different sites during the course of the study, with a clear spike in concentrations during the second survey. The site at the source of the oil spill had the highest average oil concentration in the sediment during the course of the study (site 1 = 25.73 mg/kg) compared to the other sites where the concentrations ranged between 1.6 mg/kg and 5.55 mg kg^{-1}. The oil concentration at site 1 showed a significant negative trend ($p \leq 0.05$) over time. Generally, the sunflower oil concentration in the water column decreased after the initial post spill survey after which it increased again during the third survey (Table 6.1). These concentrations then steadily decreased during the course of the study. Site 1

also had the highest average oil content in the water column during the course of the study (3.75 mg/l), whereas the rest of the sites ranged between 1.04 mg/l and 2.54 mg/l.

Table 6.1: Oil concentrations in the water and sediment samples collected during the study at the five selected sampling sites.

Sites	October 2008	January 2009	April 2009	June 2009	August 2009	October 2009
	Oil concentration in water (mg/l)					
1	7.5	1.25	6.125	4.375	2.75	0.5
2	2.64	0.125	2.125	4.625	3	0.125
3	9.5	0.125	0.825	3	1	0.625
4	6.75	0.125	1.625	4	2.625	0.125
5	0.25	0.125	0.625	3.875	1.25	0.125
	Oil concentration in sediment (mg/kg)					
1	20.4	117.5	7.5	3.875	4.625	0.5
2	0.005	5	2.125	3.5	1.875	0.625
3	0.065	27.5	0.375	3.375	1.375	0.625
4	0.085	22.5	1.375	2.25	3.875	0.625
5	0.15	2.5	0.625	4.25	1.25	0.825

The abiotic parameters measured within the sediment and water column showed different spatial and temporal trends throughout the study period (Fig. 6.2). The pH levels at all sites increased initially during the second post-spillage survey, after which it decreased again during the consecutive trips and remained relatively consistent and only increased again during the last survey. Overall, the average pH levels at the different sites varied between 7.5 at site 1 and 9.3 at site 5. The pH levels measured at these two sites were also found to be significantly different ($p \leq 0.05$). Overall, sites 1 and 2 had lower pH levels, compared to the rest of the sites, during the first couple of surveys. Although a slight increase in electrical conductivity was noticed as the study progressed, no clear

temporal trend was observed and in general the sites had similar conductivity levels which generally remained below 1 000 µS/cm. This threshold was only exceeded at some sites during the last survey. Dissolved oxygen concentrations decreased at all of the sites after the initial post spill survey after which it remained relatively constant. However, a spike in dissolved oxygen concentrations was noticed during the fourth survey at each of the respective sites, but the degree of the spike varied between the different sites. Silica concentrations showed no clear temporal trend and the average concentrations fluctuated between 3.3 mg/l at site 4 and 5.3 mg/l at site 1. These minimum and maximum concentrations recorded at the respective sites were found to be significantly different ($p \leq 0.05$). Total phosphate and total nitrogen concentrations decreased at the sites closest to the source of the oil spill (sites 1 and 2) from the initial post spill survey, after which total phosphate and total nitrogen concentrations remained relatively constant at all of the sites through the course of the study. The total nitrogen and total phosphate concentrations at sites 2 and 5 showed a significant negative trend ($p \leq 0.05$) over time. Sites 1 and 2 had higher total nitrogen and total phosphate concentrations during the first post-spillage survey when compared to the other sites. The chlorophyll *a* and *b* concentrations decreased at sites 3, 4 and 5 during consecutive field surveys, after which it remained consistent. The sites closest to the origin of the oil spill had the lowest chlorophyll *a* concentrations, namely site 1 = 4.2 µg/l and site 2 = 4.8 µg/l, and chlorophyll *b* concentrations, namely site 1 = 3.4 µg/l and site 2 = 3.7 µg/l.

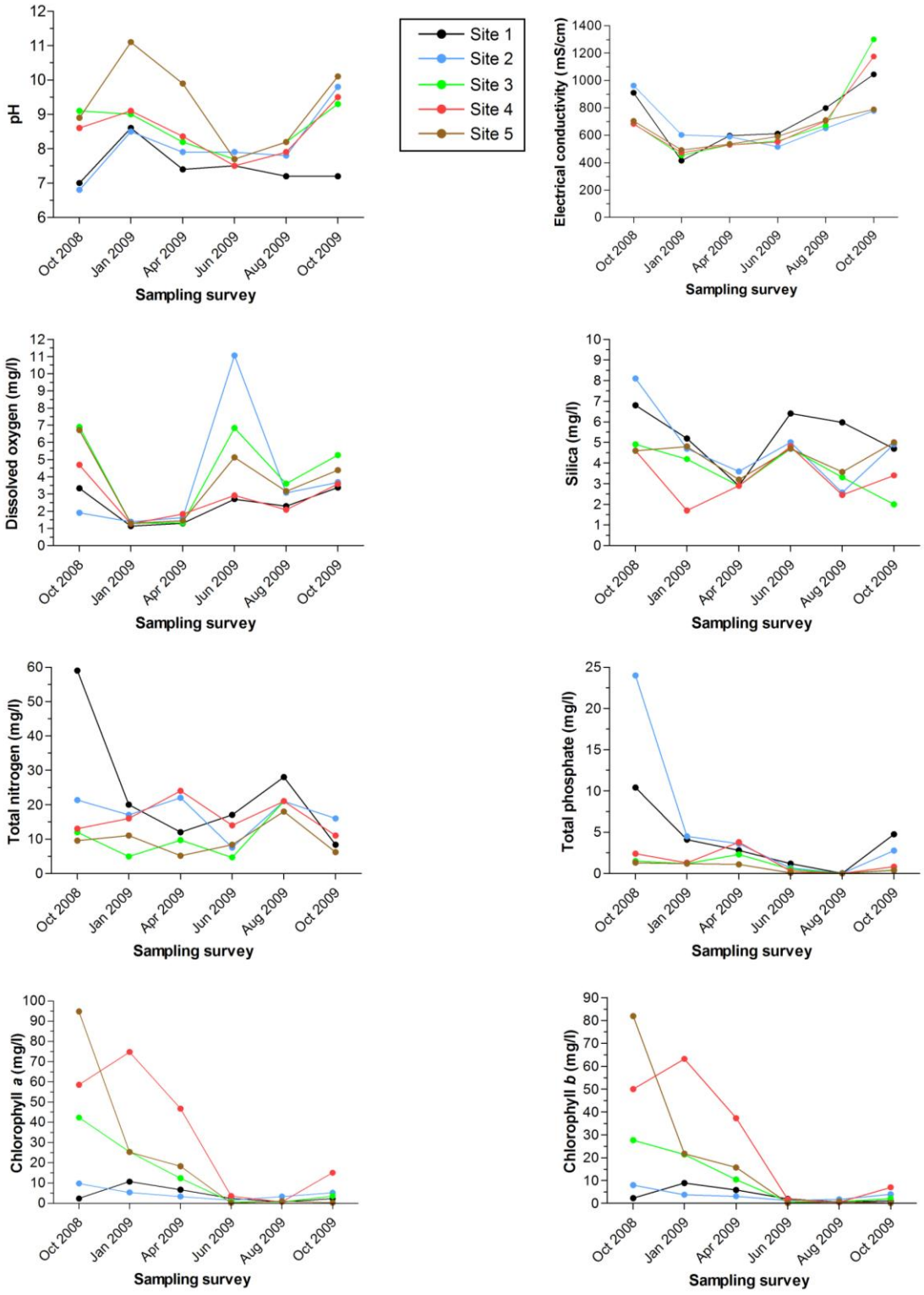

Figure 6.2: Various chemical parameters measured throughout the 12-month monitoring period in the water column and sediment.

The aquatic macroinvertebrate population was characterized by a low diversity between sampling sites with only 23 families of macroinvertebrates recorded during the 12-months study period (Table 6.2). The community was distributed amongst the orders Hemiptera, Diptera, Coleoptera, Trichoptera, Odonata, Ephemeroptera, Turbellaria, Lepidoptera, Amphipoda, two families of Mollusca (Lymnaeidae and Planorbidae) and two families of Annelida (Hirudinae and Oligochaeta) (Table 6.2).

Table 6.2: Average abundance of macroinvertebrate families at five sampling sites in the wetland Con Joubert Bird Sanctuary from October 2008 to October 2009 (n = 6). Diversity indices of each sampling site are indicated at the bottom of the table.

Orders	Families	Site 1	Site 2	Site 3	Site 4	Site 5
Diptera	Chironomidae	0	2	39	10	6
	Culiscidae	1	0	0	0	0
	Muscidae	0	0	1	0	1
	Psychodidae	4	0	8	3	1
	Syrphidae	0	0	1	1	0
Hemiptera	Belastomatidae	6	17	1	3	15
	Corixidae	8	69	1	7	46
	Hydrometridae	0	0	0	0	4
	Nepidae	1	0	0	0	2
	Notonectidae	5	2	0	0	1
Coleoptera	Dytiscidae	3	2	1	2	13
	Elmidae	4	0	1	0	9
	Hydrophilidae	8	26	6	30	7
Annelida	Hirudinae		86	3	58	89
	Oligochaeta	26	52	15	13	7
Mollusca	Lymnaeidae	10	0	0	8	21
	Planorbidae	0	0	0	2	1
Odonata	Calopterygidae	0	0	1	0	0
Trichoptera	Ecnomidae	0	0	0	7	0
Ephemeroptera	Baetidae	0	1	2	0	0
Lepidoptera	Pyralidae	1	0	2	0	0
Amphipoda	Amphipoda	2	0	0	0	0
Turbellaria	Turbellaria	0	0	0	2	0
	Shannon index (H')	1.62	1.63	1.76	1.91	1.91

Temporal changes in macroinvertebrate diversity and richness at each site are presented in Figure. 6.3. No temporal trend could be observed. The highest average diversities (H'= 1.98) and richness (d = 1.54) during the course of the study was noticed at site 3, whilst the lowest diversity (H'=1.16 and 1.19) and richness (d = 1.53 and 1.88) was found at sites 1 and 2, respectively. Figure 6.4 is an ordination plot and presents the changes in the aquatic macroinvertebrate community structures with the changes in the abiotic variables overlain. From this plot it can be seen that there is a strong relationship between the increase in oil pollution (both in the water and sediment) with the increase in Chironomidae and Pshycodidae abundances. The data generated showed that the increase in total nitrogen and total phosphate occurred along with the increase in oil pollution. These increases resulted in the grouping together of most of site 1 and site 2's early sampling points (namely October 2009, January 2009 and April 2009), indicating that these sites, especially site 1, were the worst affected by the oil pollution. It can also be observed that an increase in oil concentrations resulted in a decrease in pH, as well as chlorophyll *a* and *b* concentrations. This is due to the increase in these concentrations in an opposite direction to that of the oil concentrations.

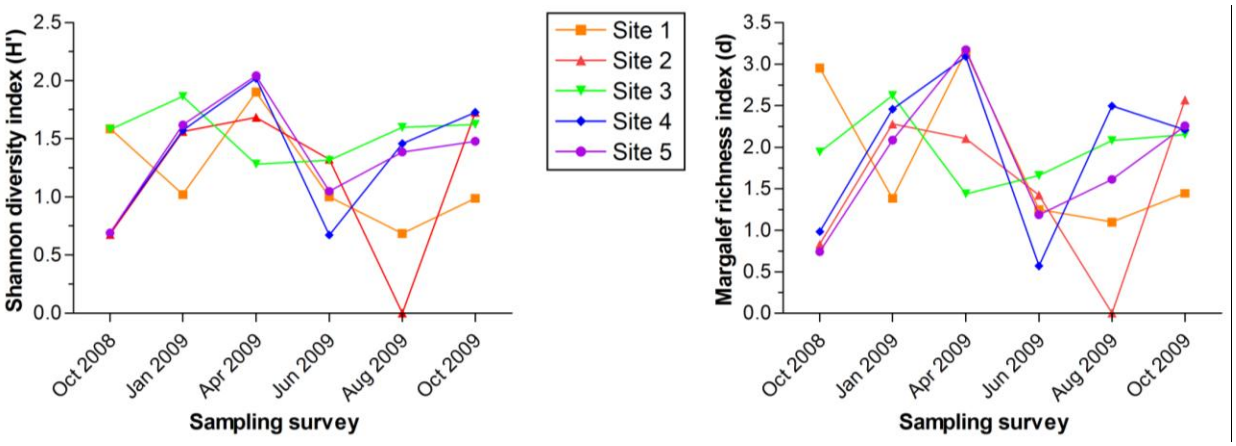

Figure 6.3: The Shannon diversity index (H') and Margalef richness index (d) of the aquatic macroinvertebrate communities present at different sampling sites (1-5).

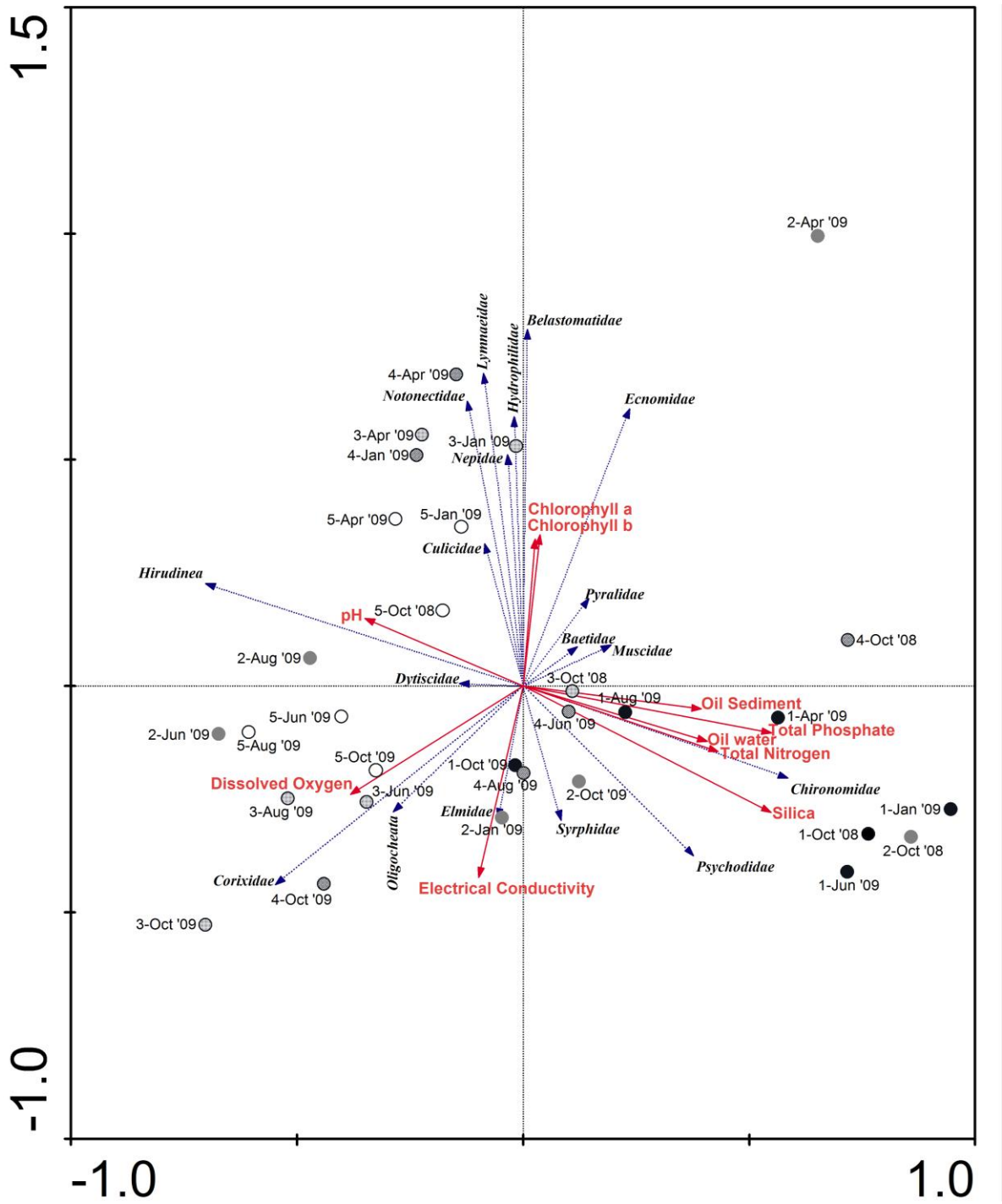

Figure 6.4: A redundancy analysis plot showing the similarity between sites based on macroinvertebrate abundance data with water quality variables superimposed (the percentage variance explained on the first axis is 34.9 % and on the second axis is 20.4 %).

6.4. Discussion

Currently there is little (if any) information available on the impacts of vegetable oil spills in freshwater wetlands. Previously, incidents of vegetable oil spills have only been reported in rivers, salt marshes and marine environments. Studies carried out on these events indicated that the spilled oil may undergo polymerization and persist for up to six years within the environment (Mudge, 1997). Thus, the physical properties of vegetable oils, as well as their environmental fate and effect, appear to be very similar to that of petroleum oils (Crump-Wiesner and Jennings, 1975). During this study the oil concentrations measured in the water was high during the first post-spillage field survey, but decreased during the second survey. This is inversely related to the oil concentrations measured in the sediment which was initially low, but spiked during the second survey. This phenomenon was due to the oils which settled out onto the sediments (Mudge, 1997; USEPA, 1997) during the second survey. Overall, the oil concentrations in the water and sediment decreased during the course of the study, although some fluctuations do occur, with the concentrations in the sediment having a significantly negative trend over time. These results indicate that most of the spilled oil settled out onto sediment relatively quickly after the spillage has occurred from where it degraded over time.

The initial increase in oil concentration measured in the water column corresponded to the initial increase in total nitrogen and total phosphate concentrations (Table 6.1 and Fig. 6.2), which correspond with the multivariate analysis of the abiotic variables (Fig. 6.4). The sites closest to the source of the spillage, namely sites 1 and 2, had the most

noticeable increase in total nitrogen and total phosphate during the initial survey, but also had the lowest chlorophyll *a* and *b* concentrations. This indicates that vegetable oil increases the total nitrogen and total phosphate concentrations in the water column and also, as in the case of crude oil, inhibits the growth of microalgae (Fabregas *et al*., 1984), which resulted in the decreased concentrations of chlorophyll. The pH levels found at sites 1 and 2 were also low during the initial survey, when compared to the other sites, but appeared to stabilize and follow a similar trend to that observed at the other sites during the course of the study. In the case of the pH values at site 1, which was the closest to the spillage, and site 5, which was the farthest from the spillage, the values were found to vary significantly. This corresponds with the decrease in chlorophyll observed at these sites as increased photosynthesis of the aquatic vegetation (including algae) results in an increase of the pH in standing water (Barkay *et al*., 1989; Mason *et al*., 1995; DWAF, 1996; Ravichandran, 2004). No clear relationship could be observed between the temporal changes in vegetable oil concentration and that of electrical conductivity, dissolved oxygen and silica concentrations and the changes observed may be as a result of seasonal variability as there would be an increase in productivity during certain seasons, which in turn will impact on certain abiotic factors, such as dissolved oxygen, etc. (Dallas and Day, 1993). Overall, it is clear that the spilled oil increased the concentrations of total nitrogen and total phosphate and decreased pH levels (either directly or indirectly) by impacting microalgae, which resulted in decreased chlorophyll concentrations.

According to Dallas and Day (1993), water quality is one of the most important factors which influence an aquatic ecosystem's integrity as the distribution of aquatic freshwater organisms is controlled mainly by water quality characteristics, including dissolved oxygen, acidity and nutrient content. Aquatic macroinvertebrates are an important component in aquatic foodwebs which link organic matter and nutrients with higher trophic levels (Yoshimura *et al*., 2006). Various environmental variables are known to influence macroinvertebrate assemblages (Collier, 1995; Wright, 1995) and result in changes in their density and/or biomass (Idyll, 1943; O'Connel and Campbell, 1953). From Figure 6.4 it can be seen that at sites 1 and 2, which were near the source of the spillage, that there is a distinct correlation between the relative dominance of Chironomidae and Psychodidae, and to a lesser extent Syrphidae, and the increase in oil within the sediment and water during the initial post-spillage field surveys. These changes highlight modifications in the composition of dominant taxa (Leland *et al*., 1989), for example shifts from sensitive to less sensitive species (Norris *et al*., 1982; Clements, 1994). The relationship illustrated in Figure 6.4 where an increase in Chironomidae and Psychodidae was observed along with an increase in oil concentrations in the sediment and water indicates their relative tolerance to this type of pollution and their ability to thrive under these circumstances. These findings confirm previous studies that noted that Chironomidae are generally linked to low dissolved oxygen concentrations and high organic matter (Ocon *et al*., 2008). These results also infer the similar environmental fate of vegetable oil pollution to that of petroleum as Oberholster *et al*. (2005) also found higher abundances of Chironomidae in a petroleum contaminated environment. This is mainly due to the fact that Chironomidae is adaptable

in various water qualities, and thus regarded as organic pollutant-tolerant organisms (Camur-Elipek *et al*., 2010). Aquatic macroinvertebrates belonging to the Chironomidae family are also able to tolerate the effects of pollution, such as low oxygen concentrations, because they contain haemoglobin (Weigel *et al*., 2002), and as a result may exploit stressed systems in the absence of competitors (Koryak *et al*., 1972).

Another taxon that also showed a relatively good relationship with that of the oil concentrations was Syrphidae. This family is known to thrive well in shallow polluted waters that permit the tip of the extended caudal respiratory tube to be projected just above the surface (James, 1979; Arimoro *et al*., 2007). On the other hand, Oligochaeta did not show any relationship with increasing vegetable oil pollution and although they are known to thrive in polluted environments, this family appeared to be more present at the less impacted, more oxygenated sites. Aquatic macroinvertebrates are especially sensitive to organic pollution (Assmuth and Penttilä, 1995) and are capable of integrating these effects during their lifetime (Ten Brink and Woudstra, 1991). Macroinvertebrate assemblages dominated by high numbers of a single invertebrate family are generally indicative of stressed ecosystems as these organisms are less sensitive to various impacts than any other major macroinvertebrate taxa (Warwick and Clarke, 1993). Thus, from the data generated it was evident that macroinvertebrate assemblage can be a useful management tool for remediation measures during post spill conditions. However, it must be recognised that the Con Joubert wetland is not an isolated waterbody, but the system was fed by urban stormwater that might have played a role in the changes of macroinvertebrate assemblages. Other abiotic factors that could have played a role as

well were the movement of the water column oil by wind action especially in the open water zones of the wetland, as well as pollutants from the incoming stormwater drainage.

6.5. Conclusions

Vegetable oil pollution in freshwater wetlands has to our knowledge not been reported previously and its environmental fate and effects are relatively unknown. Spatial and temporal changes in selected abiotic factors have been observed, and as a result total nitrogen, total phosphate and chlorophyll *a* and *b* concentrations, as well as pH levels were observed to be affected by an increase in vegetable oil pollution. A shift in the macroinvertebrate assemblages studied at the selected sites was noticed with a clear increase in tolerant invertebrate taxa noticed at the sites that was most impacted (sites 1 and 2). As a result, two core taxa, namely Psychodidae and Chironomidae, and to a lesser extent Syrphidae, were identified as potentially good indicators of changes in the macroinvertebrate assemblage during post spill conditions. These indicator taxa can be a useful management tool to determine if freshwater wetlands impacted by vegetable oil spills are recovering after employing remediation measures.

6.6. References

APHA (American Public Health Association), AWWA (American Water Works Association), and WPCF (Water Pollution Control Federation)., (1992). Standard Methods for the Examination of Water and Wastewater (19th edn) APHA, AWWA, and WPCF, Washington DC, USA.

Arimoro, F. O., Ikomi, R. B. and Iwegbue, C. M. A., (2007). Water quality changes in relation to Diptera community patterns and diversity measured at an organic effluent impacted stream in the Niger Delta, Nigeria. *Ecol. Indicators* **7**: 541-552.

Assmuth, T. and Penttilä, S., (1995). Characteristics, determinants and interpretations of acute lethality in Daphnids exposed to complex waste leachates. *Aquat. Toxicol*. **31**: 125-141.

Barkay, T., Liebert C. and Gillman, M., (1989). Environmental significance of the potential for mer (Tn21)-mediated reduction of Hg^{2+} to Hg^{0} in natural waters. *Appl. Environ. Microbiol*. **55**: 1196-2002.

Bury, R. B., (1972). The effects of diesel fuel on a stream fauna. *Cal. Dept. Fish. Game* **58**: 291-295.

Camur-Elipek, B., Arslan, N., Kirgiz, T., Oterler, B., Guher, H. and Ozkan, N., (2010). Analysis of benthic macroinvertebrates in relation to environmental variables of Lake Gala, a National Park of Turkey. *Turk. J. Fish and Aquat. Sci*. **10**: 235-243.

Chutter, F. M., (1998). Research on the Rapid Biological Assessment of Water Quality: Impacts in Streams and Rivers. WRC Report No. 422/1/98. *WRC*, Pretoria, South Africa.

Clarke, K.R., Gorley, R.N., 2006. Primer v6: User Manual or Tutorial. PRIMER-E, Plymouth.

Clements, W. H., (1994). Benthic invertebrate community responses to heavy metals in the upper Arkansas River basin, Colorado. *J. North Am Benthol. Soc* **13**: 30-44.

Collier, K. J., (1995). Environmental factors affecting the taxonomic composition of aquatic macroinvertebrate communities in lowland waterways of Northland, New Zealand. *NZ. J. Mar. Freshwater Res.* **4**: 453-465.

Cornish, A., Battersby, N.S., Watkinson, R.J. 1993. Environmental fate of mineral, vegetable and transesterified vegetable oils. *Pestic. Sci*. **37**: 173–178.

Crump-Wiesner, H. J. and Jennings, A. L., (1975). Properties and effects of nonpetroleum oils. In: "Proceedings of 1975 conference on prevention and control of pollution". American Petroleum Institute, Washington DC, pp. 29-32.

Dallas, H. F. and Day, J. A., (1993). The effect of water quality variables on riverine ecosystems: a review. WRC Report No. TT 61/93. *WRC*, Pretoria, South Africa.

De Klerk, A. R. and Wepener, V., (2011). The influence of biotope and sampling method on the assessment of the invertebrate community structure in endorheic reed pans in South Africa. *Afr. J. Aquat Sci*. **36**: 67-74.

De la Rey, P. A., Taylor, J. C., Laas, A., Van Rensburg, L. and Vosloo, A., (2004). Determining the possible application value of diatoms as indicators of general water quality: A comparison with SASS 5. *Water SA* **30**: 325-332.

Dickens, C. W. S. and Graham, P. M., (2002). The South African Scoring System (SASS) version 5 rapid bioassessment method for rivers. *Afri. J. Aquat. Sci*. **27**: 1-10.

Department of Water Affairs and Forestry (DWAF)., (1996). South African Water Quality Guidelines. 7: Aquatic Ecosystems (1st edn). *DWAF*, Pretoria, South Africa.

Fabregas, J., Herrero, C. and Veiga, M., (1984). Effect of Oil and Dispersant on Growth and Chlorophyll a Content of the Marine Microalga Tetraselmis suecica. *Appl. Environ. Microbiol*. **47**: 445-447.

Frink, L., (1994). Statement on regulatory standards for the transportation of edible oil. *Tri-State Bird Rescue Res*., pp. 30.

Gesteria, G. J. L., Dauvin, J. C. and Fraga, M. S., (2003). Toxonomic level for assessing oil spill effects on soft-bottom sublittoral benthic communities. *Mar. Poll. Bull*. **46**: 562-572.

Idyll, C. P., (1943). Bottom fauna of portions of the Cawichan River, B.C. *J. Fish. Res. Board Can.* **6**: 133-139.

James, A., (1979). The value of biological indicators in relation to other parameters of water quality. In: Biological indicators of water quality, A. James and L. Evison (eds.) A Wiley-Interscience publication, pp. 1-16.

Koryak, M., Shapiro, M. A. and Sykora, J. L., (1972). Riffle zoobenthos in streams receiving acid-mine drainage. *Water Res*. **6**: 1239-1247.

Leland, H. V., Fend, S. V., Dudley, T. L. and Carter, J. L., (1989). Effects of copper on species composition of benthic insects in a Sierra Nevada, California, stream. *Freshwater Biol*. **21**: 163-179.

Lytle, D. A. and Peckarsky, B. A., (2001). Spatial and temporal impacts of a fuel spill on stream invertebrates. *Freshwater Biol*. **46**: 693-704.

Margalef, R., (1951). Diversidad de species en las comunidades natuales. *Publicaciones del Instituto Aplicda de Barcelona* **6**: 59-72.

Marques, M. M. and Barbosa, F., (2001). Biological quality of waters from an impacted tropical watershed (middle Rio Doce basin, southeast Brazil), using benthic macroinvertebrate communities as an indicator. *Hydrobiologia* **457**: 69-76.

Mason, R. P., Morel, F. M. M and Hemond, H. F., (1995). The role of microorganisms in elemental mercury formation in natural water. *Environ. Sci. Technol*. **80**: 775-787.

Merrit, R. W. and Cummins, K. W., (1996). An introduction to the aquatic insects of North America. (3rd edn). *Kendall/Hunt, Dubuque, Iowa*, USA.

Mousavi, S. K., Primicerio, R. and Amundsen, P-A., (2003). Diversity and structure of Chironomidae (Diptera) communities along a gradient of heavy metal contamination in a subarctic watercourse. *Sci. Total Environ*. **307**: 93-110.

MPCA (Minnesota Pollution and Control Agency)., (2007). Macroinvertebrate community sampling protocol for depressional wetland monitoring sites. Biological Monitoring Program. Standard operating procedures. *MPCA*, Minnesota, USA.

Mudge, S. M., (1997). Can vegetable oils outlast minerals oils in the marine environment? *Mar. Poll. Bull*. **34**: 213.

Mudge, S. M., (1995). Deleterious effects from accidental spillage of vegetable oils. *Spill Sci. Technol. Bull*. **2**: 187-191.

Mudge, S. M. and Salgado, M., East, J., (1993). Preliminary investigations into sunflower oil contamination following the wreck of the M.V. Kimya. *Mar. Pollut. Bull*. **26**: 40-44.

Nazarova, L. B., Pestryakova, L. A., Ushnitskaya, L. A. and Hubberten, H.-W., (2008). Chironomids (Diptera:Chironomidae) in lakes of central Yakutia and their indicative potential for paleoclimatic research. *Contemp. Probl. Ecol*. **1**: 335-345.

Norris, R. H., Lake, P. and Swain, R., (1982). Ecological effects of mine effluents on the South Esk River, Tasmania: benthic invertebrates. *Aust. J. Mar. Freshwater Res*. **33**: 789-809.

Oberholster, P. J., Blaise, C. and Botha, A.-M., (2010). Phytobenthos and phytoplankton community changes upon exposure to a sunflower oil spill in a South African protected freshwater wetland. *Ecotoxicol*. **19**: 1426-39.

Oberholster, P. J., Botha, A.-M. and Ashton, P. J., (2009). Appearance of new taxa: invertebrates, phytoplankton and bacteria in an alkaline, saline, meteorite crater lake, South Africa. *Fund. Appl. Limnol*. **174**: 271-282.

Oberholster, P. J., Botha, A.-M. and Cloete, T. E., (2005). Using a battery of bioassays, benthic phytoplankton and the AUSRIVAS method to monitor long term coal tar contaminated sediment in the Cachela Poudre River, Colorado. *Water Res*. **39**: 4913-4924.

Ocon, C. S., Capítulo, A. R. and Paggi, A. C., (2008). Evaluation of zoobenthic assemblages and recovery following petroleum spill in a coastal area of Río de la Plata estuarine system, South America. *Environ. Poll*. **156**: 82-89.

O'Connel, T. R. and Campbell, R. S., (1953). The benthos of Black River and Clearwater Lake, Missouri. *University of Missouri Studies* **26**: 25-41.

Porra, R. J., Thompson, W. A. and Kriedemann, P. E., (1989). Determination of accurate extinction coefficient and simultaneous equations for assaying chlorophyll *a* and *b* extracted with four different solvents: verification of the concentration of chlorophyll standards by atomic absorption spectrometry. *Biochem Biophys. Acta* **975**: 384-394.

Poulton, B. C., Finger, S. E. and Humphry, S. A., (1997). Effects of a crude oil on the benthic invertebrate community in the Gasconade River, Missouri. *Arch Environ. Contam. Toxicol*. **33**: 268-276.

Rainio, J. and Niemela, J., (2003). Ground beetles (Coleoptera: Carabidae) as bioindicators. *Biodiversity Conserv*. **12**: 487-506.

Ravichandran, M., (2004). Interactions b4etween mercury and dissolved organic matter - a review. *Chemosphere* **55**: 319-331.

Rigger, D. 1997. Edible oils: are they really that different? In: Proceedings, international oil spill conference. American Petroleum Institute, Washington, DC, pp 59–61.

Shannon, C. E., (1948). A mathematical theory of communication. *Bell Syst. Tech J*. **26**: 379-423, 623-656.

Takahashi, M. A., Higuti, J., Bagatini, Y. M., Zviejkovski, I. P. and Velho, L. F. M., (2008). Composition and biomass of larval chironomid (Insecta, Diptera) as potential indicator of trophic conditions in southern Brazil reservoirs. *Acta Limnol. Brazil* **20**: 5-13.

Ten Brink, B. J. E. and Woudstra, J. H., (1991). Towards an effective and rational water management: The aquatic outlook project - integrating water management, monitoring and research. *EWPC* **1**: 20-27.

Thorp, J. H. and Covich, A. P., (2001). Ecology and classification of North American freshwater Invertebrates. (2nd edn). Academic Press, San Diego, USA, pp. 992.

US EPA., (1997). Oil pollution prevention; non-transportation related onshore facilities. *Federal Register* **62**: 54508-54543.

US EPA, (1994). Oil pollution prevention; non-transportation-related onshore facilities; final rule. *Federation Registration*, pp. 59.

USDOC (United States Department of Commerce), NOAA (National Oceanic and Atmospheric Administration), (1996). Damage Assessment and Restoration Program. Injury Assessment: Guidance Document for Natural Resources Damage Assessment under the Oil Pollution Act of 1990. Silver Spring, Maryland, Appendix C, Oil Behavior, Pathways, and Exposure, pps. C-1-24 and Appendix D, Adverse effects from oil, pp D-1-69.

Warwick, R. M. and Clarke, K. R., (1993). Comparing the severity of disturbance: a metal-analysis of marine macrobenthic community data. *Mar. Ecol. Prog. Ser*. **92**: 221-231.

Weigel, B. M., Henne, L. J. and Martinez-Rivera, L. M., (2002). Macroinvertebrate-based index of biotic integrity for protection of streams in west-central Mexico. *J. North Am. Benthol. Soc*. **21**: 686-700.

Wright, J. F., (1995). Development and use of a system for predicting the macro-invertebrate fauna in flowing waters. *Aust. J. Ecol*., **20**: 181-197.

Wu, J., Fu, C., Liang, Y. and Chen, J., (2004). Distribution of the meiofaunal community in a eutrophic lake of China. *Arch. Hydrobiol* **159**: 555-575.

Yoshimura, C., Tockner, K., Omura, T. and Moog, O., (2006). Species diversity and functional assessment of macroinvertebrate communities in Austrian rivers. *Limnol*. **7**: 63-74.

Zoun, P. E. F., Baars, A. J., Boshuizen, R. S. (1991). A case of seabird mortality in the Netherlands caused by spillage of nonylphenol and vegetable oils, winter 1988/1989. *Sula* **5**: 101-103.

CHAPTER 7

Summary

The occurrence, as well as the environmental fate and impact, of vegetable oil spills in freshwater wetlands have until now been unreported. Thus, the largest global vegetable oil spillage in a fresh water wetland that occurred at the Con Joubert Bird Sanctuary wetland in 2007 presented an ideal opportunity to evaluate these impacts.

The primary objective of this study was to use the succession of aquatic biota response and diversity after a sunflower oil spill in the Con Joubert Bird Sanctuary wetland over a period of one year. Response selected organisms such as *Daphnia pulex*, *Spirodela punctata, Physa acuta* and *Raphidocelis subcapitata* were used in a battery of bioassay tests in relationship with the physicochemical condition of the wetland to determine the impact of the sunflower oil spill. All the bioassay tests and field biological indicators such as planktonic phytoplankton and macroinvertebrate organisms were sampled and analyzed on a two monthly bases.

To evaluate the bioremediation potential of fertilizer usage under post-spill conditions in the Con Joubert Bird Sanctuary wetland, an investigation on the effect of the spilled vegetable oil on the biological diversity of the biota before and after biostimulation with different concentrations of fertilizer was launched. Biostimulation responses were analyzed 30 days after different concentrations of fertilizer were applied to the freshwater wetland at 3 selected sampling sites. The Con Joubert Bird Sanctuary wetland showed a high degree of contamination after the vegetable oil spill, resulting in a large volume of vegetable oil in the sediment and water column respectively. Vegetable oil contents differ

at each sampling site before biostimulation, and each site showed variable responses after biostimulation. In this study, biostimulation results displayed a higher yield of microbial activity and vegetable oil degradation at sites 1 and 2 respectively. However, the degradation of the high vegetable oil concentrations within the sediments at sampling site 3 may have been hampered or retarded by the polymerized state of the vegetable oil at this site. The phytoplankton, protozoan, macroinvertebrates and microorganisms assemblage were affected and showed little improvement at site 3 even after biostimulation with the high fertilizer concentration of 800 g/m^2 in comparison to sites 1 and 2, which showed greater biological activities and degradation of vegetable oil.

In this study it was evident that bioassay tests are adequate for the detection of potential adverse effects of complex toxic compounds at various levels of oil spills in freshwater wetlands. Organisms responded differently to the oil polluted water column and sediment samples. The sensitivity of planktonic phytoplankton biomass in the field varied greatly due to different levels of resistant oil toxicity as compared to the laboratory *Raphidocelis subcapitata* biotest. The toxicity of the sunflower oil concentrations at sites 1 and 2 were above threshold level in comparison to the control. There were no significant impacts of sunflower oil observed on the *P. acuta* and *S. punctata* bioassay tests in the wetland, while the *D. pulex* and *R. subcapitata* bioassays showed significant impacts under similar conditions. The comparison between outcomes of the bioassay assessment and analyses of physicochemical indicators showed no early warning with regards to sunflower oil contamination.

To evaluate the adverse impact of a sunflower oil spill on the diversity of planktonic phytoplankton community and their interrelationship with physicochemical parameters, a one year (28 October 2008-21 October 2009) study was conducted in the wetland. The relationships between the oil spill and seasonal variability had an impact on the planktonic phytoplankton community structure due to variation in water depth in the wetland. Planktonic phytoplankton biomass was mostly dominated by the divisions such as Bacillariophyta, Chlorophyta and Cyanophyta, respectively. Their significant increase in diversity implied that the wetland was recovering from the adverse effects of the spilled vegetable oil. Seasonality was key determinants of the planktonic phytoplankton diversity and species succession as reflected by functional groups in the wetland ecosystem after the oil spill.

To assess the impact of the vegetable oil spill on the macroivertebrate assemblage, five post spill sampling sites were selected within the wetland from which a variety of abiotic and biotic samples were collected bi-monthly over a period of 12 months. Abiotic variables included the sediment and water column oil concentrations, total nitrogen, total phosphorous, silica, chlorophyll *a* and *b*, as well as *in situ* measurements of pH, electrical conductivity and dissolved oxygen. Aquatic macroinvertebrates were chosen as biotic indicators, due to their wide applicability as water quality indicators, and collected at each site. Spatial and temporal changes in total nitrogen, total phosphorous, chlorophyll *a* and *b* concentrations, as well as changes in pH were observed. The oil spillage also resulted in an increase in tolerant invertebrate taxa, mainly Chironomidae and Psychodidae, at the sites closest to the source of the spillage. These two taxa, and to a

lesser extent Syrphidae, were identified as potentially useful indicators to determine the extent of vegetable oil contamination within a freshwater wetland. Furthermore, the monitoring of these indicators taxa can be a useful management tool to determine the recovery of freshwater wetlands after vegetable oil spills.

It can be concluded that the impact of the sunflower oil spill and seasonality were the major factors affecting planktonic phytoplankton communities, aquatic macroinvertebrate diversity and other organisms functional groups in the Con Joubert wetland ecosystem. As indicated in the thesis chapters, the lack of pre-spill data of yearly variability of the wetland ecosystem under non-spill conditions were a limitation during the analysis of experimental data. However, application of autecology of species assemblage indicated recovery of the wetland ecosystem.

For wetland management purposes during a vegetable oil spill, biostimulation is recommended as method of choice to improve microbial activity and vegetable oil degradation at contaminated sites if there is no danger of progressive eutrophication. Whilst bioassay assessment and analyses in relationship with physicochemical conditions should be performed as a method to indicate an early warning of adverse effects due to vegetable oil contamination. It should be noted that the relationships between the oil spill and seasonal variability had an impact on the planktonic phytoplankton community structure due to variation in water depth in the wetland. Most importantly, physicochemical oil spillage promoted the increase of tolerant invertebrate taxa, mainly Chironomidae and Psychodidae, in the freshwater environment.

To conclude, the vegetable oil spill in the Con Joubert Bird Sanctuary is the largest ever reported spill in a freshwater wetland, and the information in this thesis represents the first report on monitoring of succession of biota in a freshwater wetland after a sunflower oil spill incident. Therefore, the data presented in this thesis can be used as a model for restoration purposes in the future for other freshwater wetland environments globally.

Appendices

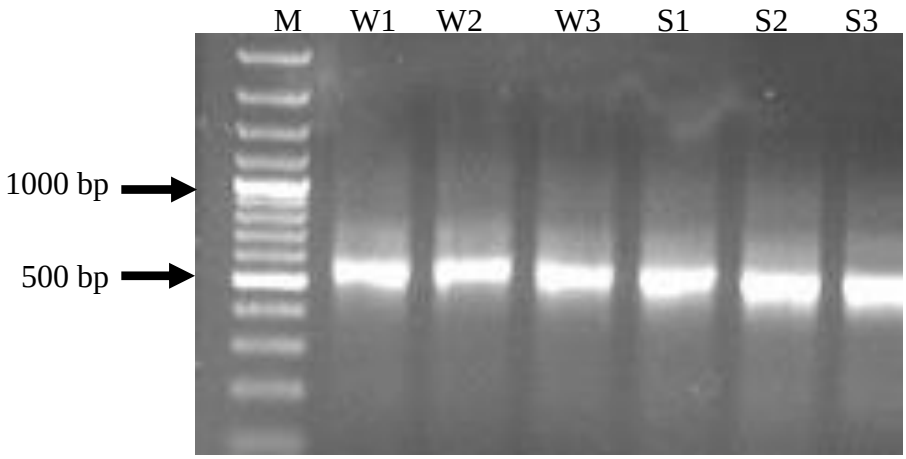

Figure S1 (Appendix): PCR product of 16S rDNA primers obtained from three sites using genomic DNA from both sediment and water column microorganisms. The gel was loaded as follows: lane M (molecular marker), lane W1 (water, site 1), lane W2 (water, site 2), lane W3 (water, site 3), lane S1 (sediment, site 1), lane S2 (sediment, site 2) and lane S3 (sediment, site 3). Each band on the gel represents lots of species diversity in a particular sampling site.

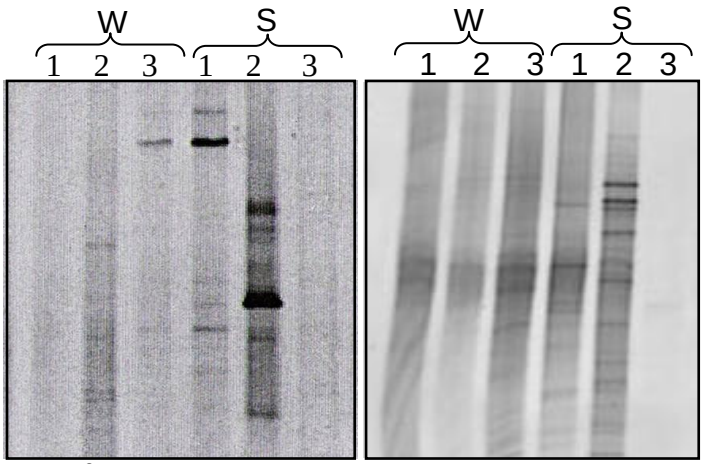

Figure S2 (Appendix): DGGE band patterns of microbial composition in each sampling site polluted by vegetable oil before and after biostimulation. The individual bands in a single well represent different species. W represent water column sample and S represent sediment sample.