# "Application of Whole-Cell Biosensor for the impact of crude oil exploration on the Environment of the Niger Delta".


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**Lancaster Environment Centre**

## Dedication

This work is dedicated to God the Father, God the Son and God the Holy Spirit

*"When men think God comes late, He comes BIG"…… Odafe*

## Declaration

This thesis has not been submitted in support of an application for another degree at this or any other university. It is the result of my own work and includes nothing that is the outcome of work done in collaboration except where specifically indicated.

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Ejenavi, Odafe Erhovwo

Excerpts of this thesis have been published in the following conference manuscripts and academic publications.

I. Jia, J., Li, H., Zong, S., Jiang, B., Li, G., Ejenavi, O., Zhu, J. and Zhang, D.*, (2016) Magnet bioreporter device for ecological toxicity assessment on heavy metal contamination of coal cinder sites, Sensor and Actuators B: Chemical, 222, 290-299.

II. Wang, X., Zhao, X., Li, H., Jia, J., Liu, Y., Ejenavi, O.E., Ding, A., Sun, Y. and Zhang, D.*, (2016) Separating and characterizing functional alkane degraders from crude-oil-contaminated sites via magnetic nanoparticle-mediated isolation, Research in Microbiology, 167 (9), 731-744.

III. Ejenavi, O. and Zhang, D.*, (2017) Evaluating oil spill contamination in the Niger Delta by magnetic whole-cell bioreporter assay, Journal of Environment Health Science and Engineering, accepted.

IV. Ejenavi, O. and Zhang, D.*, Crude oil contamination and management in the Niger Delta, Environmental Science & Technology, under review.

V. Ejenavi, O. E., Li, H, and Zhang, D. *, Simultaneous and online detection of crude oil contamination via biological-phase microextraction and biosensing (BPME-BS) device, Biosensors and Bioelectronics, in preparation

## Statement of Authorship

Statement of my contributions made to chapters 3, 4, 5 and 6 are highlighted below:

### Chapter 3

All authors authorised the publication of chapter 3 and have reviewed the final version. My role was to develop and validate the magnetic bioreporter device and measurement of sample toxicity by the device for optimal performance, to achieve the best response. I was also involved in the conceptualization, experimental design, execution, data analysis, and writing, with exception of site visits and risk analysis (section 3.2.6 and 3.3.2), which were the work of co-authors (Prof. Jia from China University of Mining & Technology, Beijing) as he helped in ICP-MS analysis of heavy metal components in the soil samples. My percentage contribution is 20 % of the entire work.

### Chapter 4

I Ejenavi, Odafe Erhovwo in my capacity as the first author of the accepted paper submitted in the journal of Environmental Science and Engineering carried out the site visits for soil and water samples collection, experimental process, execution, data analysis, writing and intellectual contribution. This constitutes 85% of the work involved with the accepted publication.

### Chapter 5

I Ejenavi, Odafe Erhovwo in my capacity as the first author of the paper in chapter 5 for preparation in the journal Biosensors and Bioelectronics, undertook the experimental design, execution, data analysis, writing and intellectual contribution. This constitutes 90% of the work involvement with the view to publish it in the near future. It is important since it is one of my key novelties of my project work.

### Chapter 6

I Ejenavi, Odafe Erhovwo contributed to the paper in chapter 6 published in Research in Microbiology, 167 (9), 731-744. I carried out the site visits for soil and water samples collection in Nigeria, involved in the conception, design, execution, analysis and writing. My involvement constitutes 30% of the total work published.

**Abstract**

The discovery of crude oil in exportable quantities in Nigeria was greeted with overwhelming ovation and high expectations in terms of the wealth generation possibilities and economic growth for the region. However, 5 decades onwards, the Niger-delta terrain and its people have become disenchanted with the ills and aggravated impacts of crude-oil discovery as a result of unsustainable patterns of its exploitation in Nigeria. The inundation of oil spills, accompanying ecological disasters and human health risks served to undermine the benefits of huge petroleum resource revenues. Nigeria has recorded over 3,324,269.28 million barrels of crude oil spills from 12,854 spills incidents as captured since 1976-2011 and the polluting process persists as a result of exploration, drilling and production activities as well as flagrant disregard for best health, safety, security and Environmental practices (HSSE). Even though these spills have been blamed on various factors, the fact remains that the people and environment of the Niger-delta desperately need state, public and private intervention to stem the menace of environmental pollution engulfing the region. This research therefore proposes urgent rehabilitative measures expedited by the Bio-reporter approach, to enhance rapid detection, toxicity and management of spills impacted sites. The Bio-reporter method and its success is hinged on its advantages over the conventional method entailing rapid detection of genotoxicity, easy operations, inexpensiveness and more importantly it provides information about bioavailability. It is thus a complementary tool to the chemical analysis methods. The novel magnetic bio-reporter device developed with the magnetic nanoparticles (MNPs) functionalization and also the device on Simultaneous and online detection of crude oil contamination via biological- phase micro-extraction and bios-sensing (BPME-BS) in the Niger Delta environment have been applied with significantly positive results. Consequently, the novel magnetic nanoparticle-mediated isolation (MMI) technology was tested on Nigerian soils for the separation and characterization of functional alkane degraders from crude-oil-contaminated sites. Essentially, the new technique's qualities of cost-effectiveness and zero risk of species invasion on the environment has become more apparent from extensive experimentation.

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# 1. Introduction

The aim of chapter 1 is to present an overview of the overall thesis, and present the background literature, as well as the overall aims and objectives of the thesis.

The specific objectives are:

- The data gap after 2000 on oil exploration and contamination via spill in the Niger Delta
- The government structure and regime on crude oil contamination monitoring management and remediation.
- Technical improvement in crude oil spill monitoring
- Implementation on the government policy and management

*1.1.Background*

Oil and gas exploratory and production activities have severe consequences on the three media of the ecosystem viz-soil, water and air. Over the past 50 years, the Niger Delta has suffered from debilitating impacts and adverse consequences in terms of the human health, food security and the environmental degradation of the ecosystem. It also directly affected the economy in Nigeria. The report of United Nation emphasized the Ogoni land as one of many spill scenarios. However, the possibility of actual environmental restoration is a conceivable accomplishment in contemporary times. It is now a realistic option for pollution management to consider biotechnical tools during the monitoring and cleaning-up process of deteriorated environment as a result of oil spills, via the analysis of the bioavailability and toxicity of hydrocarbon concentration on sediment and groundwater (Ejenavi and Zhang, 2016). Oil spills is hard to be entirely avoided, but able to be managed to the acceptable extent with sustainable standards. Whole-cell bioreporter has some significant advantages, such as rapid detection of bioavailability and genotoxicity directly over the chemical conventional method. It also proffers a more valid risk assessment practices and analysis in relation to the environment (Zhang et al., 2013). It is therefore currently in use as a complementary tool to the chemical analysis method in crude oil contamination.

*1.2 Statement of problem*

The rationale for this proposed research is borne out of the paucity of research materials and relevant intellectual literature portraying the current state of environmental degradation and neglect which has indicated an unprecedented increase from year 2000-2013 in the Niger Delta region. A lot of oil pollution incidents have occurred in the Niger Delta, one of the more recent ones include the Bonga field oil spill which occurred in 2011 and accounted for 5500 tonnes of crude oil in the Niger Delta region. There was also the Exxon-Mobil oil spill in 2010 which resulted in the release of about 90,000 – 95,000 tonnes of crude. The gravity of this situation is more appreciable when we consider that the equivalent volume of a tonne of crude oil is approximately 7.5 barrels of crude or 1,165 litres. This research work is thus targeted at capturing the economic importance,

exploration, exploitation, deprivation, degradation and excruciating poverty of the people of the region.

The research work seeks to appraise the complex environmental degradation and spillage impact on the people of the Niger Delta as a result of the exploration and exploitation of the crude oil. This research work seeks to offer reasonable suggestions and probable intellectual solutions to the problems generated as a result of the impact of crude oil exploration and exploitation on the environment of the Niger Delta.

The research questions therefore will focus on the following:

1. The data gap after 2000 on oil exploration and contamination via spill in the Niger Delta
2. The government structure and regime on crude oil contamination monitoring management and remediation.
3. Technical improvement in crude oil spill monitoring
4. Implementation on the government policy and management

This work would also attempt to trace the start of crude oil exploration in Nigeria. The project further undertakes a critical review of the Oloibiri experience and the years after it. It also gave reasons for the under-development in Nigeria by examining the perennial problems of pollution, migration of aquatic-life, water-related diseases and death. The development of explorative technology in the 19th century, arguably, represents one of the greatest achievements of our century, since it has placed at the disposal of nations an unprecedented range of plastic and dynamic facilities and potentialities that are virtually unlimited. Finding a middle ground between the wealth of nations and the effect on human life is indeed the crux of this research.

### *1.3.Data challenges*

The Niger Delta from the current literature reviews has been regarded as one of the worst areas in the world with discouraging environmental records. Field work visits occurred with the help of the community liaison officers and officials of the DPR eliminated the security fears. In furtherance to keeping pace with the project work, a second field trip was

embarked upon to achieve the planned review. Although initial concerns relating to the fragile security situation in the Niger-delta were raised by the collaborating parties in the delta and colleagues in the University of Port-Harcourt, these were overcome by the assistance provided by the Department of Petroleum Resources. Field trips work commenced from, March 2014, 3rd to 16th of May, 2015, June 14, 2015. The sites visited were Rivers, Bayelsa, Delta, Edo and Lagos States respectively. Presentations were also made at various institutions with respect to the heavy oil contamination and degradation in the region and the possibilities available through biotechnology improvement. The Universities visited were Federal University of Petroleum Resources, Effurun (FUPRE), Western Delta University, Oghara, Delta State, University of Benin, Benin City, and Department of Petroleum Resources (DPR), Lagos. The presentation at DPR is in furtherance of approvals for bioremediation pilot studies for possible monitoring and regulation of the oil and gas companies operating in Nigeria.

Official written permission was sort from the Department of Petroleum Resources (DPR) being the government official agency that regulates the activities of the oil companies operating in Nigeria, including NNPC and also for the fact that an environmental awareness being the first to use bioreporter method in the Niger Delta. The area for the purpose was selected based on the major oil companies' strength operating in Nigeria with their major operations in the communities chosen. The impacts of explorations, drilling and production in the Niger Delta region feel the impacts of OICs activities.

### *1.4.Study aims and objectives*

The actual thrust of this research work is to eliminate the huge gap beginning from 2000-2013 of the content based or contemporary texts on the environmentally dilapidated state of the Niger Delta and the exigency of its rehabilitation.

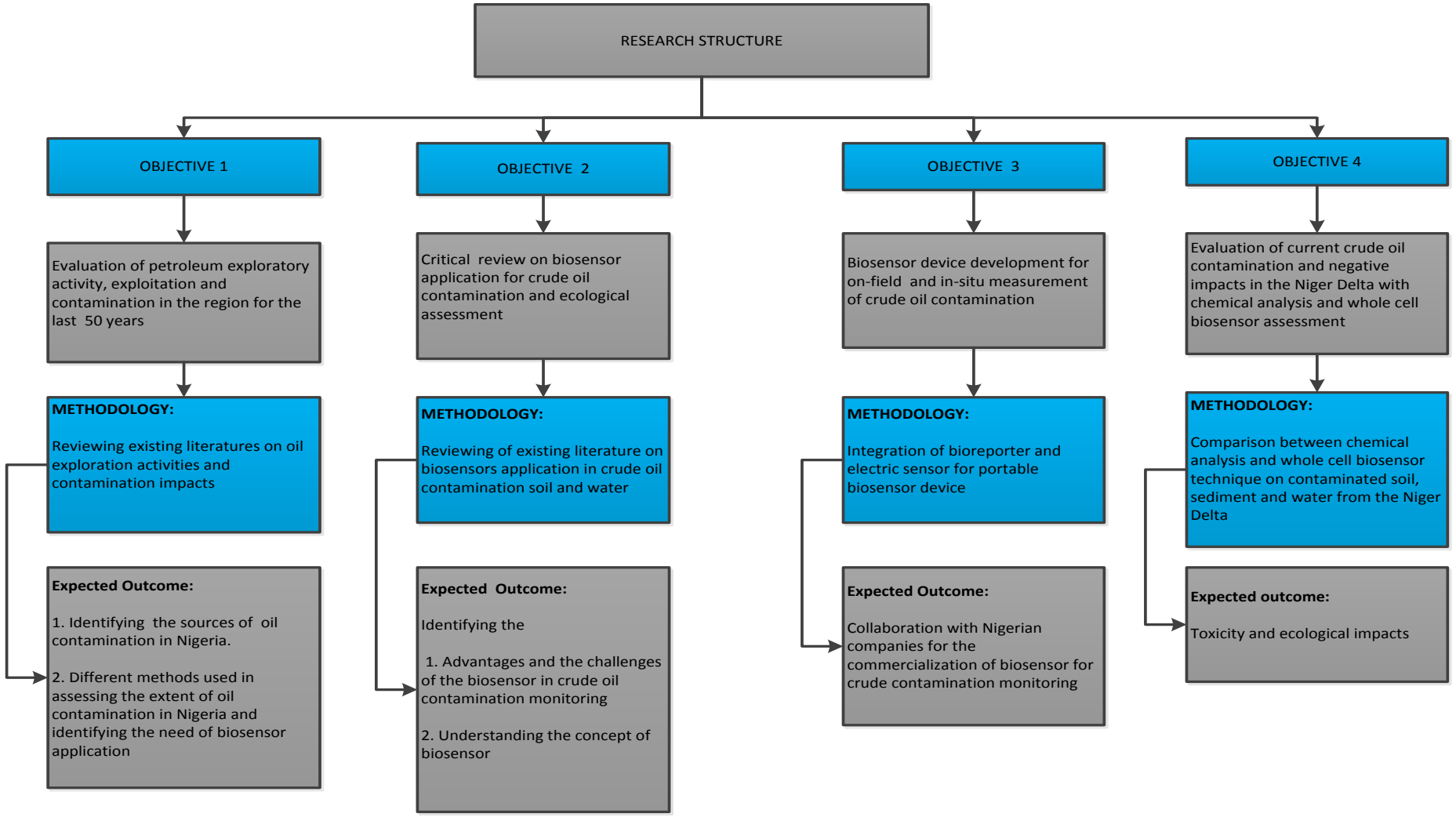


**Figure 1. 1-A** Research Structure

Following this approach, the research has four key objectives, as follows:

1. Evaluation of petroleum exploratory activity, exploitation and contamination in Niger Delta for the last 50 years.
    a) Reviewing existing literature on crude oil exploration activities and the negative impacts of crude oil contamination
    b) Identifying the sources and distribution of oil contamination in Niger Delta
    c) Revealing the structure of Nigerian government and regimes for crude oil contamination control and identifying their roles for crude oil spill control and remediation in Niger Delta
    d) Evaluating different analytical methods assessing the extent of oil contamination in Niger Delta and identifying the need for biosensor application
2. Critical review on the biosensor application for crude oil contamination and ecological assessment
    a) Reviewing of existing literature on biosensors application in crude oil contaminated soil and water.
    b) Identifying the advantages and challenges of the biosensor in crude oil contamination monitoring
3. Biosensor device development for on-field and in-situ measurement of crude oil contamination.
    a) Magnetic biosensor device development to improve biosensor response sensitivity and reproducibility in soil monitoring
    b) Integration of bioreporter and electric sensor for portable biosensor device
    c) Collaboration with Nigerian companies for biosensor commercialization for crude oil monitoring in the field
4. Suggestions on government for future crude oil spill/contamination management
    a) Fast response actions to crude oil spill
    b) Monitoring and remediation strategies at the crude oil contaminated site
    c) Policies and regulations

### *1.5.Structure of thesis*

This research work comprises of eight chapters which are inextricably linked to address the research objectives from different aspects. Their hypothesis and final deductions expedite the thesis coherence with logical conclusions.

Chapter 1 commences with an overview of research background, aims and objectives. It crucially highlights the thesis statement and research questions in a bid to convey the depth of issues forming the crux of the Niger-delta dilemma. As the world's second largest delta, the Niger Delta has huge crude oil reservoir and the oil exploration does not only bring profits but also negative consequences to local communities. The total spillage of petroleum into the oceans, seas and rivers through human activities is estimated to be at an average of 0.7 – 7 million tons per year. Due to the technical challenge, the monitoring and evaluation of crude oil contamination is not well developed in Nigeria, and the governmental structure of oil contaminated site management still needs improvement. Our research therefore addressed such technical and social challenges, and attempts to apply biological monitoring as a future solution in the Niger Delta.

## Study Area

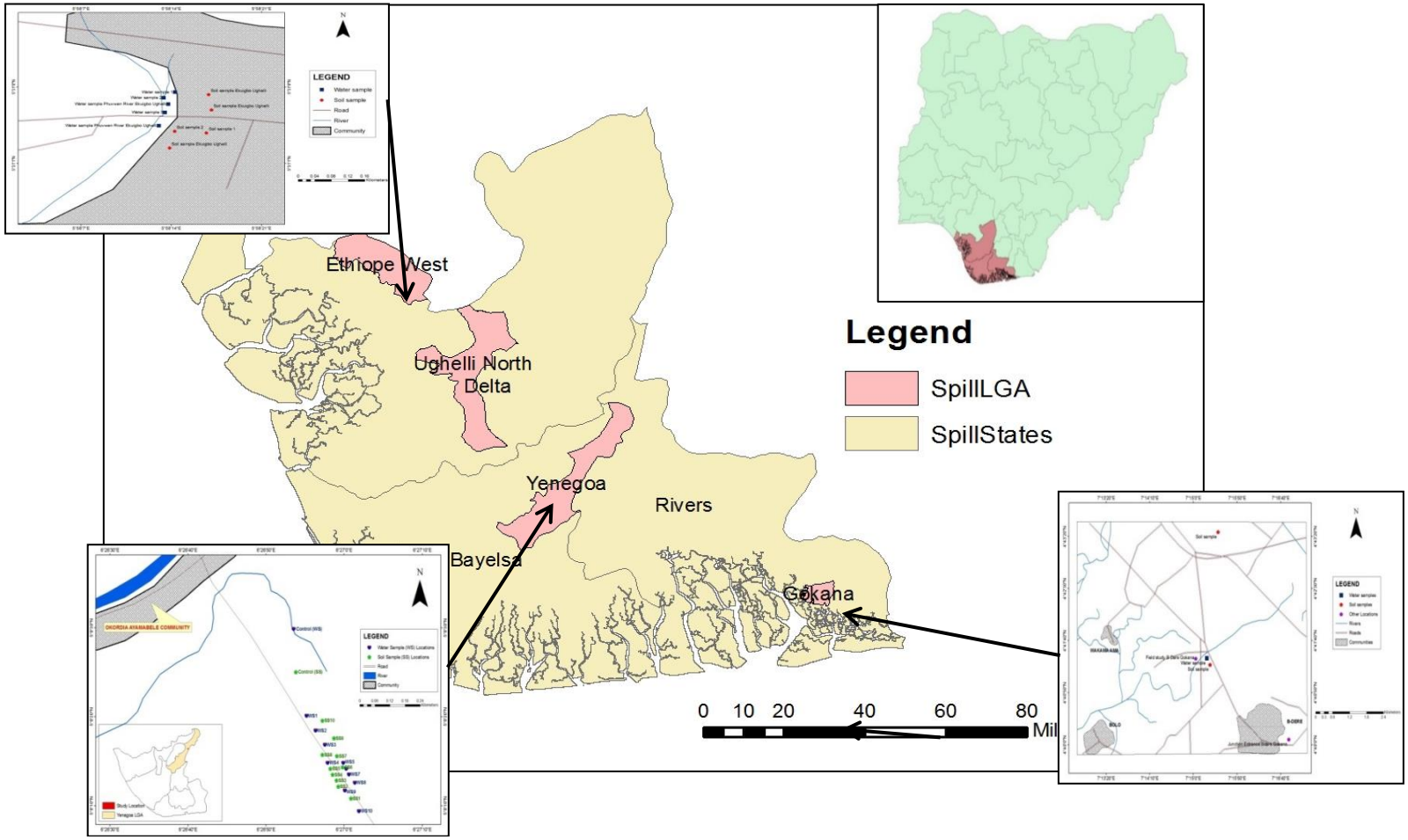


**Figure 1. 1-B** Map of the Niger Delta region, showing its states of where oil are produced

Chapter 2 is a critical overview of oil exploration history and contamination related management in the Niger Delta. In this section, the composition of crude oil and consequence of crude oil contamination is discussed with case studies, addressing how crude oil spill affects the land, water and air. Meanwhile, the highlights also include the most notable causes of oil spills and the accompanying contamination, by reviewing the oil exploration history in the Niger Delta. Thus to avert this menace and obvious threat to the existence and viability of the Niger-delta, there is as a matter of urgency a need for administrative, regulatory reforms and policy re-evaluation in the Nigerian oil and gas industry. I thus propose governmental and regulatory adjustments to ensure more proactive and coordinated approaches between governmental agencies to protect the environment, promote stringent quality controls and checks for the benefit of the Niger-delta environment. Additionally, the precautionary approaches are also proposed to spills prevention and mitigation via efficient or speedy detection and response measures. Two main approaches for crude oil monitoring in environmental media are categorized from literatures, including the conventional chemical or physical methods for non-specific and specific monitoring. Addressing their challenges, the bioreporter method is raised here as a supplementary approach to conventional analysis, which is hinged on its prime and superior advantage of rapid detection of genotoxicity and the ease of adaptability as an evaluation tool for policy and regulatory purposes.

Chapter 3 entails a preliminary study undertaken to verify and affirm the feasibility of bioreporter application in real soil samples. For most of the crude oil contaminated soils, the soil particles absorb bioluminescent signals, reducing bioreporter sensitivity and restricting its real-world application. Therefore, a novel magnetic bioreporter device was developed with the magnetic nanoparticles (MNPs) functionalization, which achieved high reproducibility with a pH value from 5.0 to 9.0, salinity from 0% to 3% and temperature from 20 $^{0}$C to 37 $^{0}$C. The data also showed the relationship between total amount and bioavailable fraction of contaminants. Our results proved that the magnetic bioreporter device can offer a high throughput biological measurement of soil contamination and is a realistic tool for crude oil contaminated soils.

Chapter 4 applied the magnetic whole-cell bioreporter device for the measurement of crude oil contaminated soils sampled from Nigeria. By reducing the interference of soil particles, the new magnetic device achieved high sensitivity, and achieved the rapid petroleum monitoring and assessment plagued by prohibitive costs and complex chemical analysis. From the in situ bioreporter assessment of the four soils and two water samples, the measurement was less than 4 hours, and the operation was easy for direct evaluating the toxicity of crude oil in soils. The soil contamination ranged from 6250.9 to 55967.6 mg/kg in soil, and the highest water contamination was 248.5 mg/L. This method thus holds the unique advantage of rapid evaluation of toxicity and bioavailability to provide environmental risk assessments at crude oil contamination sites over physical and chemical methods.

Chapter 5 further addressed the challenge of online measurement of crude oil contamination in water, and developed the biological-phase micro-extraction and bio-sensing (BPME-BS) for simultaneous and online detection of crude oil contamination in the Niger Delta. The developed passive sampler introduced alkane-chemotaxis bioreporter ADPWH_alk to seek and accumulate alkanes from water samples. The BPME-BS device achieved high enrichment factor (>4.6) and satisfactory limit of detection (0.05 mg/L) when ADPWH_alk cell was immobilized via agarose gel. The quantitative response of BPME-BS device was comparable to that of gas chromatography flame ionisation detector. The device also maintained the limit of detection under a wide range of environmental conditions, like pH between 4.0 and 9.0, temperature from 20 $^{0}$C to 40 $^{0}$C, and salinity is 0% to 3.0% sensitivity. Within 30 days storage at 4 $^{0}$C, the response of BPME-BS device was reliable, showing its feasibility in commercialization. More importantly, the BPME-BS device could detect the dynamic concentration of alkanes in water samples and 7-day simultaneous measurement proved its future application as an online alkane device. This work substantiates the theory that whole-cell bioreporter can be immobilized as a passive sampler for online diagnostic environmental contaminants.

Chapter 6 utilized the alkane bioreporter to evaluate the alkane degradation performance of soil microorganisms during bioremediation process. By introducing the novel magnetic nanoparticle-mediated isolation (MMI) technology to isolate the uncultivable-but-

functional alkane degraders from Nigerian soils, the alkane bioreporter successfully evaluated their ecological functions and influencing factors, providing valuable information about the approaches improving bioremediation performance. Our results indicated that whole-cell bioreporter is a good tool to assess the change of alkane availability during bioremediation process, and can be used as one supplementary tool to enhance bioremediation by adding appropriate carbon or nitrogen sources to encourage real indigenous oil degraders. It further showed the great potential of whole-cell bioreporter not only in contamination monitoring, but also a good approach in crude oil contaminated sites management.

Chapter 7 entailed the detailed suggestions and recommendations on crude oil spill and management in Niger Delta. From the technical aspects, the combination of conventional chemical analysis and cutting-edge biological monitoring was suggested. From the social aspect, the recommendations focused on the future management framework to improve contamination monitoring and management in the Niger Delta of Nigeria.

Chapter 8 was a comprehensive conclusion and summary of all the work of this project, with a logic structure to address the solutions on crude oil contamination management in the Niger Delta. It also proposes viable steps for governmental action or intervention. The chapter further promotes pre-emptive measures to target environmental degradation in the Niger-delta ecosystem by the implementation of policy and regulatory adjustments to inculcate best standard practices in environmental protection and management.

## Reference

EJENAVI, O. & D, Z. 2016. Evaluating oil spill contamination in the Niger Delta by magnetic whole -cell bioreporter assay. *Journal of Environment management Health Science and Engineering , accepted*.

ZHANG, D., DING, A., CUI, S., HU, C., THORNTON, S. F., DOU, J., SUN, Y. & HUANG, W. E. 2013. Whole cell bioreporter application for rapid detection and evaluation of crude oil spill in seawater caused by Dalian oil tank explosion. *Water research*, 47**,** 1191-1200.

# 2. Crude oil contamination and management in the Niger Delta

The objectives of this chapter are:

1. Evaluation of petroleum exploratory activity, exploitation and contamination in Niger Delta for the last 50 years:
    - Reviewing existing literature on crude oil exploration activities and the negative impacts of crude oil contamination
    - Identifying the sources and distribution of oil contamination in Niger Delta
    - Revealing the structure of Nigerian government and regimes for crude oil contamination control and identifying their roles for crude oil spill control and remediation in Niger Delta
    - Evaluating different analytical methods assessing the extent of oil contamination in Niger Delta and identifying the need for biosensor application
2. Critical review on the biosensor application for crude oil contamination and ecological assessment
    - Reviewing of existing literature on biosensors application in crude oil contaminated soil and water.
    - Identifying the advantages and challenges of the biosensor in crude oil contamination monitoring

## Abstract

Crude oil brings Nigeria not only the wealth, but also the ecological disasters and human health risks. Increasing more attentions have addressed the reasons and consequences of oil spill and contamination, as well as potential practical and management strategies reducing the risks. This work critically reviews the 50-year crude oil exploration history in Niger Delta, comprehensively reveals the facts and causes of oil spill incidents, and discusses the ecological and health consequence from oil spills. We also analyse the governmental structure responsible for oil contamination control and remediation, regarding the responsibilities and actions of each government agencies, and provide suggestions on further management and strategies to tackle the effects resulting from crude oil contamination, eliminating threats to food security, environmental deterioration and the human health in the Niger Delta.

**Key words:** Nigeria, crude oil, contamination, regulation, Department of Petroleum Resources

## Abbreviations

CNA: Clean Nigeria Association

DPR: Department of Petroleum Resources

EGASPIN: Environmental Guidelines and Standards for the Petroleum Industry in Nigeria

EHORECON: Environmental Health Officers' Registration Council of Nigeria

FEPA: Federal Environmental Protection Agency

FME: Federal Ministry of Environment

FRIN: Forestry Research Institute of Nigeria

HSSE: Health, Safety, Security and Environment

MPR: Ministry of Petroleum Resources

NDR: National Data Repository

NESREA: National Environment Standards and Regulation Enforcement Agency

NNOC: Nigerian National Oil Corporation

NNPC: Nigerian National Petroleum Corporation

NOSDRA: National Oil Spill Detection and Response Agency

NPS: National Parks Service

OPRC: Oil Pollution Preparedness, Response and Cooperation

PAHs: Polycyclic aromatic hydrocarbons

PEF: Petroleum Equalization Fund

PPPRA: Petroleum Products Pricing Regulatory Agency

PTDF: Petroleum Training Development Fund

HYPREP: Hydrocarbon Pollution Remediation Project

PIB: Petroleum Industry Bill

## 2.1 Introduction

### *2.1.1 Crude oil and oil spill*

Crude oil is the main energy resources for modern industry (Sorrell et al., 2010). It is a natural mixture of petroleum hydrocarbons, and the main compositions are categorized as paraffinic (saturated), naphthenic (unsaturated) or aromatics (ring structured) (Yasin et al., 2013, Odebunmi et al., 2002, Odeyemi and Ogunseitan, 1985). During the exploration, transportation, storage and manufacturing activities, a huge amount of crude oil is released into the natural environment as oil spill, consequently causing the contemporary global challenge (Ite et al., 2013a). Some main oil spill accidents are listed in Table 2.1, and the two major impacts of oil spills are often evident in the areas of ecological structure and human health (Sam et al., 2016).

### *2.1.2 Impacts of oil spill*

Oil is a form of pollution and it can either be marine or coastal. In situations where the spills occur within the coastal waters, territorial seas or oceans or both is denoted as marine oil spill. The physical and chemical property of the oil is what influences the fate and toxicity once it is released into the environment, and consequently, the effect on the environment is what affects human beings. Polycyclic aromatic hydrocarbons (PAHs) are basic constituents of crude oil. The soil gets impacted by these contaminants and makes the environment toxic. PAHs are defined on the basis of ring structure from commonly three fused aromatic rings to ten rings. In describing the polycyclic aromatic hydrocarbons in term of large or small PAHs, classified PAHs up to six rings as small while aromatics rings structures above six rings as large PAHs (Fernandez-Luqueno et al., 2011). Contamination arising from 2-rings to 6-rings of PAHs has been researched out in the Niger Delta by (Sojinu et al., 2010a) with the effluents arising from the rivers and streams caused by spillages from oil installations

**Table 2. 1** Internationally recognized oil spills.

| Date | Name of platform | Location | Oil spilled (barrels) | Reference |
|---|---|---|---|---|
| **January 19, 1991** | Gulf War/Persian Gulf | Arabian/Kuwait | 11,000.000 | (Husain, 1998, King, 2010) |
| **April 20, 2010** | Macondo Well | Gulf of Mexico, USA | 4,500,000 | (Rico-Martínez et al., 2013, McNutt et al., 2012) |
| **June 1979-April 1980** | Lxtoc-1 | Bay of Campeche, Mexico | 3,500,000 | (Jernelöv and Lindén, 1981) |
| **July 19, 1979** | Gulf Coast of Trinidad and Tobago | Trinidad and Tobago | 2,123,800 | (Husain, 1998) |
| **May 28, 1991** | ABT Summer Offshore Coast of Angola | Angola | 1,924,000 | (Etkin, 1999) |
| **March 16, 1978** | Amoco cadiz | Coast of Britany, France | 1,619,048 | (Seymour and Geyer, 1992) |
| **March 24, 1989** | Runned aground in Prince William Sound | Mediterranean Sea, off Alaska | 250,000 to 750,000 | (Seymour and Geyer, 1992) |
| **October 1986** | Abkatun 91 | Bay of Campeche, Mexico | 247,000 | (Etkin, 1999) |
| **April 1977** | Ekofisk Bravo | North Sea, Norway | 202,381 | (Dahl et al., 1983) |
| **January 1980** | Funiwa 5 | Forcados, Nigeria | 200,000 | (Odeyemi and Ogunseitan, |

| | | | | |
|---|---|---|---|---|
| | | | | 1985) |
| **October 1980** | Hasbah 6 | Persian Gulf, Saudi Arabia | 105,000 | (Lehr and Belen, 1983) |
| **December 1971** | Iran Marine Int. | Persian Gulf, Iran | 100,000 | (King, 2010) |
| **January 1969** | Alpha Well 21 | Pacific, California, USA | 100,000 | (King, 2010) |
| **March 1970** | Main Pass Block 41 | Gulf of Mexico | 65,000 | (McAuliffe et al., 1975) |
| **October 1987** | Yum II/Zapoteca | Bay of Campeche, Mexico | 58,643 | (Fingas, 2012) |
| **December 1970** | South Timbalier B-26 | Gulf of Mexico, USA | 53,095 | (King, 2010). |

Oil has severe impacts on the three media of the ecosystem viz- land, water and air. These debilitating impacts have been clearly evident on the ecosystem in recent years. Once rehabilitative actions are not carried out in time, the resulting damage of oil spill on the environment would be unimaginable and unquantifiable (FME, 2006). It would entail the entire system's total loss of biodiversity and degradable horizon. This has requested for a re-evaluation of the past 50 years following the appreciable impact and the adverse consequences on human health, in addition to the environmental degradation of the ecosystem.

Basically, three main environmental organic contaminants namely hydrocarbons, chlorinated and nitroaromatic compounds are released into the environment as a result of pipelines leakages, sabotage and crude and its products transportation which threaten ground and surface water quality (Holliger et al., 1997). The composition or constituents of crude oil released into the environment is detrimental to both ecosystem and human health (Ordinioha and Sawyer, 2010). These compositional constituents of petroleum consists primarily of hydrocarbon and other hundreds of substances that include benzene, toluene and xylene (Ayotamuno et al., 2011, EPA, 2011).

Crude oil leakages have deleterious impacts, and often along with damaging to natural ecosystems, inhibition of native plants growth, reduction of soil fertility, and pollution of groundwater which poses risks to human beings when consumed. The consuming poisonous substances can increase the risks of cancer and related issues (Ordinioha and Sawyer, 2010) and relevant diseases are dermatitis, fetal abortions, fungal infections, headaches and nausea (Adesodun et al., 2008, Azibabua et al., 2013, Orimoogunje and Ajibola-James, 2013a). With long history of the refineries effluents due to the various chemical processes (fractional distillation, desalination and demineralization) involved in the separation of different chemical products from the region, chemical pollutants are being released into the environment which posed challenges with best global practices. These chemical pollutants cause severe damage to our bio system and negative impacts on flora and fauna, as well as wetlands and aquatic or land habitats. Furthermore, it deteriorates human health and causes environmental hazards like oil spillage, water pollutions, ocean acidification, acid rain, ozone layer depletion, thus disturbing the stability of living environment. Thus, this discharge of untreated toxic substances into the environment has

effectively created a conundrum for the whole society and the inhabitants living within the region.

From the research on aquifers, streams and rivers in 18 oil fields in the Niger Delta (Agbalagba et al., 2013), we can find serious concerns of extreme oil pollution. The acidic level of oil drill wastes and highly pressurized crude oil pipelines discharges reaches a pH 4.2±5.6, which altered the soil content undoubtedly (Odeyemi and Ogunseitan, 1985, Osuji and Adesiyan, 2005a, Wegwu et al., 2011). The concentration of toxins contained either in drinking, bathing and fishing water is much higher than the safety limits set by the US Environment Protection Agency and the negative impacts on a socio-economic scale as evidenced in the life and the environment of oil bearing local communities include; forest destruction and biodiversity loss, health hazards and untreated waste disposal from refineries contain toxic chemicals constitute a high level of land, water and air pollutants (Kadafa, 2012a) and consequently necessitated a court ruling in the Netherlands against Shell for the pollution in the Niger Delta on January 30$^{th}$ 2013 at the International Court of Justice, in Den Haag (Ridderhof, 2013).

### *2.1.3 Research on oil spill in Nigeria*

Since 1978, 407 papers were published with 2357 citations on crude oil spill and contamination in the Niger Delta, from the data on Web of Science. The 60% of them were published since 2005. About 182 world largest spills statistics in world with an average rate spillage in the last 10 years was presented (Fingas, 2012) showing the negative impacts globally. These topical issues and general public opinion of authors regarded the Niger Delta as a serious environmental degradable system (Onduku, 2001). The main focuses are the monitoring, remediation and management of crude oil spill. The adoption of specific corrective actions through bioremediation and management as a tool for Nigeria's government to improve the ecosystem and curtail further ecological deterioration as well as restore the environment, and monitoring will be the essential or viable approaches for the environmental improvement in Nigeria (Onu, 2003). Consequently, the main research area in relation to oil spills is to consider the extent to which exploratory activity has negatively impacted on the sources of livelihood of the indigenes of the Niger Delta. Assessing the strategies to monitoring and the management of oil spills contamination by the adoption of

biosensor techniques as well as recommending specific corrective actions through bioremediation management forms a fundamental aspect of this work. This research also proposes feasible recommendations for the Nigerian government relating to eco-friendly options for the Niger-delta environment in order to curtail further ecological deterioration; it also serves to eliminate the huge sources and data gap evident between 2000-2013 as evident from previous literatures and contemporary texts relating to oil spillage data and environmental dilapidation in the region, whilst advocating urgent steps for the rehabilitation.

## 2.2 Crude oil exploration history in Nigeria

### *2.2.1 Nigerian crude oil resources*

Crude oil resources are huge in the Niger Delta and predominant in Nigerian economy. The government's bulk revenue is from crude oil exports which accounts for about 90% total export and 95% of foreign exchange earnings. Nigerian crude oil is being regarded as "sweet crude" because of his small amount of sulphur it contain and as such has a high price value internationally (Dickson and Udoessien, 2012). From data in 2011, Nigerian oil reserves were 37 billion barrels. This translates to about 2.9% of global oil reserves.

Bitumen another crude oil resource in Nigeria entails very large volumes of conventional oil formed by natural process acted upon by bacterial activities (Amigun et al., 2012) and demand necessitated aggressive exploitation for local consumption and enhanced the economy (Adeniran, 1999). Its composition is related to the type of original crude and its degree of inspissation. It can be waxy solid hydrocarbon or asphaltic solid hydrocarbon. Chemically, there is a degradation process from the paraffinic oils to waxy residues with a carbon range of $C_{22}$ and $C_{29}$ and hydrogen of 14 to 16 respectively. Its origin is paraffinic oil with increasing degradation of naphthenic oil (Hunt and Jamieson, 1956). In the research of Selley (1985), the probable reserves of bitumen in Ondo State of Nigeria is estimated to be about 16 billion barrels, while tar-sands resources are estimated to be 42 billion barrels (KPMG, 2012). These quantum reserves boast the Nigeria economic and make the sustainable supply of energy important to the continuous economic growth of the nation which drives socio-economic activities. Similarly, the oil and gas reserves places Nigeria as the sixth world largest exporter of crude (Kadafa, 2012d)**.** The oil reserve as at

1st January, 2015 is 37,448 MMbbls of oil and 187.99 of gas respectively. Nigeria, as a key player in the global oil and gas supply chain, like other countries, should respond to growing global energy demands by increasing its hydrocarbon reserves to meet up with local and foreign energy demand and supply obligations (Gbadebo and Okonkwo, 2009, Onuke, 2014, Oyedepo, 2009).

### *2.2.2 Oil exploration activities*

The majority of oil reserve was located in the sedimentary basins in the Niger Delta. Nigerian oil exploration activities started in east Lagos in 1908 by Nigerian Bitumen Corporation from the oil seeps in Okitipupa. Interrupted by the World War I, sporadic oil exploration activities were operated by Shell until the discovery of a commercial quantity of oil reserve in 1956 at Oloibiri. The cumulative country production from 1958-2010 is 29,803,198,120 barrels, with highest annual production of 920,017,277 barrels in 2006. However, the current production as at September 2011, average daily production stands at 2,439,000 barrels with deep offshore production accounting for a third of the Nigeria's current production. From the summarized country production (Akpomudjere, 2011), the whole history of oil exploration in the Niger Delta can be categorized into 4 stages, and the oil reserves and number of exploration wells drilled are shown in Figure 2.1. Figure 2.1 and Figure 2.2 show Nigerian huge potential for exploration and production activities, the oil reserves has been experiencing low level thresholds for decade. This is attributable to the onshore and shallow water fields attaining their peak production as well as well as the reduction in exploration efforts by operators in Nigeria and consequently, a steady rise of oil and, condensate and gas from 1976-2009, and decline slightly until 2010-2011.

**Initial oil exploration (1908-1956):** The initial exploration activities started with a modest production of 5,001 barrels per day (Nwaobi and TERRITORY, 2005). Prior before 1956 where commercial quantity was discovered at Oloibiri, the search for oil exploration prospecting was interrupted abruptly by the World War I in 1914 and later World War II in 1939-1945 which terminated initial exploration by the Shell D'Arcy (Now Shell-British Petroleum-BP), however, BP started drilling a number of oil exploratory wells in 1937 to 1951, enjoying an initial monopoly of the oil exploration activities between 1938-1955.

**Aggressive oil exploration (1957-1983):** When oil was discovered in 1956, aggressive drilling occurred in 1957 to 1969 and was slightly interrupted by the Nigeria civil war in 1967-1970. In 1961, offshore concessions were granted to Shell/BP, Gulf (now Chevron), Mobil and Amoco overseas (now Texaco). Since then, several new comers have explored oil in those areas. In 1964, Okan field was discovered and explored by Chevron, thus it became the first offshore field in Nigeria. Idaho oil field was discovered and explored by Mobil in 1965. Since then, aggressive exploration and exploitation has plagued the Niger Delta. Currently, over seventeen oil companies are still active in oil explorative operations (Odularu, 2008). Since the discovery of Oloibiri field in 1956, the nation has steadily grown its oil reserves base at an annual average rate of 11.3 percent, thereby enjoying positive annual average net addition of 676 million barrels (Akpomudjere, 2011). The oil boom that kicked off in the 70s lead to the creations of 3 refineries. These refineries were built in 1978, 1980 and 1989 creating economy wealth.

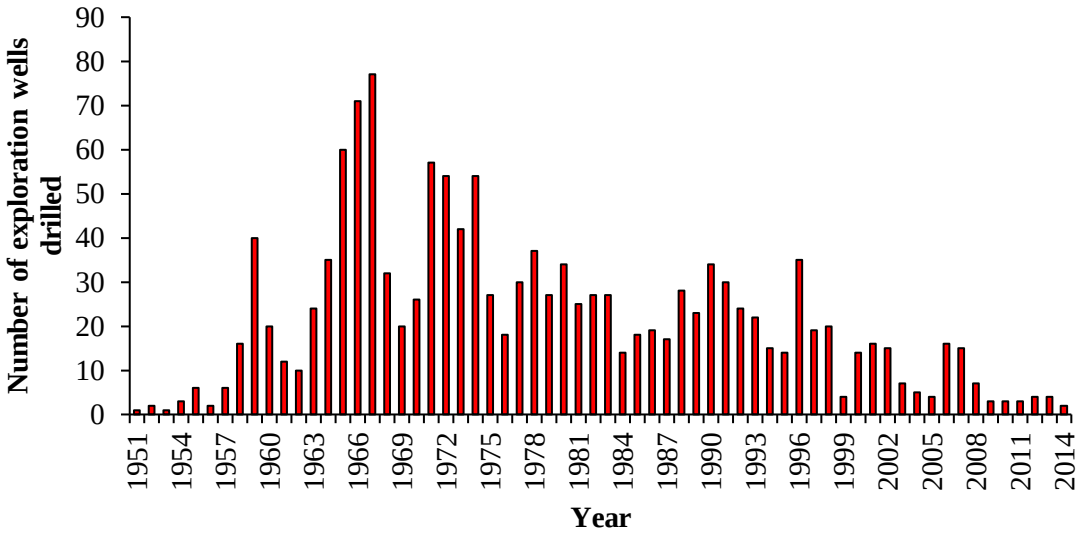


**Figure 2. 1**Number of exploration wells drilled in the Niger Delta (1951-2014).

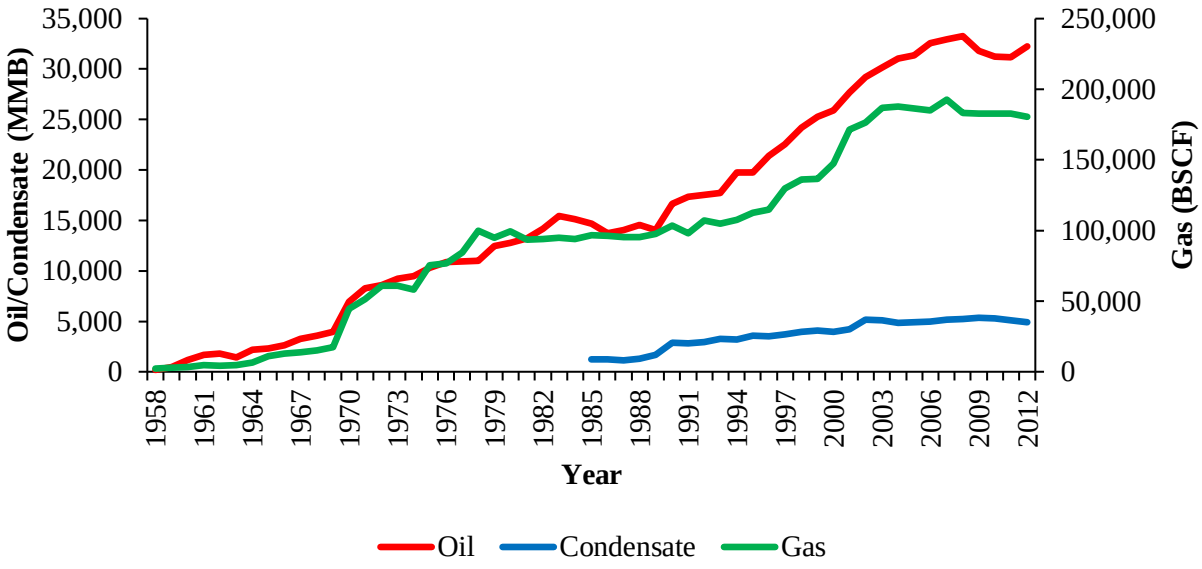


**Figure 2. 2** Oil and gas reserves in the Niger Delta (1956-2012). Data are from DPR.

**Further development (1984-1998):** After 1970 oil crisis, production began to drop significantly in 80s as a result of economic downturn and host communities unrest -Niger Delta militancy. The pressure group heightened up when an environmental activist Ken Saro-Wiwa was killed. Between 1992 and 1998, approximately 382,866.34 million barrels of oil was spilled into the environment resulting from about 2,911 incidents (Figure 2.3), the spill increased from 225 times to the highest value of 846 times incidents in the period. The multinationals decided to move from the continental shelf to the deep offshore as a result of the pressure and unrest from the pressure group which has resulted to all manners of kidnapping and social vices in the region. Thus, oil reserves of the nation are currently at an all-time low declining at an annual average rate of 11 percent without adequate replacement in the last decade. This is attributable to a general reduction in exploration efforts by all operators, while at the same time about 70% of onshore and shallow water fields are at decline phase with an average depletion rate of 2.7%. It is the reason that the nation has not be able to meet up with the daily oil production of 4 million barrels and the oil reserve of 40 billion barrels aspiration of 2010 (Akuru and Okoro, 2011).

**Current stage (1999-now):** Democracy was returned to Nigeria by the long protracted military rule that has placated the country of development for a long time. During this period, a total of about 718,317.49 million barrels resulting from 7, 107 times was spilled

into the environment with an all times highest peak of vandalization in 2006 of 3,674 incidents and the quantity of oil spilled was 535,624 barrels. Nigeria being a key player in the oil and gas industry has to join its international peers in this quest by developing hydrocarbon reserves growth policy framework in order to meet up with both its local and international energy supply obligations (Khusanjanova, 2011, KPMG, 2013). Data from the Department of Petroleum Resources (DPR) revealed a total of 673 wells drilled by Class and well drilled by Contract and Class from 2010-2014 and these exploratory activities are evident in spillage occurrences.

## 2.3 Crude oil spill during exploration and contamination in Niger Delta

Crude oil spill represents the accidental discharge during exploration, transportation, storage and manufacturing process that directly or indirectly impact on the environment (Adelana et al., 2011b). The spill may either be minor, medium, major or attain disaster status (Ite et al., 2013a). The oil spillage can either be due to negligent actions or accidents occurring during operational activities or a deliberate act of theft called sabotage. These effluent discharges into the environment effectively introduce contaminants to the environment. The estimated Nigerian crude oil spillage into the ocean is 0.7-7 million tons per year (Kadafa, 2012d). Although, the number of oil spill incidents significantly increased, the historical data in Figure 2.3 illustrated that the quantity of oil spill has decreased from 0.15 million barrels per year (2.1 million barrels in 2,796 incidents from 1976 to 1990) to 0.06 million barrels per year (1.2 million barrels in 12,854 incidents from 1991 to 2011) (FME, 2006).

The spills constitute pollution to the environment. Pollution is the introduction of elements or compounds of higher concentrations that affects humans, the environment alike and or the biological components (Scullion, 2006). Table 2.2 lists some key oil spill disasters in the Niger Delta region where the disaster affected a large expanse of land, destroyed about 836 acres of mangrove forest, polluted fresh and swamp forest, aquatic animals, crops and vegetation on land and polluted even the Atlantic Ocean polluting the marine environment. The Niger Delta Resource Damage Assessment and Restoration project in 2006 estimated a damage of approximately 355 hectares of impacted area of oil spillage incidence which destroy freshwater swamp forest, barrier forest island, and freshwater swamp. Many of

these spilled occurred before the establishment of National Oil Spills Detection and Regulatory Agency (NOSDRA) in 2006. Oil spills are regulated by NOSDRA and DPR. The legislation governing Acts oil spills make it mandatory to report all oil spills; whether small, minor, medium or disaster spills resulting from oil companies.

Over 10,000 crude oil spill incidents were reported in Nigeria, with a total oil spillage of around 9 to 13 million barrels, 50 times more than Exxon Valdez oil spill that occurred on 24th March, 1989.In September, 1979, Oshika village in Rivers State witnessed a spill of 500 barrels of crude oil which destroyed lakes and sediment, killed crabs, fishes and shrimps with high mortality in embryonic shrimp and reduced reproduction after 8 months (Kadafa, 2012d). Similarly, another major oil spillage incident occurred in one of the oil wells in Mobil oil company covering a distance of 200 kilometers that affected fish town in Bayelsa State on 12th January, 1998 and estimated 40,000 barrels oil spilling into marine environment (Aghalino and Eyinla, 2009). The Funiwa-5 oil well Blow-out spilled into the environment on the 17th January, 1980, is being classified as one of the largest international oil well blowouts by volume (King, 2010).

From 1976 to 2011, a total number of 12,854 incidents occurred in the Niger Delta and resulted in approximately 3,324,269.28 million barrels of crude oil spilled into the environment (Figure 2.3). Major oil spills occurred in the eastern zone of the Niger Delta at the SPDC Forcados terminal, of about 580,000 barrels were accountable for the Texaco Funiwa-5 oil blowout of 1980 (Nwilo and Badejo, 2001). Figure 2.1 clearly illustrates the lowest number of oil spill in 1989, and the highest ones were found in 1978, 1979 and 1980 accounting for 552 incidents with a total quantity of approximately 1,783,975 barrels. An obvious increasing number of oil spill incidents from 1990 to 2011 can be observed which might be attributed to many factors, e.g. aging pipelines, failure from oil company facilities, sabotage, wastage accruing from operational activities and negligence arising from transportation of crude. Figure 2.4 shows oil spill distribution along pipelines in the Niger Delta.

**Table 2. 2** Key oil spill disasters in the Niger Delta.

| Time | Location | Spill amount (barrels) | Terrain | Reference |
|---|---|---|---|---|
| **1908** | | Not available | Land | (Tolulope, 2004) |
| **1979** | Forcados, Delta State | 570,000 | Polluted swamp forest and aquatic animals and environment | (Tolulope, 2004, Ukoli, 2005) |
| **1980** | No.5 well, Funiwa Field, Rivers State | 421,000 barrels destroyed 836 acres of mangrove forest within six miles. | Ocean, sea and swampy areas. | (Tolulope, 2004, Aghalino and Eyinla, 2009, Gabriel, 2004) |
| **1980** | Oyakama, Rivers State | 30,000 | Land and freshwater | (Ukoli, 2005) |
| **1980** | Rivers State | 200,000 | 340 hectares of mangrove forest | (Nwilo and Badejo, 2005a) |
| **1983** | Oshika, Rivers State | 5,000 | Forest and lake | (Gabriel, 2004, Kadafa, 2012a) |
| **1995** | Etiama Nembe of Ogada- Brass pipeline, | 24,000 | Freshwater, swamp forest and brackish water mangrove | (Gabriel, 2004) |

| | | | | |
|---|---|---|---|---|
| | Bayelsa State | | swamp | |
| **1997** | Ogbodo-Isiokpo pipeline, Rivers State | Large expanse of land | Destroyed crops and vegetation on land | (Osuji and Adesiyan, 2005a) |
| **1998** | Eket, Awka Ibom | 40,000 | Atlantic Ocean | (Nwilo and Badejo, 2005b) |

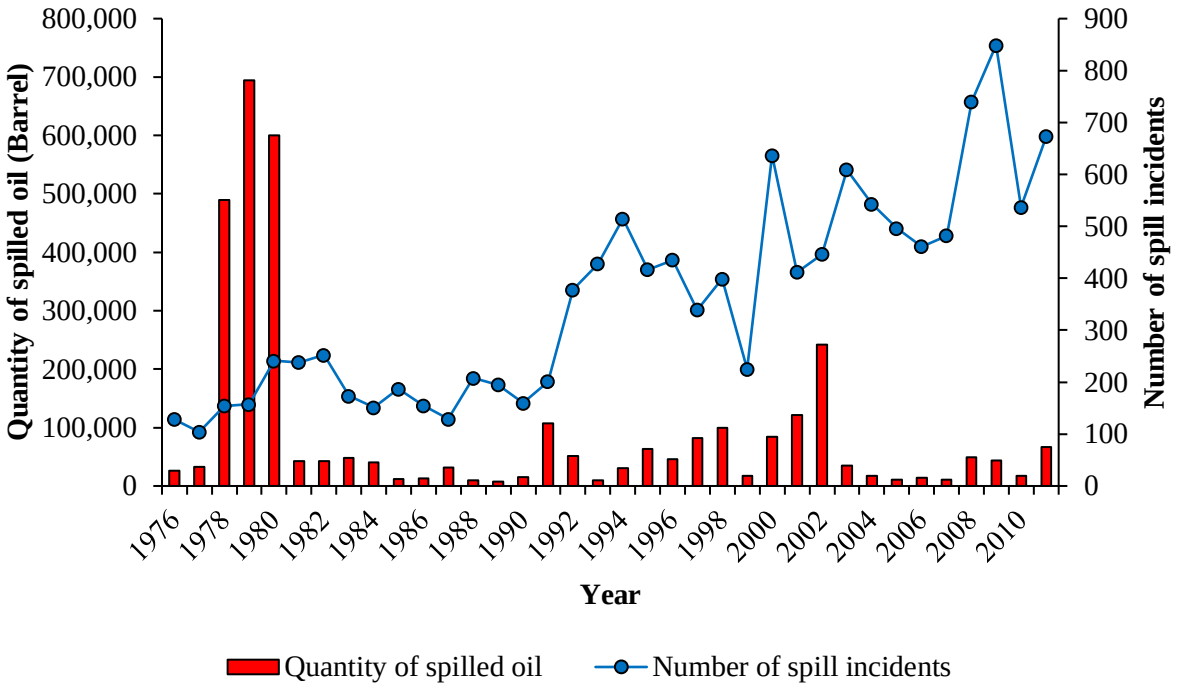


**Figure 2. 3** The quantity of spilled oil and number of spill incidents (1976-2011). Data are from the Nigerian Petroleum Development Corporation (NNPC) & DPR.

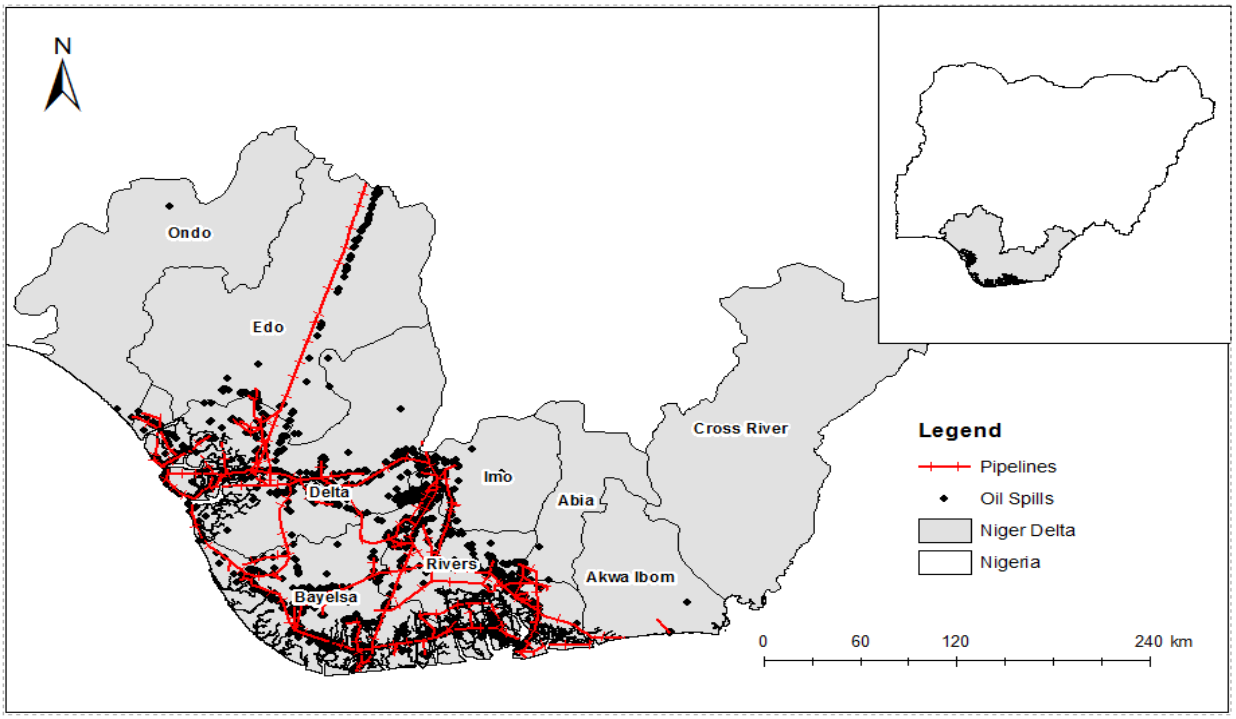


**Figure 2. 4**.Niger Delta oil pipelines and oil spill coverage area (NOSDRA, 2016).

*2..3.1 Oil spill during exploration activities*

Oil spillage arising from crude oil operation activities in Nigeria is usually from oil exploration, drilling and production activities. Data from Table 2.3 shows minimal spills from exploration and production while the bulk of spillage is transportation through pipelines, trucks and sabotage. The quantity of crude spilled during crude oil operational activities is negligible as result of high level of Health, Safety, Security and Environment (HSSE) compliance.

**Table 2. 3** Spill incidence relating to Production Activities

| | **Number of Incidence** | **Quantity spilled (Barrels)** |
|---|---|---|
| **2008** | 58 | 674.43 |
| **2009** | 69 | 86,341 |
| **2010** | 81 | 7,669.52 |
| **2011** | 18 | 210.21 |
| **2012** | 0 | 0 |
| **2013** | 2 | 0.06 |
| **2014** | 128 | 82.62 |
| **2015** | 84 | 387.77 |
| **2016** | 97 | 375.19 |

Data from DPR.

This spillage due to the unwarranted non- adherence to best HSSE practices occurred frequently and quite often through crude oil theft, vandalism, sabotage by communities claiming for compensation, aging and corrosion of pipelines, transportation and loading of products mode, operational errors, dumping and natural occurrences. These factors have led to the degradation and ecosystem destruction and thus placed Nigeria as a country that is negligent in its ecosystem or environment protection (Audu et al., 2016). Sabotage resulting from oil spills is often deliberate or malicious acts targeted at undermining oil industry facility or infrastructure. The essential purpose is to circumvent the effective administration and distribution of petroleum to storage facilities for personal or group gains. These acts affect exploration activities and are basically subversive acts to prevent the effective

operations of the oil and gas industry in such areas. This is quite distinct from human error resulting in accidents, which may be attributed to personnel lack of concentration and distractions in the process of executing legitimate tasks, as (Aprioku, 2003) attributed three reasons for sabotage Firstly, the relevant individuals demanding for compensation for the damage either of farmland or the environment was not paid. Secondly, the individuals who cut the pipelines ultimately extort exorbitant sums for the imagined or actual loss or damage. Thirdly, the certain individuals or groups of persons who cause production disruption compel oil companies to deliver social conveniences to the localities where oil companies operate.

### *2.3.2 Oil spill during distribution and refinery process*

From tankers, trucks, rails and large ships like oil bunkers, or discharge as a result of drilling activities or neglectful actions of oil industry operators, pipelines break and ruptures trends from 2003-2012 clearly depicts the seriousness of the environment degradation as recorded by the Nigerian National Petroleum Corporation (NNPC). It is studied that the origin of the pollution from oil explorations is mainly petrogenic and pyrogenic from continuous oil pipelines spillage leakage over time in the region with gas flaring activities on the surface soils. The Jesse (Delta State) pipelines fire disaster that engulfed a town resulted in a loss of thousands of lives was attributed to oil spills. The explosion was described as the most deadly in the history of Nigeria as it was discovered that the ruptured pipelines that was responsible for the spills belonged to NNPC. The oil spill from engineering drills and machines failures holds only 1% of total spill amount (Egberongbe et al., 2006). Operations through transportation and marketing generate oil spills and emissions of hydrocarbons; this is also a major culprit in the oil spills dilemma in Niger-delta environment. Oil spillage through transportation often has far reaching effects despite that the majority of them comprise of accidental occurrences.

The pipeline distribution network and pipeline breaks indicate a steady rise in the number of oil spill incidents from 1976 to 2011 due to pipelines failures, sabotage and pipeline ruptures. In the year from 2003 to 2012, a fluctuation in the number of vandalization and rupture vis-à-vis the quantity of oil spilled is found (Figure 2.5). The year 2005 recorded the highest quantity of oil spilled which was necessitated by increased in vandalization

activities as compared to the previous year 2004. Vandalization got to its peak in 2006, when amnesty programme was introduced in the Niger Delta till 2010. Vandalization went high again in the 2011 when the programme was temporary suspended resulting in further agitation of ownership of the resources. The numbers of vandalization increased from 2003 to 2006 with increased in the quantity of crude oil spillage while from the year 2006 to 2010, the vandalization activities reduce due to effort of government and public enlightenment strategy of the negative impacts of oil spillage. However, the number of rupture units along the pipeline does not correspond to number of vandalization which further shows support to one percent (1%) mechanical failures (Egberongbe et al., 2006). This suggests that aging of the pipeline does not correspond to volume of crude spilled but to the number of activities of vandals. Furthermore, the vandalization is an external force on the pipeline that affects the operation and thus affects the quantity of crude spillage. On the other hand, the rupture activities are related to the state of the pipeline and more operational in nature. The vandalization reduces with increase and participation of government in social services and provision of amenities to the people of the Niger Delta. Also, the inclusion of the Niger Delta in governance has a significant impact in the reduction of vandalization activities between the years 2006 to 2010. However with the enlightenment and social corporate responsibilities by some multinational, the vandalization activities reduce in 2011 and 2012.

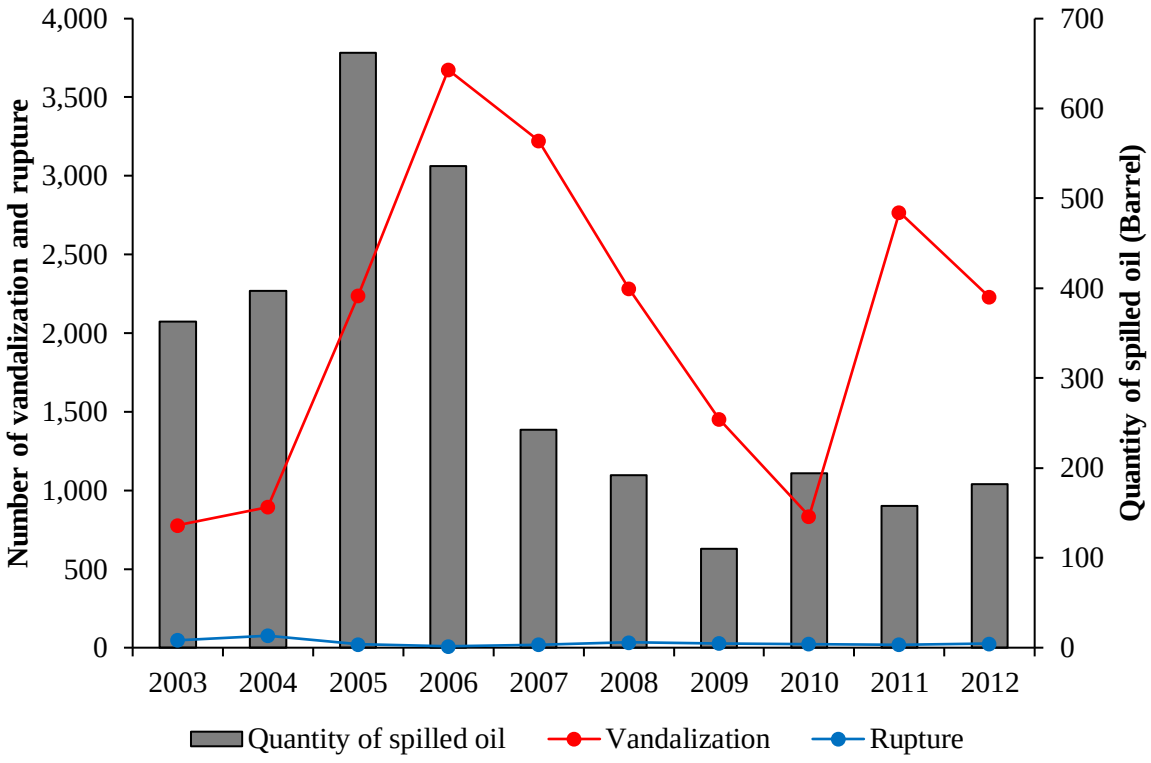


**Figure 2. 5**.Number and quantity of spilled oil from pipelines breaks (2003-2012). Data are from NNPC/DPR.

## 2.4 The ecological and health and impacts of crude oil production in Nigeria

### *2.4.1 Environmental threats*

The effects of contamination spillages over time are hard to be quantified, but its effect has caused environment threat issues in the Niger Delta. The critical study of danger posed by spills on mangrove ecosystem of the Niger Delta revealed that it will take about 14 years to recover an affected tree in a non–remediated land and about 7 years to restore that same tree in a remediated land (Orimoogunje and Ajibola-James, 2013a). Furthermore, the study also evaluated the changes in the land cover of the mangrove ecosystem in Niger Delta from 1986 to 2008. This is the underlying reason that advocated the need for strong regulatory provisions to be put in place to protect mangrove trees (Imoobe and Ironro, 2009, McGenity, 2014). Consequently, a review of the chemicals in mangrove ecosystems decline was attributed to environmental pollution and the concentrations of some organic

chemicals including petroleum chemicals have been reported comprising trace metals, the effect of pipeline explosion and its antecedent threat to the loss of farmland, pollution of ground water sources, abdominal pain and ecological effects was well investigated (Omodanisi et al., 2014). This highly degraded land and the accompanying pollution is a visible proof of hazards to human health.

The extensive degradation of the region has suffered a setback ecologically with an average yearly spill of 115,000 barrels which make the ecosystem the most oil-impacted in the world (Ndifon, 1998). Further research work using the GIS and remote sensing technologies to assess the spills and physical impact shows that oil spreading is continued unless proper clean-up or remediation process is undertaken, as there has been an increase of contamination from 5.14% in 1987 –2002 and 17.64% from 2002- 2004 indicating a total spread of 22.78% from 1987-2004 (Ajide and Isaac, 2013).

Drilling fluids materials contained organochloride pesticides (OCPs) and corrosion inhibitor has adverse effect on plants species (Sojinu et al., 2012). They were banned/ restricted in 1980 by the developed countries while developing countries, like Nigeria, still encourage its use because of its low cost for insects and pest control (Sojinu et al., 2012). The extent of polycyclic aromatic hydrocarbons contamination (Olajire et al., 2005) in the sediments of Niger Delta agreed with the work of (Sojinu et al., 2010a). Consequently, based on combined chemical analysis and bioassay toxicity, the assessment of sediments of the Niger Delta revealed that the soil contained PAHs from petrogenic and pyrolytic origin (Olajire et al., 2005).

Spill incidents include intermittent discharges of crude oil affecting the ecosystem from both land-based wastewaters and ballast water (Benson and Essien, 2009). The non-biodegradable pollutants contrary to most pollutants have protracted impacts on the soil and adsorption properties from a large number of metallic compounds (Iwegbue et al., 2009). The spills effectively damage both flora and fauna within the environment of the contaminated soils citing an example of the Ogbodo-Isiokpo spill in the Niger Delta of 1997, the discharges from the highly pressurized crude-oil pipelines were evaluated with respect to the overall or total organic matter and total organic carbon conclude the acidic level of pH 4.2±5.6 as compared to the soil that was not polluted. This hydrocarbon

contamination indication was within the range of 2.71±3.48 mg/kg (Osuji and Adesiyan, 2005a), and the severely pollution of the oil drill wastes pumped from the crude oil well on the terrestrial and aquatic ecosystems and the contamination extent from the sources of PAHs (Odeyemi and Ogunseitan, 1985). Consequently, (Olajire et al., 2005) made an assessment of the sediment of petrogenic and pyrolytic sources using the bioassay and chemical analysis combined to indicate non polar aliphatic compounds of the major cause of toxicity in the sediment. The chronic and acute effects of wastes linked to the moderate and humid aquatic environmental processes which were associated with petroleum mining in the offshore regions was reviewed (Holdway, 2002) and credible solutions and applicable contingence plans were proposed by (Okogu, 1994) for the petroleum industry. He opined that the management approach to the problem should be systematic in taking advantage of Clean Nigeria Association (CNA) which is a consortium of eleven oil companies inclusive of NNPC as the main objective is to combat spills of liquid hydrocarbons and general pollutants (Nwilo and Badejo, 2006). By this measure, the ecosystem will be preserved and waste lands will be geared towards productive use (Anyakora et al., 2011).

### *2.4.2 Human health*

There has been a dearth of figures and statistics rates in Nigeria but research carried out by (Chukwuma, 2006b) link PAHs to cancer risk among Nigerians as a result of pollution., and decreases in sperm count and fertility to humans and animals, many miscarriages could be attributed to the effects of oil crude oil exploration in the region (Azibabua et al., 2013) and the conducted research on the Histopathological effects of the Nigerian Bonny Light Crude Oil on the Ovaries and Fallopian Tubes of Pregnant Rats. Previous studies also show a causal link to cancer related issues and infertility and shows prevalence in childhood malnutrition on children at a rate of 24% increase (Azibabua et al., 2013). The petrochemical plants at Alesa Eleme in Portharcourt constitute a channel for environmental pollution which include products obtained from the various chemical processes. This threat necessitated a studies of 54 samples collected and analysed from streams and rivers of communities with public taps show contamination of aquifers (Agbalagba et al., 2013). Dispersants of effluents into the environment raises serious concerns in the society and

shows danger of mitochondrial dysfunctions and apoptosis in mammalian cells (Zheng et al., 2014), possibly contributing to cell death. Exploratory activity in parts of the Niger Delta with sediments or soils containing polycyclic aromatic hydrocarbons posed real threats to the ecosystem in some areas of the two rings PAHs dominant Imo rivers and Oginni canal (Sojinu et al., 2010a), a similar work carried out in the area also revealed the carcinogen substances (Ordinioha and Brisibe, 2013a).

The contaminant is a grave source of concern as a result of the PAHs determination that was cancer related and other effects of oil spill lead to lipid oxidation as a result of ammonium metavanadate or crude oil or co-exposure to both, which has been tested with male albino rats (Mahmoud et al., 2012), and confirmed similar studies by (Sunmonu and Oloyede, 2012) on exposure to contaminated water consumption by rats with monocrotohos. This in turn leads to functional damage of the hepatocytes in activities of the transaminases and liver to the body weight ratio. There is no doubt whatsoever, that oil spillage has effect in the concentration of some inorganic cations and anions which impairs the sources of water for domestic and industrial purposes if not treated (Onyeike et al., 2002)**.** In conclusion, the findings and present studies have shown that the Nigerian bonny light crude oil is toxic to the reproductive system of rats and this toxicity is expressed by progressive and significant ($p \leq 0.05$) decreases in weight and loss of pregnancy and also confirmed by (Orisakwe et al., 2004) on research carried with the Bonny light crude oil and its adverse effects on the kidney of an animal using adult albino rats, after 7 days, the result revealed highly damaged kidney cells as fluid intake was measured daily.

### *2.4.3 Food Security*

Oil exploration and production has created food challenges in the Niger Delta and the destruction of farmland which in turn brings poverty and hunger. The traditional means of livelihood which comprises of farming and fishing has been comprised due to lose of soil fertility, pollution of rivers and loss of forest wildlife (Abejide, 2014). The situation has gradually undermined the source of livelihood of the people and placed the region in a precarious state. Summary of loss of farmland area may be hard to quantified, however the environmental impact assessment of Ogoniland from the United Nation report (UNEP, 2011) on 76 sites (land and ground water) and the Niger Delta restoration report (FME,

2006) shows a clear example of a heavily polluted region due to oil spills (Omadjohwoefe, 2013). Soil organic content is a major requirement for healthy farm crops produces hence any reduction in pH, organic content, Nitrogen and Phosphorous affect soil fertility (Mmon and Deekor, 2010). The crude oil contaminant affects the soil fertility when spills occur; apart from the attendant effects of destruction of farmland, change in biodiversity, there is a general loss of ecosystem. Food is important for the survival of humanity, however most people in the Niger Delta live on less than $1 per day (Bationo et al., 2007). The rapid depletion of soil organic carbon is the resultant effect of continuous cultivation of the arable land for the rapid growing population of Niger Deltas which was as a result of declining food production in the last 20 years. Long–term and acute effects on human health as a result of oil spills has been well articulated (Ordinioha and Brisibe, 2013a) that oil spills reduces household food security, as it affects both vegetables and cassava production by 40%.

In alleviating rural poverty, deficiencies in households and environmental exploitation, the need for transformation of agriculture in West Africa agro-systems with expansion of production capacity is imperative coupled with soil productivity restoration and maintenance as there is a linkage between soil fertility and soil organic matter. The environmental protection for the agro-system based should be encouraged and maintained through sustainable land management for improved soil fertility. Sustainable land management is an index of soil organic which is being used to determine the response to nitrogen and phosphorus fertilization (Bationo et al., 2007, Nandwa, 2001, Woomer et al., 1994).

## 2.5 Policies and management regimes in the Nigerian oil industry

Ministry of Petroleum Resources (MPR) deals with the political activities of upstream, midstream and downstream sector of the petroleum industry regarding oil spill management, comprising of DPR, NNPC, Petroleum Products Pricing Regulatory Agency (PPPRA), Petroleum Training Development Fund (PTDF) and Petroleum Equalization Fund (PEF) in Nigeria (Ambituuni et al., 2015). Of them, DPR and PPPRA are government regulators, while NNPC, PEF and PTDF are commercial, marketing and training arms respectively. Figure 2.6 shows the governmental structure for petroleum and non-

petroleum agencies, which have specific functions and responsibilities, as summarized in Table 2.4. Likewise, Federal Ministry of Environment (FME) is the governmental agencies dealing with oil spill management from environmental aspect, comprising of National Oil Spill Detection and Response Agency (NOSDRA), National Environment Standards and Regulation Enforcement Agency (NESREA), Forestry Research Institute of Nigeria (FRIN), the National Parks Service (NPS) and Environmental Health Officers' Registration Council of Nigeria (EHORECON). However, both NOSDRA and DPR have the oversight functions of oil spill management and remediation as contained in the Environmental Guidelines and Standards for the Petroleum Industry in Nigeria (EGASPIN) of 1992 and 2002. This duplication of functions has made it difficult to tackle oil spill promptly hence it hampers oil spill management. A detailed description of the agencies and functions will be attempted.

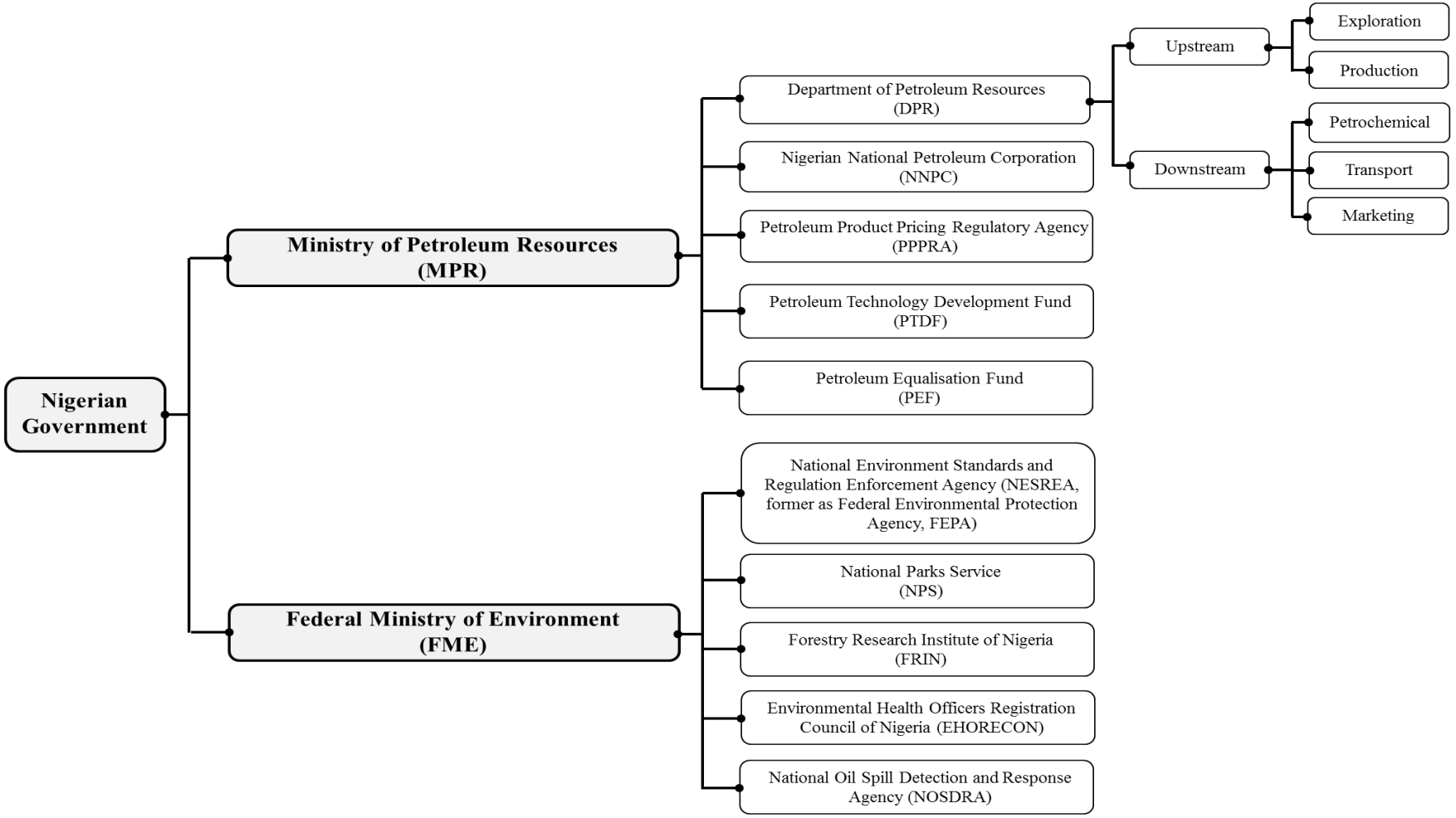


**Figure 2. 6** Nigerian governmental structure for petroleum industry and oil spill management.

**Table 2. 4** Nigerian government structures for petroleum industry and oil spill management.

| | Agencies | Functions | Challenges |
|---|---|---|---|
| **MPR** | **DPR** | Technical regulator for the oil and gas industry, supervising all the upstream, midstream and downstream activities of petroleum industry. | Duplicated functions with NOSDRA. |
| | **NNPC** | Government state owned corporation with 13 subsidiaries, covering all the upstream, midstream and downstream activities of petroleum industry. | Duplicated functions with DPR and PPPRA. |
| | **PPPRA** | Downstream commercial petroleum regulator to determine the pricing policy of the petroleum products and regulate the supply and distribution of petroleum products for improved transparency. | Duplicated functions with DPR and NNPC. |
| | **PTDF** | Training and education of Nigerians in the petroleum industry: 1) Human capacity development; 2) Institutional capacity building; 3) Research and development for technology enhancement | - |
| | **PEF** | To ensure the mechanism of uniform pricing for oil industry. Charged with the primary responsibility of reimbursing petroleum marketing companies for any losses suffered by them, solely and exclusive, as a result of sale of petroleum products at uniform prices | - |
| **FME** | **FEPA** | Defunct Agency. Repealed by the NESREA Act, 2007 | - |
| | **NESREA** | Leading environmental protection agency for environmental standards and enforcement. | Duplicated functions with DPR and NOSDRA. |

| | | | |
|---|---|---|---|
| | **NPS** | To preserve, enhance, and protection of plant life and wild faunae management in the National Reserves as a result of petroleum activities. | - |
| | **FRIN** | To conduct research relating to wild life management, agroforestry or utilization of forest yields. | - |
| | **EHORECON** | For environmental health officers to ensure that the environment is devoid of hazard and threat to human lives | - |
| | **NOSDRA** | To coordinate and implement the national oil spill contingency plan. | Duplicated functions with DPR. |

**Table 2. 5** Acts and regulations superintended by DPR.

| Acts and regulations | Functions |
|---|---|
| **Oil pipelines Act (1956)** | Provide for licenses granted for the maintenance of oil pipelines, including supplementary provisions in relation to oil fields, oil mining and pipelines. |
| **Oil in Navigable Waters Act (1968)** | Implementation of the International convention for the prevention of the pollution of the sea by oil and also makes provisions for such prevention in the navigable waters of Nigeria |
| **Petroleum Regulations Act (1967)** | Prohibition of discharges waste water into water sources |
| **Petroleum Act (1969)** | Grants the rights for exploration from the territorial waters and Nigeria's continental shelf |
| **Petroleum (Drilling and Production) Regulation (1969)** | Regulation setting out requirements for oil prospecting license and oil mining lease. The regulation also helps to control and prevent production pollution |
| **Petroleum Refining Regulations Act (1974)** | Regulation of the petroleum refining observation within the refining industry. |
| **Petroleum Production and Distribution (Anti-Sabotage), Act (1975)** | Offence and penalty of sabotage in respect to production and distribution of petroleum products in Nigeria. |
| **Petroleum Equalization Fund (Management Board), Act (1975)** | Reimbursements in relation to losses suffered by petroleum marketing companies within Nigeria as a result of sales of petroleum products at uniform prices across the country |
| **Associated Gas Re-Injection Act (1979)** | Phase out gas flaring in Nigeria. |
| **Associated Act Reinjection Regulation** | Prohibition of gas flaring and promote the re-injection of associated gas |

| | |
|---|---|
| **(1980)** | accruable from petroleum production |
| **Crude oil (Transportation and Shipment) Regulations (1984)** | Prohibition of topping and prescribe checks for vessels, ships, tankers, in transportation of crude oil engagement |
| **Petroleum Products (Uniform retail prices) Order (1986)** | Prohibits non-uniform retail price across the country and therefore negates products prices driven by the market forces |
| **Petroleum (Drilling and Production/(Amendment) Regulations (1988)** | Set the right and granting licences with respect to drilling and production matter, practices and working ethics, conservation on the field development |
| **Petroleum (Amendment) Regulation (1989)** | Sharing formula between NNPC and oil operating companies. |
| **Petroleum (Amendment) Decree (1996)** | Amended to add marginal fields |
| **Petroleum Products (Prices of automotives and lubricating oils) Order, (1996)** | The bill seeks to address the pricing policy of automotive and lubricating oils |
| **Mineral oils (Safety) Regulations (1997)** | The regulation deals with the handling of mineral oil safely. Prescribes safely standard and imposition of penalty with respect to (OMLs)Oil mining lease) and (OPLs) Oil prospecting license |
| **Deep offshore and Inland Basin Production Sharing Contracts Decree (1999)** | It is about certain fiscal incentives granted to the oil and gas companies operating in the deep offshore and inland basin areas underproduction sharing contract with NNPC |
| **Deep offshore and Inland basin production Sharing Contracts (Amendment) Decree (1999)** | Basically among other things, the Act gives effect to certain fiscal incentive of oil and gas companies in the deep offshore and Inland Basin area under the production sharing between the oil companies and NNPC on behalf of the |

| | |
|---|---|
| | government |
| **Petroleum (Drilling and Production (Amendment) regulations (2001)** | Amendment discourages abandonment of existing wells |
| **Deepwater Block Allocation to Companies (Block–in-Rights) Regulation 2003** | The regulation applies to oil mining leases and oil prospecting for deep water blocks except those issued to NNPC |
| **Oil Prospecting licenses (conversion to oil mining leases, etc) Regulation (2003)** | The Act deals with the regulation effect on an oil prospecting license issued under the petroleum Act and therefore converted to oil mining lease after satisfying all conditions specified by the regulator (DPR) |
| **Marginal Fields operations (Fiscal Regime) Regulations (2005)** | Applicable to fiscal regimes for marginal fields operations. |
| **Petroleum (Drilling and Production (Amendment) regulations (2006)** | The provisions for the prohibition of waste disposal of products from refining activities are the provisions set out by the Act of 2006 |

### *2.5.1 DPR*

DPR was established from the Nigerian National Oil Corporation (NNOC), as an affiliated department to handle the commercial operational activities in the oil industry on behalf of the Federal government of Nigeria (DPR, 2014). DPR became fully autonomous in 1975, subsequently to ministry and later renamed as MPR in 1985. As the Inspectorate it was removed from NNPC and transferred to MPR as the technical arm and finally renamed as DPR. Sequel to the Decree 33 of 1977 merged MPR and NNOC to form NNPC, DPR was under NNPC as a regulatory body. The subsumed functions of DPR include:

1) To supervise all the operations in petroleum industry carried out in Nigeria, related to license, lease and permit;
2) To apply field monitoring of petroleum industry operations and ensure national aspirations with respect to gas supply obligations and limiting gas flaring;
3) To ensure that all the operations in petroleum industry meets with the international regulations of health, safety and environment;
4) To record oil reserves, production, exports, licenses and leases during petroleum industry operations;
5) To advise Nigerian government and other national agencies on techniques and public policies related to petroleum exploration activities and administration;
6) To charge rents, royalties and other revenues timely and accurately on behalf of Nigerian government;
7) To administrate and maintain National Data Repository (NDR).

DPR released series of regulations and actions to control and mitigate the consequences from crude oil spill and contamination which necessitated the government to put in place different number of legal and policy instruments to address the issues of pollution in the oil industry since 1956. Table 2.5 summarized Acts and Regulations of the government for which the DPR exercises oversight responsibilities. The Petroleum Act of 1969 (as Amended), provides regulations for the safe working of operations in the petroleum industry coupled with the prevention of pollution of water sources and conservation of

petroleum resources. It however lacks transparency in the process for the grant or award of petroleum leases, licenses and permits, as the sole responsibility is vested on the Petroleum Minister. Conversely, the drilling and Production Act of 1969, as amended in 1988, 2001 and 2006 provides for land protection, environmental protection, decommissioning of wells and procedures for abandonment wells. It also makes provisions for the licensees and lessees to mandatorily keep accurate records of the crude extracted. However, the confidentiality clauses in the lease agreement restricts transparency and accountability. In 1999, the Deep Offshore and Inland Basin Producing Sharing Contracts Decree, 1999, 2003, and marginal field operations (Fiscal Regime) Regulation 2005 was enacted as a result of the Nigerian governments interest in promoting investments in the deep off shore, the Act therefore served as an incentive scheme to expand oil and gas investments off shore. The Associated Gas re-injection Act became effective in Nigeria as part of the commitment to the Climate Change Convention (UNCCC, 1992); (Oppenheimer and Petsonk, 2005) to delimit carbon emissions and climate change. The political will to stop gas flaring is however grossly lacking. The Act mandates the submission of proposals from oil producing companies in Nigeria to submit schemes for the re-injection of all associated gas. Gas flaring has been a burning issues in the Niger Delta, in 2012 the Nigerian government signed an agreement to stop gas flaring by 2020 with the UN but the enforcement to delivered. However the dates for implementation of cessation of flaring failed and re-injection Act of the associated gas serves to weaken the Act and undermine the gas flaring cessation process and thus encourage incessant environmental pollution due to failure of enforcement.

### *2.5.2 FME*

The Nigerian Federal Republican Constitution of 1999 by virtue of Section 20 has environmental policy as a core provision and it entails that the powers to protect, safeguard the three media; land, wet lands or water bodies, atmosphere, forest and wild life in order to improve the environment reside in the Federal Government. This is articulated in the provisions of the empowerment act of the FME (FME, 2013) this immediately came into force during the civilian administration of Chief Olusegun Obasanjo. This was in the aftermath of a protracted period of military rule in Nigeria. The objective was to reconcile

Nigerian legislation and environmental law implementation with the obtainable standards in the global society. The structure of FME has some parastatals or agencies under its supervision as Federal Environmental Protection Agency (FEPA), NOSDRA, NESREA, NPS, FRIN and EHORECON. This is in addition to DPR. The ultimate challenge generated is that despite these regulatory bodies, the intransigent issues relating to the environment is still very much a concern. The problems of spill need to be taken seriously if Nigeria wants to meet up with the developed world and be part of a committee of environment protection. Table 2.7 shows Acts and regulations superintended by FME.

### *2.5.3 NOSDRA*

NOSDRA is a non-petroleum governmental institution, which was legally approved by parliament in 2005 and started implementation since 18$^{th}$ October in 2006. NOSDRA has a clear vision of sustaining a zero tolerance for any incident of oil spill in the Nigerian environment, to also create and nurture ecocentric practices during exploration and exploitation. NOSDRA has thirteen clear objectives of protecting the environment:

1) A national operational organization to adequately monitor major oil pollution in a timely, effective and appropriate manner.
2) To identify and clean-up high-risk areas.
3) Monitoring of resources to save lives, with a view to protect the environment in line with best practices.
4) To collaborate with other bodies like CNA to ensure maximization of facilities for implementation and assist when spills occur.
5) The use of combating materials and equipment and functional network system for effective response in cases of major oil pollution incidents.
6) To manage with a good training and drill exercise for oil pollution readiness.
7) Liaison with the African sub region by requesting for cooperation, technical services and consequently for cooperation in responding to major oil incidences.
8) Cooperation with International Marine Organization (IMO) in view to combat oil pollution via a modern technology response to oil spill monitoring and evacuation.

9) Training in the area of Research and Development (R&D) to help even local incidence pollution.

10) An agreement establishment with other African countries sharing common border delineation for rapid movement of equipment and other materials for activities arising from emergency oil spills.

11) New strategies for fighting oil spills and responses

12) To develop an entire audit plan for implementation.

13) Carry out other duties as specified in the functions or mandate.

The NOSDRA functions are summarized into surveillance, compliance, reports, coordinates, formulation, implementation, removal of substances that are hazardous to the environment by carrying out the set time objectives (NOSDRA, 2014). The establishment is subdivided into upstream and downstream operations with three levels or tier response system that are determined by the quantity of barrels spilled which can be summarized in the Table 2.6. The table shows the size of the oil spills with the agents accountable for the spill. The table also shows the coordinating body (NOSDRA) providing strategies and oversight for oil spills incidents via the establishment of a National Response and Control Centre. By the section 5 of the Act of NOSDRA, the Agency mandate among others things is to ensure timely, safely and effective and appropriate response to oil pollution and the identification of high risk and cleanup areas which is at the top priority.

### *2.5.4 NESREA (formerly FEPA)*

The FEPA Act was replaced by the administration of FME, NESREA Act 2007 (NESREA, 2013). Its regulation is on National Effluent Limitation Regulation, Federal Solid and Hazardous Waste Management Regulations (1991), National Environment Protection (Pollution Abatement in Industries and Facilities producing Waste) Regulations (1991) which requires industry facilities to have anti-pollution equipment for the treatment of effluents, the agency has custodian of submission of industry's treated effluents, prohibition of substances that are hazardous into the air, land or water released in Nigeria beyond the approved limit as set by the Agency. The report of industries discharges and submission of comprehensive list of chemical used for production are submitted to the Agency. It

regulates the solid hazardous wastes of industries which are dangerous to public health and the environment and the possibility of recycling by research. Finally, any discharge of hazardous waste must be notified by industries to the Agency. The Agency also has the power to imposed penalty.

A similar related organization is the NESREA which is an organization under the FME and its does not have any link with oil spill management but its mandate was to enforce the relevant environmental laws or guidelines as well as policies, standards and regulations in Nigeria and also enforce compliance with the International treaty obligations, and conventions reached since Nigeria became signatory.

**Table 2. 6** Categories of oil spill and agencies responsible for operations.

| Level of Tier | Spill volume (barrels) | Body Responsible |
|---|---|---|
| **Tier 1** | <50 | Individual body or Oil Pollution Preparedness, Response and Cooperation (OPRC) |
| **Tier 2** | 50-5,000 | A mutual cooperation of local bodies like CNA. |
| **Tier 3** | >5,000 | Both national and international cooperation which may be either close or in a remote area from the company facilities. |

**Table 2. 7** Acts and regulations superintended by FME.

| Acts and regulations | Functions |
|---|---|
| **Cap 165 LFN in 1990** | Harmful waste disposal prohibition. |
| **Cap Act (1968)** | The conservation of nature and natural resources reached in Africa. |
| **Cap 108 LFN 1990** | Endangered species protection. |
| **Cap 131 LFN 1990** | General guideline for federal environmental protection. |
| **Cap 1972** | Prevention of marine pollution damage. |
| **Cap 1971** | Compensation for oil pollution damage. |
| **Oil Pollution Act 1990** | Oil pollution prohibition by preventing, mitigating, cleanup and liability. |

### *2.5.5 FRIN, NPS and EHORECON*

FRIN, NPS and EHORECON are not directly linked to oil spill monitoring, but have the responsibilities to preserve, enhance and protect the plant and wild fauna in the National Reserves as a result of petroleum activities, and also conduct research relating to wild life management, including all facets of forestry, agroforestry or utilization of forest yields.

## 2.6 Challenges and perspectives

### *2.6.1 Policies and regulations*

This resultant effect of spills eventually lead to the destruction of farmlands, groundwater and soil contamination which generally poses a threat to human health and destruction of biodiversity (Nwilo and Badejo, 2006). One major challenge in Nigeria earlier discussed is the lack of effective regulatory and management policies to address the serious issue of oil spillage. However, the government and the people acknowledge the danger that poses to the environment and the ecosystem. The pertinent question is what steps has the government taken to tackle it. The main challenges in tackling the crude oil contamination are the cost, facilities and technician's handicap to tackle the oil spill and crude oil monitoring compared to the developed society. The governments need to provide adequate fund for these agencies to operate efficiently, effectively, independently and can exercise effective technical oversight. Both DPR and NOSDRA of government bodies responsible for the monitoring of the oil spill and remediation lack resources as they suffer from shortage of senior staff and experienced staffs that lack understanding of the oil industry especially in spill management (UNEP, 2011). The monitoring and assessment activities, which are necessary aspects of the downstream over-sight functions, require expert and environmental scientists. This is crucial for an enhanced or more professional and specialized inspection of all oil spills and the duration of the clean-up. This will go a long way in curbing subsequent spills in the Niger Delta. Crude oil-contaminated sites can be monitored through self-potential processes i.e. geophysical method (Giampaolo et al., 2012). The groundwater and soils contamination by hydrocarbons is not just an environmental problem but a source of concerns, and the need for regulation and monitoring is imperative since Nigeria has been regarded as one of the worst nations in the world with discouraging environmental records (Ezeonu et al., 2012). The seriousness of oil spill in the Niger Delta could be

associated with the non-identification of the source. How spill happens and its occurrence is a serious challenge.

Initially, the region of the Niger Delta comprised of the three states (Rivers, Delta and Akwa-Ibom) with similar geographical location (Abejide, 2014). The people livelihood is basically farming, trading, lumbering, fishing, palm wine tapping, manufacturing etc. until oil exploration and production activities which destroy the livelihood, altered biodiversity and environmental degradation. For more than 48 years since oil was discovered in commercial quantity in 1956 and commercial production in 1958, there has been no wholesale reform of the Nigerian oil and gas sector legal and regulatory framework to cater for the petroleum sector apart from segmented laws and Acts. Apart from the creation of NNPC in 1977, the Minister of State Petroleum Resources held a consultation meeting called '7 Big Wins' on 14$^{th}$, January 2017 in Aberdeen, Scotland to set a new charter for the industry to meet up with global standards and best practices. Oil-led development countries have been characterized by negative consequences on their social, political, and economic structures (Karl, 2007). The effect of the social and environmental at the regional and local levels were properly presented and articulated. The purport of the article was that a country dependent on oil as the primary revenue source was characterized by high levels of poverty, inequality, with slow growth rate, lack of employment, hindrances to diversification of economy, high corruption, negative health and environmental consequences at various levels, high incidences of conflict and war. This true fact presentation of (Karl, 2007) was strengthen by (Kadafa, 2012a) as many host communities has been relocated as a result of the oil spillage.

The challenges of the current policies and regulations despite the laws that were promulgated could be attributed to the following:

Poor Standards of enforcement by the regulatory bodies due to conflict of roles. A sound regulatory body with standard practices, equipped technical facilities, knowledgeable human and financial capacity will help to reduce the crude oil contamination in the region (Ladan, 2014). Conflicts of roles of regulatory bodies are one major handicap of quick and prompt response to oil spill management.

Exorbitant cost of procuring and maintaining equipment: The equipment facilities for oil spillage containment is expensive to procure, and also the cost and culture of maintenance of already procured facilities has not been encourage by government in oil spillage management

Visionless and narrow-minded attitude: The passions of the agencies employees negate the visions of the laws and Acts establishing same, as assignment are not given due diligence. The government should trained and equipped agencies through exchange of technology by ensuring adequate budgetary allocations every year.

Ignorance of the impacts of contamination due to oil spillage caused by individual, corporate or government on the short and long term effects. There should be an educational campaign of the negative impacts nationwide at the federal and state level.

Corruption and lack of transparency is a serious impediment to the enforcement of laws and regulations in Nigeria. The inability of international bodies to combat the oil spillage in conjunction with local regulatory agencies is because of hostile attitude of the host community and contribution.

A recent study by the United Nations revealed that an effective clean-up technique for spills in the Niger Delta will take about 30 years. This assessment or evaluation, however, is not taken into cognizance subsequent or future spills. The most challenging issue with the companies was that the monumental costs of clean up and land remediation after spills have occurred have resulted in outright neglect of the environment. This has resulted in unabated ecosystem degradation. More worrisome, however, is the unsavoury practice of the holding indigenous corporations and government agencies to ransom in lieu of compensation. The peculiarity of the terrains was what made the British Government in 1958 to propose special Federal Territory for the Niger Delta. After 50 years of exploration activities, the environmental degradation of the region has become worrisome and the livelihoods of the Niger Delta people threatened turning the region into wasteland and soil infertility because of oil spillage and crude oil contamination (Gbadegesin, 1997).

### *2.6.2 Oil spill control and monitoring*

The environmental problems plaguing the Niger Delta is a re-current issue which needs urgent government attention. Presently, there are no viable environmental policies for effective ecosystem protection. In the same vein, oil spill monitoring has not been effective despite the roles of DPR and NOSDRA who have oversight regulations and EGASPIN (1992) operational guidelines, rather these agencies are concerned with data collections and also lack the technology of measuring the volumes of oil spilled. For example the unattended frequency of occurrence of the spills in the Niger Delta is as a result of lack of enforcement of the regulations relating to spill management and in most cases lack technology-know –how to combat spill. A substantial number of legislative enactments have been passed by the Nigerian Government to regulate the environment, as well as crude oil pollution arising out of industrial activity. The nagging issues that capture our attention is, despite the grand sounding names of these Acts and governmental agencies, when spills do eventually occur, how quick is the response of these Agencies? What are the instruments used to monitor these spills, how contemporary is the assessment process of these environmental risks or hazards? What are the standards or yardsticks for the measurement of the levels of pollution or degradation, what are the preventive measures that have been put in place?

Crude oil production activities have resulted in the release of petroleum effluents from the four refineries in the region. The complex constituents of crude oil and its associated products are detrimental to the ecosystem. This informed the provisions of the Oil Drilling, Production and Petroleum Exploration Act of 1969 with respect to exploration and production activities and vis-à-vis the preservation of natural resources as contained in the Act. The roles of government bodies monitoring the crude oil industrial activities have minimal impacts to the environment as result of issues affecting the duplication of regulatory bodies and more importantly, the overlapping roles (Nwilo and Badejo, 2006). NESREA, NOSDRA and DPR have oversight functions of effluents discharges from the refineries and petrochemical.

The two main approaches for crude oil monitoring are specific and non-specific methods (Wang et al., 1999). Specific determination of oil contamination, including Gas

Chromatography Mass Spectrometry (GC-MS), Gas Chromatography Flame Ionization Detector (GC-FID), High-Performance Liquid Chromatography (HPLC), Two-Dimensional Gas Chromatography (GC×GC), Isotope Ratio Mass Spectrometry (IRMS), Supercritical Fluid Chromatography (SFC) and thin-Layer Chromatography (TLC). All these methods can characterize the fingerprints of oils, extremely sensitive and highly automated (Wang and Fingas, 2003), but suffer from the exorbitant price and laser-heated source, low speed up analysis, and cost of analysis per time. On the other hand, the non-specific monitoring methods include infrared spectroscopy (IR), flourescence spectroscopy, ultraviolet (UV) and gravimetric analytics. They have the advantage that preparation and analytical time is short and cheap, and used in screening of sediments for aromatic and saturated compounds. It is also applicable for the measurement of total petroleum hydrocarbons (TPHs), contamination site assessment, petroleum products type and determination of its presence and existence in water or degradation. The disadvantage or drawdown is the lack of detailed individual component of data generated and the information of the non-specified source. This method has great limitations due to its inherent tendency of inaccuracy. Crude oil contamination will further undergo more complexity on the ecosystem due to weathering processes, microbial degradation, evaporation, photo chemical oxidation, dispersion, water oil emulsification, dissolution as the spills released into the environment. All aromatic hydrocarbons in different crude or oil have different numbers of aromatics (Wang and Fingas, 1997). Because of the inaccuracies of the non-specific monitoring method, this work will not recommend it for quick and rapid approach in crude oil monitoring in the Niger Delta.

Whole cell bioreporter is proposed as a wholly indispensable technique for application in all cases of crude oil contamination and ecological assessment. As a simple, sensitive and inexpensive method (Hansen, 2008), whole-cell bioreporter aids in compilation of information and data handling for environmental management and environmental studies in environmental sciences. Consequently, the complexity of contaminants of environmental samples thus makes it impossible for just physical or chemical interactions of chemical analysis which are unpredictable. Moreover, since risk assessment is related to bioavailability, the ability to respond rapidly, sensitively, ease of usage and cost-effectiveness are the constituents that makes it unique (Rodriguez-Mozaz et al., 2005).

Whole-cell bioreporters has very unique advantages as a result of the ease of toxicity determination and bio-availability (Zhang et al., 2013). The development of the bacterial whole cell biosensors uses live cells for sensing performance, notwithstanding its limitation of robustness and inability to reproduce (Song et al., 2012). The principle is composed of nuclei acid or proteins that are made up of biological elements consisting of regulatory genes(s), constitutive or regulated promoter(s) and the gene(s) reporter (Perumal and Hashim, 2014). The construction of the bacterial whole cell biosensors are based on behavioral protein changes or spectral properties in signal response which is known as "post-translational" biosensors. Whole –cell or tissue based biosensors are very stable and can stay for 8 weeks (Li et al., 2013, Song et al., 2009a). As such this work will recommend the use of bioreporter due to the advantage of rapid detection of genotoxicity directly over conventional chemical method and oil content in the determination of environmental pollution (Zhang et al., 2013). This is being used as a complementary tool to the chemical analysis method. Acinetobacter baylyi ADP1 are alkane degrader which can be used in the Niger Delta to sense alkanes because of his wide range with the well characterization of gene regulation (Zhang et al., 2012c). Some good examples can be found in fresh water, seawater and soil samples similar to the Niger Delta environment (Zhang et al., 2013). Owing to its significant advantages in crude oil spill management as fast and cost-effective approach, whole-cell bioreporter can also be used to curtail environmental degradation and thus act as the tool for environmental sustainability. Biosensor is defined as biotechnological tool that detects, transmit and record physiological or biochemical change (D'souza, 2001). The advantages of this tool over the conventional methods is in terms of its ability to monitor relevant toxicity, its associated swiftness and cost effectiveness (Ron, 2007). On the other hand, analytical techniques measures only concentration, while biosensors can measure toxicity because they are biologically based. The recent application and principles of biosensor (Michelini et al., 2013) and its deployment has attracted much attention through the use of smarts support, bio-inspired materials (confinement of living cells), the cost–effectiveness and portability. It requires smaller samples and can be analyzed in complex mixtures.

### *2.6.3 Petroleum contaminated land clean up*

There have not been cases of contaminated land remediation in the Niger Delta as the legislative law of EGASPIN 1999 (and 2002 revised) has no clear-cut of responsibility. The duplicity of responsibility of both DPR and NOSDRA has created loop hole for multinationals to take advantage of non-compliance in carrying out operational activities.

Remediation entails the process of treatment of contaminated land and restoring it to its original functionality. This process could involve physical, biological or chemical treatment. Different techniques have been applied in the Niger Delta but with little result, hence it is important to adopt the bioremediation approach which is socially, economically viable and eco-friendly. Details of these approach are discussed in the next section with recommendations, however the bioremediation approach is the viable option for the Niger Delta region based on many similar studies to the Niger Delta terrains and its permanent ability to remove contaminants successfully (Zabbey et al., 2017, Mandal et al., 2014, Wang et al., 2016b, Xu et al., 2016, Sarkar et al., 2005, Sánchez-Arias et al., 2013).

Nigeria have no appropriate current legislation for remediation and clean-up although it was mentioned in EGASPIN documents, Nigeria by virtue of the Section 19 of the NOSDRA Act CAP 157 Laws of the Federation of Nigeria empowers the oil spills detection Agency to advise the government on the impacts of oil spills on the health of the people and undertake appropriate remedial and restorative actions on the environment (Ezeibe, 2011) but not until June 2016 when FME in consonance with the Federal Government of Nigeria's Environmental Renewal and Development Initiative (ERDI) proposed to undertake the Hydrocarbon Pollution Remediation Project (HYPREP) to remedy the effects of environmental pollution and degradation due to oil production activities in Ogonilands and its environs (Sam et al., 2016, Zabbey et al., 2017). This may serve as a bench-mark for all remediation activities of all contaminated land oil clean-up in the future. Remediation is the process of restoring land to its original functionality via clean-up (Antizar-Ladislao, 2008), and the approach could be physical, chemical or biological (Zabbey et al., 2017). The Physical/chemical method is aimed to stabilize (stabilization), evaporate (soil vapour extraction and thermal desorption), elute (soil washing and solvent extraction), or transform (advanced oxidation) of the residual crude oil

in the soil. It has the advantage of complete crude oil removal and a short operation time but the major setback is the high cost of operation (Ferguson et al., 2004). Further remediation is needed in the enriched crude oil area through evaporation and elution treatment (Nkeng et al., 2012, JC and Mbogu, 2013, Zamani et al., 2014) which makes the option not viable in the Niger Delta region because of its complex terrains (covered by clays), carbon dioxide and greenhouse gases emissions into the surrounding environment from the chemical treatment method. None of the conventional methods are reliable than the biological methods since they are environmentally unfriendly, hence the need for drastically review of the biological method and hydrocarbon waste remediation. For example, during the Nigeria civil war in 1969, about 255ha of farm land in Ejama-Ebubu, Eleme of Rivers State was contaminated with hydrocarbon and is yet to be clean-up since there were no specific methods proved to be effective for the region (Zabbey et al., 2017, Giadom and Tse, 2015). The biological methods have attracted many attentions and there are many practical examples in the Niger Delta which includes bio-stimulation, bio-augmentation, phytoremediation and many more. Bioremediation is defined as the natural removal, reduction or transformation of organic and inorganic pollutants by living microorganisms (Wilson and Jones, 1993, Cohen, 2002, Das and Chandran, 2010). How successful is the bioremediation is dependent on the inherent biodegradability of the pollutants. The advantage of bioremediation is the comparative cost effectiveness with no deteriorating impact on the environment (Pasumarthi et al., 2013). Different methods of bioremediations application in the Niger Delta and their advantages are discussed.

Biostimulation is the approach of adding nutrients and oxygen to boost the activities and functions indigenous oil degraders. The nutrients and oxygen are the major constituents the microbes needed to create the necessary enzymes to break down contaminants. Research depict that “organic matter content and soil-nutrient status with a 400kg/ha rate of poultry manure application” was effectively applied in the studies of crude-oil pollution soils to ameliorate its content which was successful, however, this research was done in small scale (Ogboghodo et al., 2005). Similarly, a related work was carried out (Adesodun and Mbagwu, 2008, Orji et al., 2012) and was only successful in small scale. The fertilizer applications applied to the farmland has the disadvantage of toxic nature to waterways, humans and marine ecosystem because it contained tributyltin compounds. The

antifoulingpaint was banned in 2008 because of its negative impact (Du et al., 2014). Minimal results were achieved by the use of fertilizer at 2 ton/ha rate for about 5 weeks in the oil contaminated soil, which indicates a possible application of bioremediation (Chorom et al., 2010), however, the application of fertilizers increased the concentrations of nutrients and enhanced the biodegradation in the petroleum-polluted agricultural soils. This processes was remediated (Ayotamuno et al., 2006) in the areas that was contaminated by major spills as the physicochemical parameters are altered leading to the environmental degradation. In remediating the land, the bio-stimulation method with fertilizers and moisture content for an agricultural research (Ayotamuno et al., 2006). The result revealed a decrease in total petroleum hydrocarbons after six weeks remediation, except for the control cell which was due to anaerobic conditions as the rate of fertilizer affect the rate of degradation. This research revealed that effective bioremediation for agricultural soils should be done preferably in a dry season of the Nigerian climate with Nitrogenous-based fertilizers in an applicable range of 4.7 and 12.5 ton/ha.

Alternatively, bioaugmentation increases the oil degradation performance by adding indigenous or exogenous petroleum degrading microorganisms into the contaminated sites. This process is to assist the stress microorganisms that is not capable of biodegrading the contaminants. Case studies were presented of different countries where bio-augmentation has been applied and its recommendations with strict regulation. The process recommends a combination of bio-stimulation and bio-augmentation and hope will be a viable option in remediating contaminated sites in the Niger Delta (Adams et al., 2015) .

Plant can be also introduced as an approach to promote oil degradation as phytoremediation. Naturally, in the ecosystem, plants acts as filters and metabolized substances generated by nature. Phytoremediation is an emerging technology but have a disadvantage of high cost and long period of operation. This method has been applied in the Niger Delta using the water hyacinth (*Eichhornia crassipes)"* treatment which show positive result of remediating crude oil contaminated soil at 1 and 3% but has disadvantage of prevailing factor in the whole process of remediation and cannot be used for large scale contamination (Udeh et al., 2013).

Enhanced natural attenuation process (ENAP) is another method applied in Niger Delta (Akpan et al., 2013), and the subcritical process for remediation was tested in lab-scale studies. The experimental results show that a high efficiency remediation of hydrocarbons like “lubricating oils, diesel fuels and polycyclic aromatic hydrocarbon (PAHs)” removal in a range of 77% and 91-99% was observed respectively under temperature influence, and pressure. The RENA method operated by industry operators and regulators in Nigeria is the popular method adopted for the Niger Delta terrains. The lithology challenge of sites in the Niger Delta makes it difficult for effective means of remediation especially where the spilled oil has percolated the soil beyond 5m and the aquifers of the groundwater has been altered (Zabbey et al., 2017, Ebuehi et al., 2005, Orji et al., 2012). However, only small farm settlement remediation was successful (Ebuehi et al., 2005) but it is quite time consuming and it requires strict monitoring during the process. Adherence strictly to standard practices and the mitigation measures for all the cases against RENA is documented in some reviews especially in (Orji, 2012).

Bioreactor is a process that treats contaminated soils and sludges in a bioreactor by extraction and biodegradation (Riser-Roberts, 1998). Bioreactor method is an ex-situ treatment and more friendly environmental approach but has a limitation of capital because it requires soil evacuation. Studied described the technique of bioreactor based treatment as having advantage over all other treatments through phytoremediation, bioventing, composting, bio-filtration, land-farming, bio-sparing and bio-piling of the land with a success and also the advantage of putting the environment in an optimum controlled condition for the hydrocarbon biodegradation. The enhancer (namely NPK fertilizer, poultry litter and urea fertilizer) for the biodegradation of the pollutants in the seven stirred tank bioreactors was used for a similar research to test the scale of remediated hydrocarbons in a degraded mangrove swamp using nutrients of cow dung on one of the site (Chikere et al., 2012, Orji et al., 2012). The method has a similar disadvantage of inadequate transfer of technology on an industrial scale. The restoration of mangrove swamps through bioremediation technology will give hope to the people of the region when a large scale pilot is researched in the future. The degrading harmful organic component is being achieved by the spontaneous movement of the activity impacted by bacteria, algae, fungi which produce the enzymes. The improvement of the soil pH, original soil status, soil

fertility and soil quality was introduced by (Adekunle et al., 2012) using a locally resourced material which was designed to fit the heterogenous complexities and multiphasic of the Niger Delta contaminated environment. This use of the bioremediation agent (Ecorem) applied in solving and assessing the effect of remediating in an oil polluted soil assessed the importance and benefits of (1) Looking at petroleum product spill on the soil pH with his attended effect, (2) The ability to predict the purposes of the products-soil ratio simulation on the soil pH, (3) the product–soil ratio on soil pH influences and (4) the ability and impact to remediate the soil using localized product on the soil pH while comparing it to original status of the soil and remediated matrix. In carrying bioremediation studies, an enrichment culture is a choice of separation of microorganisms present in the contaminated soil. In the treatment of oil-contaminated samples, the enrichment culture shows a great potential as culture that can be used. However, it has been recently acknowledged that in the subsurface oil biodegradation, the primary agent is an aerobic bacterium which occurs in oil/water contacts. (Taylor et al., 2001) draw his conclusion that an increase biodegradation and concentration of phenol reduction $C_0$+$C_1$ are predominantly typical of Nigeria oils. The research shows that crude oil samples of alkanes range from $C_{12}$ to $C_{42}$ with occurrence of lighter alkanes indicate that the spillage is recent. The pattern of degradation of the alkanes is such that the rate of degradation decreases with increase of the carbon number. The higher the alkanes number of carbon, the longer it takes to degrade the alkane because the solubility of hydrocarbons decreases with the increase in molecular weight as the resultant effect of *P. aeruginosa* and *E. fergusonii* that was present in crude oil showed great potential in degrading hydrocarbon. The biological treatment of soil (bioremediation), in–optimum environmental conditions, is essential in the removal of hydrocarbon as a result of spills resulted from blowouts, vandalization or equipment failures. A computer-based system was used for investigation and selection of remediation technologies in petroleum-contaminated soils in Romania. The ability for the computer-based system to assess the pollutants, the estimated migration and the preliminary soil investigations provided that the recommendation has a high advantage (Dunea et al., 2014), although this methodologies are not quite popular in the Niger Delta despite the common usage of the software in the region. However, the ability of specialists to classify it into six major areas of decision support makes it a viable option. The six major classifications are

nature and contamination extent, data from site characterization, remedial action, data worth, risk from human health and economic cost/benefit, since no single technique/technology may be considered as a solution or panacea for all contaminated sites problems (Riser-Roberts, 1998).

The disadvantages of the physical/chemical methods make the application of nanoparticles in environmental bio-sensing and bioremediation solution to remediate the Niger Delta environment with a view to commencing pilot studies, as research intensifies. Bio-sensing and bioremediation have been regarded as the most cost effective and reliable techniques to solve such problems. With the recent development of nanotechnology, the combination of nanoparticles and biological process is successful in enhancing measurement accuracy, improving bioremediation efficiency and broadening biochemical application in environmental research (Zhang et al., 2012c). This serves to propose risk assessment and bioremediation as crucial strategies for the Nigerian government to curtail further environmental devastation and promote ecosystem rehabilitation.

### *2.6.4 Sustainable development*

The World Commission on environment and development and subsumed in the Brundtland Commission report (1987) is hinged on sustainable development. This report defines sustainable development as '*The development that meets the needs of the present without compromising the ability of future generations to meet their own needs*'. This idea comprises of two remarkable concepts. First, the concept of **needs**, in particular the essential needs of the world's poor, to which overriding priority should be given. Secondly, the idea of **limitations** imposed by the state of technology and social organization on the environment's ability to meet present and future needs.'

All definitions of sustainable development require that we see the world as a system connecting both space and time. Based on the above concept of sustainable development, the Niger Delta region belongs to the world's poor and therefore requires special attention on all three pillars of sustainability so as to achieve environmental protection and enhancement in the region. Lack of enforcement of environment policy which is aimed at achieving sustainable development creates an insecure environment and at such secured the inequality environment for the well beings and health status of the Niger Delta.

Going by the enormous environmental impacts caused by oil spills, the government should include or modify the proposed Petroleum Industry Bill (PIB) to contain stiff penalties with a view to serve as major deterrents in the oil spill prevention policies. For example, the cost implication of Deep Water Horizon oil spill at the Gulf of Mexico is above $28 billion, as the penalty and claim for compensations (50%) and clean-up operations (50%) (Ramseur and Hagerty, 2013). It would also be necessary to include clauses in the bill requiring petroleum industries to remediate the environment of future spill occurrence caused by the oil exploration and Production companies. As derived from the PIB (PIB, 2012) the responsibilities of the downstream sector should contain parts of the bill that deals with the remediation areas as part of the core downstream operations which would include the full implementation of environmental policies and objectives circumscribed in HSSE initiatives. This is crucial for an enhanced or more professional and specialized inspection of all oil spills and the duration of the clean-up thus curbing subsequent spills in the Niger Delta.

The 2015 bill envisages that Downstream Petroleum Regulatory Agency (DPRA) would maximally acquire the responsibilities of the downstream subsector. One of the core areas of the downstream operations would include the full implementation of Environment policies and objectives as circumscribed in HSSE initiatives. One of the strong objectives of DPRA in Post PIB is effective and ecocentric downstream operations in order to promote environmental interests and concerns as necessary agents in achieving sustainable development and viable economy. As a regulator, the DPRA will be saddle with the responsibility of formulating and enforcing policy which will cut across environmental, marketing and other areas of operations within the industry (pages 31- 42 of the proposed PIB), especially pages (33d-e, 42e), on Downstream Petroleum functions in (V- page 115, emphasis on pages 121b section 232, 124d section 240, 126b section 244, and 134 c-d section 260). All of these are now subsumed in the 2015 bill that proposed ONE petroleum regulatory body.

More importantly, at some points there will be collaboration between the Inspectorate and the Agency with FME and other relevant government agencies where environmental issues and policies standout in Part VII- Health, Safety and Environment section 289-293. Income shall be generated from Players that circumvent the law from environmental impact assessment reports and evaluation and other related environmental matters for the Agency

(page 42e). Environmental quality management as in the upstream sector in page 102, 1- 8 as integration between both sectors of the government arm. These actions if implemented will protect and preserves the environment in the near future.

The current strategies of monitoring crude oil contamination is based on Joint Investigation Visit and lack credence when subjected to SWOT analysis (Rim-Rukeh, 2015) which does not embrace the visual observations of Ultrasonic Thickness Measurement currently being use by some multinational. The failure of JIV has created doubt in the communities, since most time the communities were not involved in the process from the onset due to observed poor governance.

In securing the effectiveness of oil spill management, third party involvement is crucial and public awareness through social involvement in environmental protection against oil exploration and contamination. This public awareness campaign both from the side of the government and stakeholders, and effective communication similar to what is obtainable in the UK will curb the challenges of lacking technology and stakeholder engagement (Cundy et al., 2013, Geaves and Penning-Rowsell, 2016). Also, the defects in government structure, complexity of the terrain and dynamic nature of the environment for an effective land remediation in the region of the Niger Delta will be overcome.

## 2.7 Review on bioreporters (Biosensors)

### *2.7.1 Introduction*

Bioreporter has the advantage of rapid detection of genotoxicity directly over conventional chemical method and oil content in the determination of environmental pollution and asses the risk that is associated with the environment (Zhang et al., 2013) which is being used as a complementary tool to the chemical analysis method. Acinetobacter baylyi ADP1 are alkane degrader which can be used in the Niger Delta to sense alkanes because of his wide range of lengths (carbons range from 7- 36) with the well characterization of gene regulation (Zhang et al., 2012c). Typical examples of this method have been used in fresh water, seawater and soil samples as illustrated by (Zhang et al., 2013). The use of Whole cell biosensor is becoming popular for the detection of toxicity and environmental pollution

and also for the monitoring and evaluation of oil spills. It can also be used to curtail environmental degradation and thus act as tool for environmental sustainability.

(Ron, 2007) highlighted the advantages of this tool over the conventional methods in terms of its ability to monitor relevant toxicity, and its associated application swiftness and cost effectiveness. On the other hand, analytical techniques measure only concentration, while biosensors can measure toxicity because they are biologically based. The application and principles of reviewed on biosensor technique is a welcomed development because in recent times, the deployment of biosensor has attracted much attention by the use of smarts support and bio-inspired materials to the confinement of living cells (Michelini et al., 2013). This design has the advantage of cost –effectiveness and portability.

The signals used in the biosensors could be thermal, electrical or optical detector with specific biochemical reactions capacities, using whole cells, organelles, immunosystems, tissues or isolated enzymes to detect chemical compounds. Certain parameter makes the biosensor development for on-site analysis challenging, whether for food, environmental, or clinical, since its requires rapidity as relating to real-time analysis, high sensitivity, selectiveness, robustness, simple to operate, non-preliminary sample treatment when incorporated in the development of whole-cell biosensors. Despite that in chemical and drug sample analysis, living cells are used in the detection of physiological changes with response to the sample concentration, hydrocarbons likewise response to similar physiological changes with regards to different analyte, or its classes. That notwithstanding the development of whole cell biosensors to meet up all the features the above detailed properties will be nearly impossible (Michelini et al., 2013). Therefore, integration of living cells into a device with a close contact of traducer using a high detector in optical biosensor by immobilization or encapsulation is what whole biosensors requires.

The whole- cells biosensors is different from other biosensing configurations because of the use of cells as biorecognition elements which enable information obtainability about the bioavailability of chemicals and the corresponding effect. The application of whole-cell biosensor in the environmental monitoring has also be researched by(Elad and Belkin, 2012, Zhang et al., 2012c, Jouanneau et al., 2012) and most recently (Michelini et al., 2013) took into consideration the advantage of genetically modified bioluminescent (BL), and

enabling biosensors integration for the use of portable field deployable device with excellent analytical performance. Notwithstanding, the short comings of the commercial production of whole- cell biosensors and their poor robustness in response to temperature and pH which are very important for each enzyme to work/function maximally in the active center of the enzyme ionized state i.e. where there is proton donor or proton accepting group which occur inside the range for which the binding substrate is possible. With the use of photomultiplier tubes, biolumininescene has the advantage of high detectability of genetically modified bioluminescent (BL). However, the problem of immobilize or encapsulate BL Whole-cell biosensors as a consequence of long- term storage before integration into portable analytical devices must be critically examined. (Michelini et al., 2013) has been able to demonstrate how bacteriophages recently used in living biosensors for field application after an 8 month storage period as bacteriophage-based detection has excellent biosensing potential. However, the limitation is the degradation of the shell that cannot be controlled since spore germinates. In the design and fabrication of field-deployable devices, Genetic modified organisms( GMOs) must be taken into consideration, as regulatory framework for whole-cell biosensors may be a source of concern in the field – deployable device in order to maximize the risk of GMOs spreading as cautioned by the European Union(French et al., 2011). Improving the device for effective performance was well presented in a review by (Michelini et al., 2013). (Ron, 2007) has shown the instrument's advantages over chromatographic methods applied in the measurement of the total pollutant, rather than measuring changes on-line processes coupled with the detection of only the biological active pollutant and toxicity which is the inverse response of the biological active pollutant.

The recent whole-cell biosensor that makes use of the electrochemical expression gene monitored of the pollutant in online and in-situ that are suitable unlike the "whole cell" man-made 'biosensors that is constructed by fusing a pollutant-responsive gene promoter. This biosensors technology is man-made based on DNA recombinant such as the Escherichia Coli E- 12 strain which is used for the aromatic hydrocarbons detection.

### 2.7.2 Biosensors Construction and Development

When a bioreceptor is combined with a transducer, a biosensor is made. Two main constituents are important, the promoter and the reporter gene, being the basic requirements for the construction of biosensors. The bioreceptor is a biomolecule that recognizes the target (analyte), whereas the traducer converts the recognition event into a measurable signal. Whole-cell man-made biosensors entail the combination of a promoter and a promoterless reporter gene and the choice of choosing a promoter is of a primary important. Transducers used primarily in the construction of biosensors are thermometric, acoustic devices, photometric and electrochemical. Of all of the above mentioned transducers, amperometric biosensors have been very dominant in both commercial applicability as well as in research as a result of flexibility and simplicity. In choosing a responsive promoter, the key consideration is being sensitive and specifically knowing that the systems that are biological based are highly stimulus (sensitive). Bacterial gene promoters do not only detect groups of compounds, but also specific since hydrocarbons can be detected by vapor as well as heavy metal in concentration detection by the bacterial promoters gene as low as part per billion (Biran et al., 2000, Ron, 2007).

The other constituents have enzyme that are usually encodes and catalyzes the reaction, thus enabling an easy monitoring process. In this case, enzymes are useful indicators of good reporters in biosensors construction, owing to the catalytic nature of the enzymatic reaction. This make the choice of gene expression for on-line in-situ monitoring for electrochemical measurements highly recommendable due to its ability to employ a compact analyzer, highly sensitive, reproducible and disposable electrodes viable option. Other advantages are in the measurement of crude or turbid solution and its wide spread applicability in the monitoring of gene expression in yeasts, bacteria, and mammalian tissue cultures(Ron, 2007). Similarly, the bacterial choice is dictated by its population size, rapid growth rate, reduced expense and maintenance cost, coupled with the ability to tailored them makes it an option for pollution monitoring. (Dennison and Turner, 1995), showed how the use of different enzymes for the construction of biosensors for environmental monitoring of effluents can be categorized into three phases of the environment, i.e. soil, water and air. The construction of the biosensors was not only portable but its mass production potential leads to low manufacturing cost. One area of biosensors which is very

important in biosensors development is the immobilization of cells. The immobilization methods entails physical adsorption or chemical that is entrapment that is within a membrane or gel physically, covalent binding or molecules that is cross linking attached to the sensor surface as a biological recognition element, with instances reported in (Rogers, 2006).

(Belkin, 2003) reviewed whole cell using microbial sensing systems of pollutants in the environment. An approach that complements the biosensors with the traditional method of chemical or physical analysis. The reporter gene that has been generally used for several years are the environmental microbial sensor system as reporting elements, however, the versatile fluorescent has played a very noticeable role in the past few years as a protein genes(Southward and Surette, 2002, Zhang et al., 2002) with increases of popularity and acceptability. Bioluminescence bacterial genes has played conspicuous role over the years in an environmental sensor as a reporting elements. The similarity of bioluminescence and fluorescence was well captured as bioluminescence has much sensitivity detection capacity and faster rate than fluorescence in relation to the targeting analyte, as the enigmatic activities was a measure of bioluminescence as opposed to the fluorescence that has the protein presence quantified.

### 2.7.3 Various Types of Biosensors

Different biosensors with contrasting applications have been reviewed by (Ezeonu et al., 2012), based on their sensing component. The three basic biosensors are classified accordingly are molecular, cellular and tissue with basic advantages and disadvantages bearing in mind, the requirements. The Enzymes –based biosensors, Antibody-based biosensors (immunosensors) and the DNA-based biosensors.

#### *2.7.3.1 Enzymes –based biosensors*

These enzymes –based biosensors were first used in the 1960s by Leyland Clark and consist of glucose oxidase. The enzymes are immobilized by an oxygen electrode for blood glucose sensing. (Ripp et al., 2010) shows its wide application which is quite lucrative in the medical diagnosis. The ability of the enzymes to act as organic catalyst helps the reactions transformation from substrate into products; (Ezeonu et al., 2012) however, the enzymes based biosensors are not always steady as a result of instability of enzymes. The

requirement of the enzymes reactions where complex co-factors exits are critical, regeneration of these factors thus becomes enigmatic which can only be resolved by circumvention of using enzymes that are stable at high temperatures which are naturally available from thermophilic microorganisms (Luong et al., 1988).

*2.7.3.2 Antibody – based biosensors (immunosensors)*

Antibody –based biosensors (immunosensors) make use of antibodies as recognition elements. Their advantages are simply seen in its binding stability, highly specific and target analytes (substance or antigens) is very strong (Ripp et al., 2010). Its application cut across polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and other pollutants. Their wide use of environment monitors as effective detector is traceable to their functions. The Automated Water Analyzer Computer Supported System (AWACSS) is the best introduced antibody–based biosensing in environmental monitoring system which is applicable as unattended, remote and continuous check of hydrocarbons pollutants for control in water quantity (Ezeonu et al., 2012). To be used for targeted groups of contaminants, AWACSS applies optical evanescent wave transducer and polyclonal antibodies flourescently labelled for multiplexed detection, as the pre-incubation step is usually short in about 5 minutes. Antibody-based biosensor are more versatile than enzyme-based biosensors because of their bonding range of affinities, however, their limitation is seen for environmental monitoring application that has an assay that are complex and the number of reagents are also specialized (Rogers, 2006). Other aspects of application of antibody–based biosensors are monitoring of environmental substances in the areas of pesticides, herbicides, and their analytes (target) are considerably broadened over the years include explosives (TNT and RDX), phenols, toxins like microcystin, endocrine disruptions and pharmaceutical compounds, with application technique of microbial biosensor summarized in (Su et al., 2011).

*2.7.3.3 DNA- based Biosensors*

These are biosensors that monitor a change in the nuclei acid's structure using a transducer. It usually occurs when target chemical is exposed with the changes in the structure which brought about mutagenic nature of the chemical as a result of mutations, and with the attachment of nuclei acid by the chemical's ability to non-covalently or covalently bonding

(Ezeonu et al., 2012, Ripp et al., 2010). The biosensor immobilization is a function of nuclei acid as a recognition layer on the transducer surface. The provision of overall potential harmfulness in terms of genotoxicity, carcinogenicity and cytotoxicity chemical is what nuclei acid biosensors is, thus are generally non-selective. These DNA electrochemical biosensors has been used for environmental monitoring, as (Wang et al., 1997), reviewed different effort in copulation of nuclei acid recognition layers with the electrochemical transducers. Its stability advantage and sythesization for repeated use by regeneration over the enzymes or antibodies biosensors, and played a major role in the environmental monitoring assessment. Example of DNA biosensors of specific analyte microbial and viral pathogens are the chronopotentionmetric hybridization biosensors. An illustration of a conventional DNA biosensor with a double-stranded DNA immobilization on a single –use disposable screen. These biosensors used to screen soil samples make use of battery-powered potentiostat electrochemical cell operating system (Bagni et al., 2005, Sassolas et al., 2008) and the workability of the DNA biosensors and its application in PAHs detection in the bile of fish using the accumulation of PAH compounds as was monitored in the contaminated water by (Lucarelli et al., 2003) was presented in (Ezeonu et al., 2012).

## 2.8 Conclusion

The Niger Delta requires thorough oil spill management to curb its high detrimental impact on the people and the environment. This work gives a details summary to the Acts and Regulations functions superintended by DPR and 13 years gap of crude oil spillage data, summarize oil spill disaster in the region, analyzed government structure for petroleum industry and agencies with challenges of duplication of functions. Consequently, the Nigerian oil and gas regulatory framework and FME must have a clear-cut function without duplications of responsibilities in order to overcome the challenges crude oil spillage monitoring is currently facing in the region. Most importantly the EGASPIN rules and guidelines issued in 1992 and re-issued in 2002 as operational working documents for environmental issues by both government agencies (DPR and NOSDRA) must be revisited by the National Assembly to give clear interpretation to those grey areas. The proposed bill before the parliament must be passed without delay to solve the lingering problem of crude

oil contamination and also to overcome the challenges of functions duplication. The effects of oil spillage is real threats to food security, social and environmental deterioration, mangrove ecosystem decline, loss of livelihood, potential cumulative effects of carcinogenic related diseases and reduction in life expectancy will be mitigated through education and public enlightenment by both the multinational and the government. Bio-sensing and bioremediation have been regarded as the most cost effective and reliable techniques to solve such problems with zero impacts to climate change. With the recent development of nanotechnology, the combination of nanoparticles and biological process will be successful in enhancing measurement accuracy, improving bioremediation efficiency and broadening biochemical application in environmental research. This combination serves as risk reduction in Niger Delta environment oil spillage management assessment and bioremediation which is crucial strategy that can be adopted by the Nigerian government to curtail further environmental devastation and promote ecosystem rehabilitation.

Whole –cell or tissue based biosensors are very stable within 8 weeks and considering the distance of biosensor application between the laboratory and the field work, it can unravel that or can offer information about bioavailability and ecological toxicity uniqueness for the monitoring and assessment of the crude oil contamination in the Niger Delta. The Whole -cell bioreporter (biosensor) will serve as a revolutionary approach for crude oil contamination and ecological assessment in Nigeria and this can be further modified for purposes of encouraging technical support and expediting regulation and policy measures in the Niger Delta.

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# 3 Magnet bioreporter device for ecological toxicity assessment on heavy metal contamination of coal cinder sites

The aim of this chapter is to assess the feasibility of bioreporter application in real soil samples.

The specific objectives are:

- To develop a novel MNPs device and apply bioreporter in contaminants in toxic soil samples that has not been used elsewhere.
- Assess the advantages of biosensor in alkane and toxicity detection in contaminated soil samples.
- Examine the factors that hinder field application of bioreporter device for long-term monitoring.
- Pioneer research on magnetic biosensor to solve the problems associated biosensors application for soil toxicity assessment.

## Abstract

A novel magnet bioreporter device was developed in this research for soil toxicity assessment, via magnetic nanoparticles functionalized whole-cell bioreporters. The whole-cell bioreporter ADPWH_recA kept response capability to DNA damage after magnetic nanoparticles (MNPs) functionalization, and could be harvested from soil samples by permanent magnet to reduce the soil particle disturbance. Compared to conventional treatments applying bioreporter directly in soil-water mixture (SW-M treatment) or supernatant (SW-S treatment), MNPs functionalized bioreporter via the magnet device (MFB) treatment achieved high sensitivity to evaluate the toxicity and bioavailability of chromium contamination in soils from 10 mg/kg to 5,000 mg/kg soil dry weight. The MNPs functionalized bioreporter also achieved high reproducibility with pH value from 5.0 to 9.0, salinity from 0% to 5% and temperature from 20°C to 40°C. A case study was carried out on the ecological toxicity assessment of heavy metal contamination at the coal cinder site via the magnet bioreporter device. The heavy metal toxicity declined with the increasing distance to the coal cinder point, and a significant accumulation of heavy metal toxicity was observed along the vertical distribution. No direct link was found between the pollution load index (*PLI*) and heavy metal toxicity, and the results suggested the bioreporter test monitored the toxicity of heavy metals in soils and was an important approach for ecological risk assessment. Magnet bioreporter device also offered the high throughput biological measurement and was feasible for *in situ* monitoring.

**Key words:** whole-cell bioreporter, magnetic nanoparticles (MNPs), magnet bioreporter device, heavy metal, toxicity

## 3.1 Introduction

Heavy metals are the key anthropogenic environmental contaminants, mainly caused by industrial activities (Li et al., 2000, Loska et al., 2004). All around the world and particularly in China, numerous heavy metal contaminated sited are found due to the improper disposal of various chemical wastes (Smith, 2009), including coal cinders (Alizai et al., 2003), and the key pollutants include chromium, mercury, arsenic, lead, cadmium, manganese, cobalt, copper, nickel and zinc. They have high mobility through the leachate and further contaminate the biospheric soils (He et al., 2006, Dang et al., 2002), with respective carcinogenic, teratogenic and mutagenic effects (Valko et al., 2005). The high level of heavy metal in soils threatens the ecological system (Giller et al., 1998), poses potential risks to human health (Jarup, 2003) and draws attention on early warning for potential cancer induction (Farre et al., 2005). Due to the complex composition and synergetic effects in soils, traditional chemical and physical analysis only provides the amount information (Smith, 2009), but the toxicity and bioavailability of heavy metal contamination from coal cinder are hard to be evaluated.

Recently, whole-cell bioreporter has become initiative and legislative tool for environmental monitoring, with capability to sense the bioavailability and toxicity of contaminated water and soil samples (Belkin, 2003). With genetically engineered bacteria, yeast, fungi, or animal cells, the biological signals of whole-cell bioreporter are initiated by phenotypic color (*lacZ*), fluorescent (*gfp*/*yfp*) or bioluminescent (*luc*/*lux*) genes (Van Dyk et al., 2001, Meighen, 1994, Sanseverino et al., 2005). It offers highly sensitive, rapidly analytic, easy operation and cost-effective feasibility for *in situ* pollutants assessment (D'Souza, 2001). Some whole-cell bioreporter specifically senses the heavy metal molecules (Rasmussen et al., 2000, Ivaska et al., 2002) or their cytotoxicity/genotoxicity (Rodriguez-Mozaz et al., 2006).

Though the application of whole-cell bioreporter in water sample is successful, it suffers from the heterogeneous features of soils (van der Meer and Belkin, 2010). Exposed to whole-cell bioreporter, the soil particles will absorb the bioluminescent signal (*lux* or *luc*) or give strong fluorescent interference (*gfp*). Some recent work has assessed the bioavailability and toxicity of copper (Corbisier et al., 1996), cobalt and nickel (Tibazarwa

et al., 2001) via direct exposing the whole-cell bioreporter to the soils (Song et al., 2014), but the biological sensitivity and specificity are significantly reduced. Some pre-treatments, like water extraction or ultrasonication, are therefore applied to transfer contaminants into aqueous phase for biological analysis (Liao et al., 2006). Particularly for heavy metal, the aqueous extraction has been used for whole-cell bioreporters to sense the bioavailability of chromium (Jiang et al., 2015), mercury (Rasmussen et al., 2000), lead and cadmium (Turpeinen et al., 2000, Fritze et al., 2001) in soils. Nevertheless, the main drawback is the neglect of the real occurrence of pollutants in the porous soil (Ivaska et al., 2002). Technically, a new type of bioreporter device is required to sense the soil contaminants *in situ* and effectively separate the living reporter cells from the soil particles for biological signal detection. Magnetic nanoparticles (MNPs) functionalization offers the feasibility of magnetic remote control and is biocompatible for whole cell bioreporter (Zhang et al., 2011a). Its equipping and portability for *in situ* monitoring is still under development and required further research.

In this work, a novel magnet bioreporter device was developed and optimized for effective monitoring and assessment of coal cinder contaminated soils. With whole-cell *Acinetobacter* ADP1_recA reporter (Song et al., 2009b), the magnet device effectively reduced the impacts of soil particles and improved the sensitivity and reproducibility, comparing to the direct exposure of bioreporters to the soils. The MNPs functionalized bioreporter was able to evaluate the ecological toxicity of heavy metal contamination, via the high throughput and easy operation magnet device. This work showed the feasibility and potential of *in situ* environmental risk assessment via whole-cell bioreporter for the coal contaminated sites.

## 3.2 Material and methods

### *3.2.1 Bioreporter strain and incubation*

In this research, the *Acinetobacter baylyi* ADPWH_recA whole-cell bioreporter was introduced for environmental ecological toxicity evaluation (Song et al., 2009b, Zhang et al., 2013). Compared to other plasmid based or *Escherichia coli* hosted toxicity bioreporter, the reporter gene was located on the chromosomal with high stability and *Acinetobacter* was soil bacterium to tolerate the ambient soil environment and achieve high sensitivity.

After cultivation in Luria-Bertani (LB) medium overnight at 30°C, the 10.0 mL ADPWH_recA cells were harvested by 3,000 rpm centrifugation for 10 minutes. The bioreporter pellets were further washed by deionized water and resuspended in 10 mL deionized water for magnetic nanoparticles functionalization or 10 mL fresh MMS medium for toxicity measurement. The 1.0 litre MMS medium contained 1.0 g $(NH_4)SO_4$, 2.5 g $KH_2PO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 0.005 g $FeSO_4 \cdot 7H_2O$, 0.25 g nitrilotriacetic acid (NTA), 0.55 g NaOH, 3.24 g sodium succinate (20 mM) and 1 ml Bauchop and Elsden solution.

### *3.2.2 Direct toxicity measurement on soil samples*

The direct toxicity measurement of soil samples was conducted for the supernatant of soil/water mixtures (SW-S) and the bulk soil/water mixtures (SW-M) respectively. For SW-S treatment, the 100 mg soil sample was suspended in 1 mL deionized water and homologized by 150 rpm shaking for 10 min. The 20 μL supernatant and 180 μL bioreporter suspensions were added into each well of a black clear-bottom 96-well microplate. For SW-M treatment, the 1.0 mL bioreporter suspensions were added into 100 mg soil sample, and the mixture was directly transferred into the microplate. The incubation and induction was conducted at 30°C for 4 hours, in the Spectra M5 Plate Reader (Molecular Devices, USA). Three biological replicates were carried out for each sample and the bioluminescent signal was measured every 10 minutes with 5 seconds shaking before each reading.

### *3.2.3 Magnetic nanoparticles synthesis and functionalization*

All the chemicals in this study were analytical grade and purchased from Sigma Aldrich (UK) without specific statement. Magnetic nanoparticles synthesis followed chemical deposition method (Zhang et al., 2011a). The 12.5 mL NaOH (1.5 M) was added dropwisely into the mixture of 1.0 mL $FeCl_3$ (2 M in 2 M HCl) and 0.5 mL $FeCl_2$ (1 M in 2 M HCl) with 40k Hz ultrasonic homologization, until the appearance of dark iron oxide precipitates. The precipitates were further separated by permanent magnet and washed with deionized water until the supernatant reached pH=7.0.

For bioreporter functionalization, 10 mL bioreporter suspensions (in deionized water) were mixed with 1 mL MNPs suspension, incubated at room temperature for 10 minutes with 150 rpm shaking. The MNPs-bioreporter was subsequently harvested by a permanent

magnet and washed twice by deionized water. The magnetized bioreporter was finally suspended in 10 mL MMS medium for soil toxicity assessment via the magnet bioreporter device.

### *3.2.4 Magnet bioreporter device and operation*

The magnet bioreporter device contained the magnet probe assay and plastic cover for bioreporter strain transfer, as illustrated in Fig. 3.1a. The magnet probe assay was assembled by 96 magnet probes (1 cm length and 3 mm id), fixed on the plastic base and patterned (20.66 mm distance between each magnet probe) for the high throughput bioreporter measurement on the 96-well microplate. For MNPs functionalized bioreporter via the magnet device (MFB treatment), the 1.0 mL bioreporter suspension was mixed with 100 mg soil samples and transferred into each well of the 96-well microplate (Fig. 3.1 b-1). For the determination of the best cultivation time before magnetic separation, the bioreporter cells were magnetically harvest at 0, 15, 30, 45, 60, 75, 90, 105 and 120 min. The recovered MNPs functionalized bioreporter was counted by plate count and the bioluminescent response was also measured.

After incubation at 30°C for 1 hour as the optimal cultivation condition, the magnet probe assay (with plastic cover) was emerged into the reaction system for 30 seconds (Fig. 3.1 b-2). The bioreporter cells were then separated from the soil suspension and attached on the plastic cover by magnetic field. The magnet device was transferred and emerged in another 96-well microplate, supplemented with 200 μL fresh MMS medium (Fig. 3.1 b-3). Removing the magnet probe, the plastic cover and microplate was incubated at 30°C for 5 minutes with 150 rpm shaking (Fig. 3.1 b-4). The bioreporter cells were resuspended in the fresh MMS medium and the bioluminescence was further measured on the Spectra M5 Plate Reader (Molecular Devices, USA). The detection and data analysis followed the same instruction for the direct toxicity measurement on soil samples.

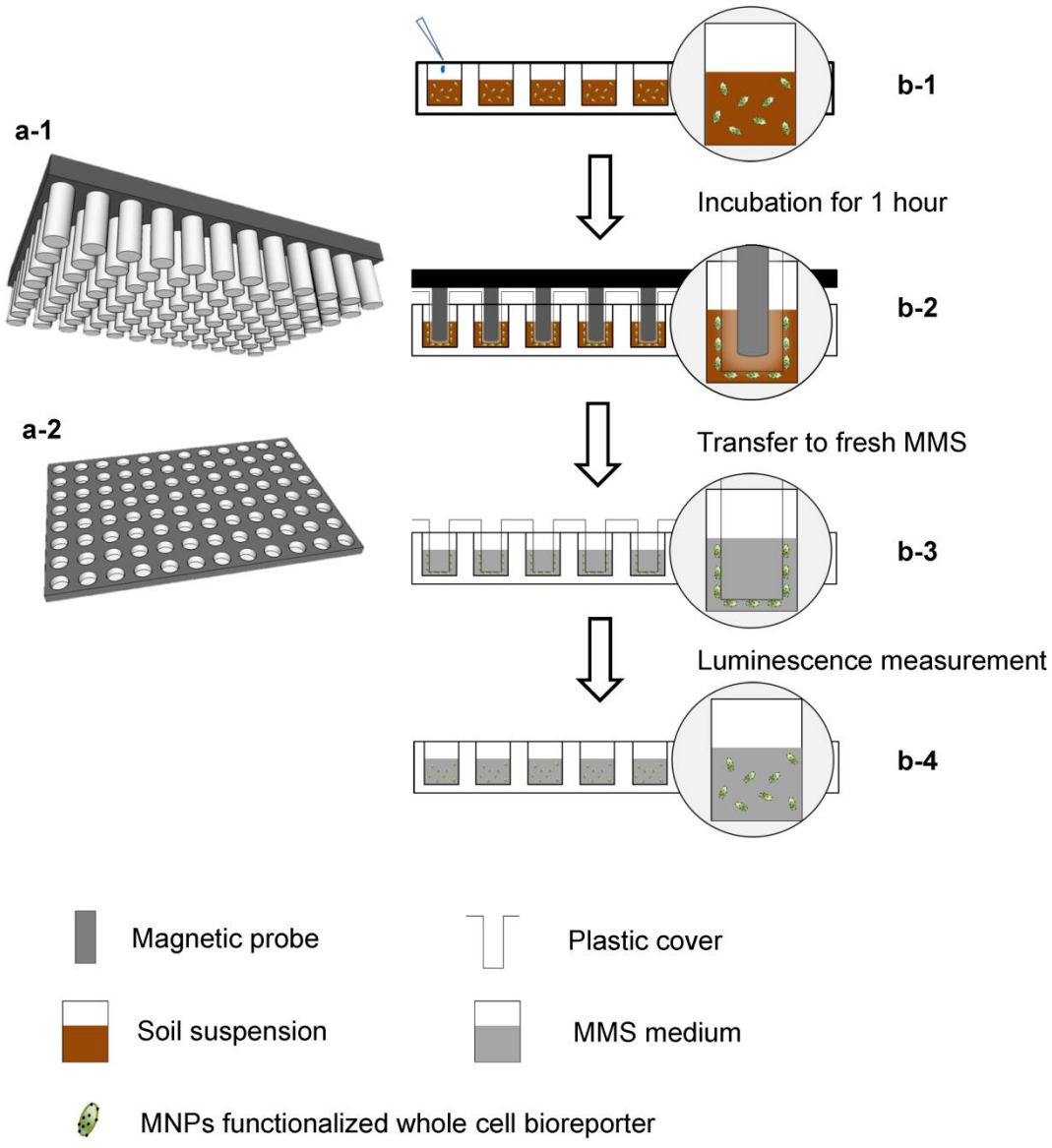



**Figure 3.** 1 **Schematic instruction for magnet bioreporter device.**

(a-1) and (a-2) for magnetic probe assay and the 96-well microplate respectively. (b-1) The 1.0 mL MNPs functionalized bioreporter was mixed with soil samples and further incubated at 30°C for 1 hour; (b-2) separation from soil suspensions via magnetic probe. (b-3) Resuspension in fresh MMS medium; (b-4) incubation and bioluminescence measurement 30°C for 3 hours.

For reproducibility test, MNPs-bioreporter was applied to sense the toxicity of 100 mg/kg chromium contaminated soils in the medium with different pH values and salt contents. The pH value in the induction medium was adjusted by 1.0 M HCl or 1.0 M NaOH solution as 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The series of salty medium was prepared by adding NaCl into the MMS medium with the final concentration of 1%, 2%, 3%, 4%, 5% and 10%. As for the temperature influence, the temperature during induction period was controlled by the Spectra M5 Plate Reader at 10°C, 15°C, 20°C, 30°C, 37°C, 40°C and 45°C. To evaluate the life-time of MNPs-bioreporters, the bioreporter suspension was stored at 4°C and taken out for direct toxicity measurement without any pre-treatment.

### *3.2.5 Toxicity model for heavy metal contaminated soils*

For the calibration of heavy metal toxicity in soils, chromium was chosen at the target analyte since it was the main heavy metal contamination at the coal cinder site. The artificial chromium contaminated soils were prepared by mixing 1.0 g uncontaminated soil with 1 mL potassium bichromate solution with the concentration of 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5,000 mg/L. The soil slurry was air dried in the laminar hood. The chromium contamination in soils was 0, 0.1, 0.2, 0.5, 1, 2, 5, 10, 20, 50, 100, 200, 500, 1000, 2000 and 5,000 mg/kg soil dry weight, respectively.

The genotoxicity of hexavalent chromium in soil was identified as several mechanistic processes, including mutagenic effects on DNA and direct inductive immunological responses (Nickens et al., 2010). Such genotoxic effects consequently caused the accumulation of single stranded DNA (ssDNA) and the activation of SOS response for damaged DNA repair. The SOS response (bioluminescent response of ADPWH_recA) is the function of LexA-like SOS repressor (*LSR*, cell$^{-1}$) and can be simulated by the previous gene expression model (Al-Anizi et al., 2014a, Zhang et al., 2012d), as shown in Equation (1).

$$SOS_{r,s} = 1 + \left(\frac{k_{dSLR}}{2\cdot(1+k_{ssDNA})}\cdot[LSR]\right)\cdot\frac{[Cr]}{(K_{mLSR}\cdot K_{Cr}\cdot k_{ssDNA}\cdot k_{Cr})^{-1}+[Cr]} \quad (1)$$

Here, $SOS_{r,s}$ (SOS response ratio) is dependent on the hexavalent chromium contamination level in soils ($[Cr]$, mg/kg). $K_{Cr}$ is the isotherm equilibrium of chromium-DNA adduct

(DNA phosphodiester backbone with chromium) and $k_{Cr}$ represents ssDNA generation constant from the chromium-DNA adduct (L/(cell·g) chromium). $k_{dSLR}$ represents the equilibrium coefficient of *LSR* dimer ($dSLR$, cell$^{-1}$) and monomer ($mLSR$, cell$^{-1}$) and $k_{ssDNA}$ represents the cleavage reaction constant of *LSR* dimer. $K_{mLSR}$ is the dynamic gene expression (SOS response) level activated by *LSR* monomer.

### *3.2.6 Sites description*

A total of 16 soil samples were taken from the methanol plant of Yulin Energy and Chemical Industry, Yanzhou Coal Corporation, China (Figure 3. 2).

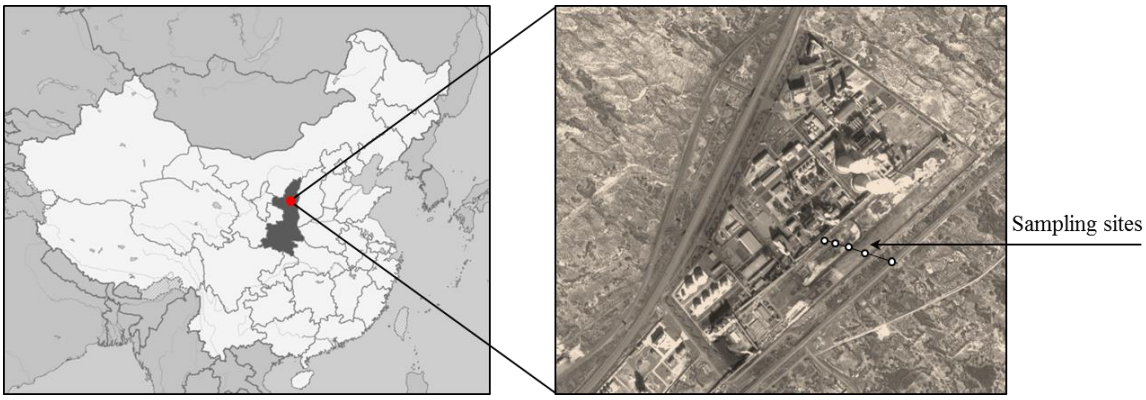



**Figure 3. 2** Location of research area in Yulin and the sampling sites.

The site (698,000 m$^2$) was located in Yulin Shaanxi Province (N38°34'41.9'', E109°55'50.4''), in the junction of Maowusu Sandy Land and the Loess Plateau. The annual coal consumption was 31,200 tonnes and the soils have been seriously contaminated by the coal with high heavy metal content. The sampling sites were designed along the leeward direction of the cinder heap, with the distance of 0, 10, 50, 80 and 150 m. The uncontaminated soil sample was collected in the living area of the plant, 500 m away from the heap. At each point, the soils were sampled at different depth of 0-20 cm (surface soil), 20-35 cm (middle soil) and 35-50 cm (bottom soil) to evaluate the toxicity profiles caused by the trace metal transportation.

### *3.2.7 Chemical analysis*

Before chemical analysis, all of soil samples were seized by 200 mesh. Mercury was determined by DMA-80 Hg analyzer (Milestone S.r.L., Italy). For other trace elements, the

samples were digested in an UltraCLAVE microwave high pressure reactor (Milestone S.r.L., Italy), containing 330 mL distilled $H_2O$, 30 mL 30% $H_2O_2$ and 2 mL 98% $H_2SO_4$ as the digestion solution (Dai et al., 2011). With 50 bars initial nitrogen pressure, the microwave digestion program was listed in Table S1. Further digestion for 50 mg soil sample was conducted in 5 mL 40% HF, 2 mL 65% $HNO_3$, and 1 mL 30% $H_2O_2$ (Dai et al., 2012). The inductively coupled plasma mass spectrometry (ICP-MS, X series II, Thermo Fischer Scientific, USA) was used for the determination of the trace elements in a pulse counting mode (three points per peak). In this study, the multi-element standards (Inorganic Ventures, CCS-1, CCS-4, CCS-5, and CCS-6) were referenced for the calibration of trace element concentrations. As and Se were determined by ICP-MS with collision cell technology (CCT) due to their volatility (Li et al., 2014). Polyfluoroalkoxy volumetric flasks were used without drying on electric hot plate to avoid As/Se volatile loss. With the 1 μg/L tuning solution, the torch position and ion lenses were optimized before real sample measurement. The optimal parameters of the ICP-CCT-MS and calibration curves of As/Se were listed in Table 3.2 and 3.3.

**Table 3. 1** Microwave program for soil sample digestion.

| Step | Time (min) | Temperature (°C) | Pressure (bar) | Microwave power (Watt) |
|---|---|---|---|---|
| **1** | 12 | 60 | 100 | 1000 |
| **2** | 20 | 125 | 100 | 1000 |
| **3** | 8 | 160 | 130 | 1000 |
| **4** | 15 | 240 | 160 | 1200 |
| **5** | 60 | 240 | 160 | 1000 |

**Table 3. 2** Optimal instrumental parameters for ICP-CCT-MS.

| Items | Values/status | Items | Values/status |
|---|---|---|---|
| **Plasma RF power** | 1400 W | Collision gas | Mixture of $H_2$ and He |
| **Nebulizer gas flow** | 1.00 L/min | Collision gas flow | 4 ml/min |
| **Auxiliary gas flow** | 0.8 L/min | Pole bias | -16 V |
| **Cool gas flow** | 13.0 L/min | Hexapole bias | -19V |
| **Sampling depth** | 130 steps | Number of main runs | 3 times |
| **ICP-MS interface** | Nickel Xt | Dwell time | 10 ms |
| **Peristaltic pump speed** | 30 RPM | Acquisition mode | Peak jumping mode |
| **Nebulizer** | Teflon Nebulizer | Resolution | Standard |

**Table 3. 3** Calibration curves and method detection limit (MDL) of As and Se.

| Element | Isotope | Linearity (µg/L) | Determination coefficient | MDL (µg/L) | RSD (%) |
|---|---|---|---|---|---|
| **As** | 75 | 1-100 | 0.999982 | 0.024 | 1.654 |
| **Se** | 78 | 1-100 | 0.999936 | 0.095 | 1,996 |

### *3.2.8 Data analysis*

The bioluminescence response was calculated by averaging the bioluminescent signal from the 7 time points between 180 and 240 minutes for each well. The relative bioluminescence response ratio was the specific value of the bioluminescence response of contaminated soil samples to that of the uncontaminated soils. The heavy metal profiles in soil samples were statistically analysed by SPSS software (Version 15.0 for Windows) via Principal Component Analysis (PCA). The equality and normality of data were tested by Brown-Forsythe and Shapiro-Wilk test respectively, and the null hypothesis was rejected for $p<0.05$.

Contamination factor (*CF*) is defined as the ratio of the heavy metal concentration in the sample soil to the baseline concentration in background soil, as shown in Equation (2) (Bhuiyan et al., 2010). Pollution load index (*PLI*) is determined as the $n^{th}$ root of the *n* CF in Equation (3) (Bhuiyan et al., 2010). The *CF* and *PLI* are empirical indices to evaluate the level of heavy metal contamination, and the higher values indicate heavier contamination of individual and multiple heavy metals respectively.

$$CF = \frac{[Heavy\ metal\ in\ sample\ soils]}{[Heavy\ metal\ in\ background\ soils]} \quad (2)$$

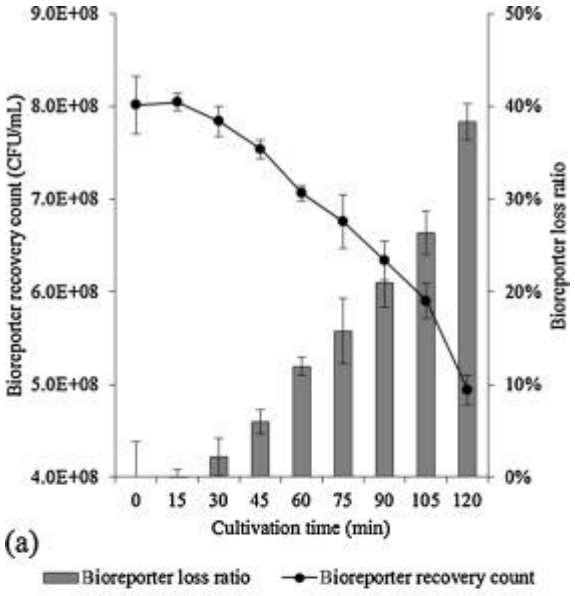
$$LI = (CF_1 \times CF_2 \times \cdots \times CF_n)^{1/n} \quad (3)$$

## 3.3 Results and discussions

### *3.3.1 Higher sensitivity and reproducibility of magnet bioreporter device*

The MNPs functionalized bioreporter could be magnetic remote controlled for effectively separation from the soil particles. The MNPs were biocompatible, and the viability and bioluminescent signal of whole-cell bioreporter remained over 99% comparing to the native bioreporter cells (Zhang et al., 2011a). With the strong electrostatic attraction between the negative iron oxide ($Fe\text{-}OO^-$) and positive amino-groups ($\text{-}NH_3^+$) on bacterial membrane, the separation effectiveness by magnetic field was above 99.6% and the synthesized MNPs had neither cytotoxicity nor genotoxicity on bacterial bioreporter cells (Chen et al., 2013). MNPs functionalized whole-cell bioreporter therefore had the feasibility to sense the toxicity of soil samples *in situ* and subsequently isolated for bioluminescent signal measurement.

Due to the cell division, the MNPs functionalized bioreporter gradually lost their magnetic capacity (Zhang et al., 2015b). Though longer incubation with soil samples could improve the chemical uptake by bioreporter cells for higher responsive ratio, the less recovery rate consequently resulted in lower bioluminescent signal and lower sensitivity. Figure 3.3 illustrated that, within 45 minutes incubation, over 90% living bioreporter cells were isolated from the soil/water mixture based on plate count.



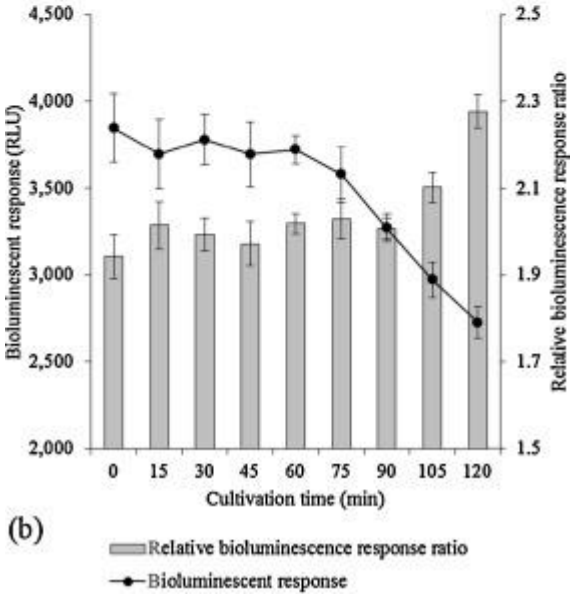



**Figure 3. 3**. The cell recovery rate (a) and bioluminescent response (b) of whole-cell bioreporter against the incubation time with soils.

Over 90% of living bioreporter cells were successfully harvested from the soil/water mixture within 45 minutes incubation. The relative bioluminescence response ratio ranged between 1.90 and 2.00 when the incubation time was less than 75 minutes. The 60-minute incubation was identified as the optimal time for sufficient bioreporter cell recovery and high response sensitivity.

The results fitted with previous study that about 12% free bioreporter cells were observed after 120 min cultivation in rich medium (Zhang et al., 2011a). As for the bioluminescence and relative response ratios, the bioluminescent signals were stable from 3600 RLU to 3800 RLU when the incubation time was less than 75 minutes, and the response ratio ranged from 1.90 to 2.00. The results suggested a highly reliable responsive period between 30 and 70 minutes. The optimal incubation time for sufficient bioreporter cell recovery and high response sensitivity was identified as 60 minutes, and it was further applied in the following work on calibration curve and real soil sample assessment.

The summarized features of the magnetic ADPWH_recA whole-cell bioreporter were listed in Table 3.1 from the reproducibility test.

**Table 3. 4** Analytical characteristics of magnetic ADPWH_recA whole-cell bioreporter.

| **Characteristics** | **Description** |
|---|---|
| **Limit of detection** | 1 mg/kg chromium(VI) in dry soil |
| **Linear range** | 1-100 mg/kg chromium(VI) in dry soil |
| **Pre-incubation time** | 1 hour |
| **Detection time** | 4 hours |
| **Reproducibility** | High reproducible when pH value is from 4.0 to 9.0, salinity ranges from 0% to 3%, and temperature is from 20 °C to 37 °C |
| **Storage time** | 30 days |

After 1 hour pre-incubation of the MNPs functionalized bioreporter, the cells were captured by permanent magnet and resuspended in fresh medium without soil disturbance for another 4 hours. As a soil bacterium, ADPWH_recA had strong tolerance to the environmental variations and maintained high reproducibility under different pH, salinity and temperature condition. The relative bioluminescent response ratio maintained stable (1.44 to 1.51) when pH value ranged from 5.0 to 9.0, dramatically dropping to 1.25 at pH=4.0 and 1.12 at pH=10.0 (Fig. 3.4a). The results were similar to previous research on the pH influence on *Acinetobacter baylyi* ADP1 that *Acinetobacter* based bioreporter could tolerate large pH variation (Li et al., 2009). Fig. 3.4b also illustrated the good responsive performance of MNPs functionalized ADPWH_recA at 20°C (relative bioluminescent response ratio=1.47), 30°C (relative bioluminescent response ratio=1.50) and 37°C (relative bioluminescent response ratio=1.49). The tiny reduction of bioluminescent response at 15°C and 40°C attributed to the less bacterial activities at inappropriate temperatures, and the response was very weak under even lower (10°C) or higher (45°C) temperature conditions. Salinity did not significantly affect the reproducibility of ADPWH_recA and the relative bioluminescent response ratios were above 1.45 when the salinity was no higher than 3%, but were gradually suppressed at higher salinity level (Fig. 3.4c). Therefore, the MNPs functionalized bioreporters had high reproducibility under the normal pH value, salinity and temperature conditions of natural soils and no specific pre-treatment was required for real soil sample assessment. High activity and responsive sensitivity of MNPs functionalized whole-cell bioreporters was also observed after 30 days storage at 4°C (Fig. 4d). Without any pre-treatment, the stored bioreporter cells could be directly applied for soil assessment and the relative bioluminescent response ratio was above 1.45 for chromium contaminated soils of 100 mg/kg soil dry weight. The life-time of MNPs functionalized bioreporter was the same to the original *Acinetobacter* based bioreporters (Zhang et al., 2012a, Song et al., 2009b), indicating that MNPs functionalization had minimal impacts on the bacterial activities and was an appropriate approach to expand its application in soil contamination assessment.

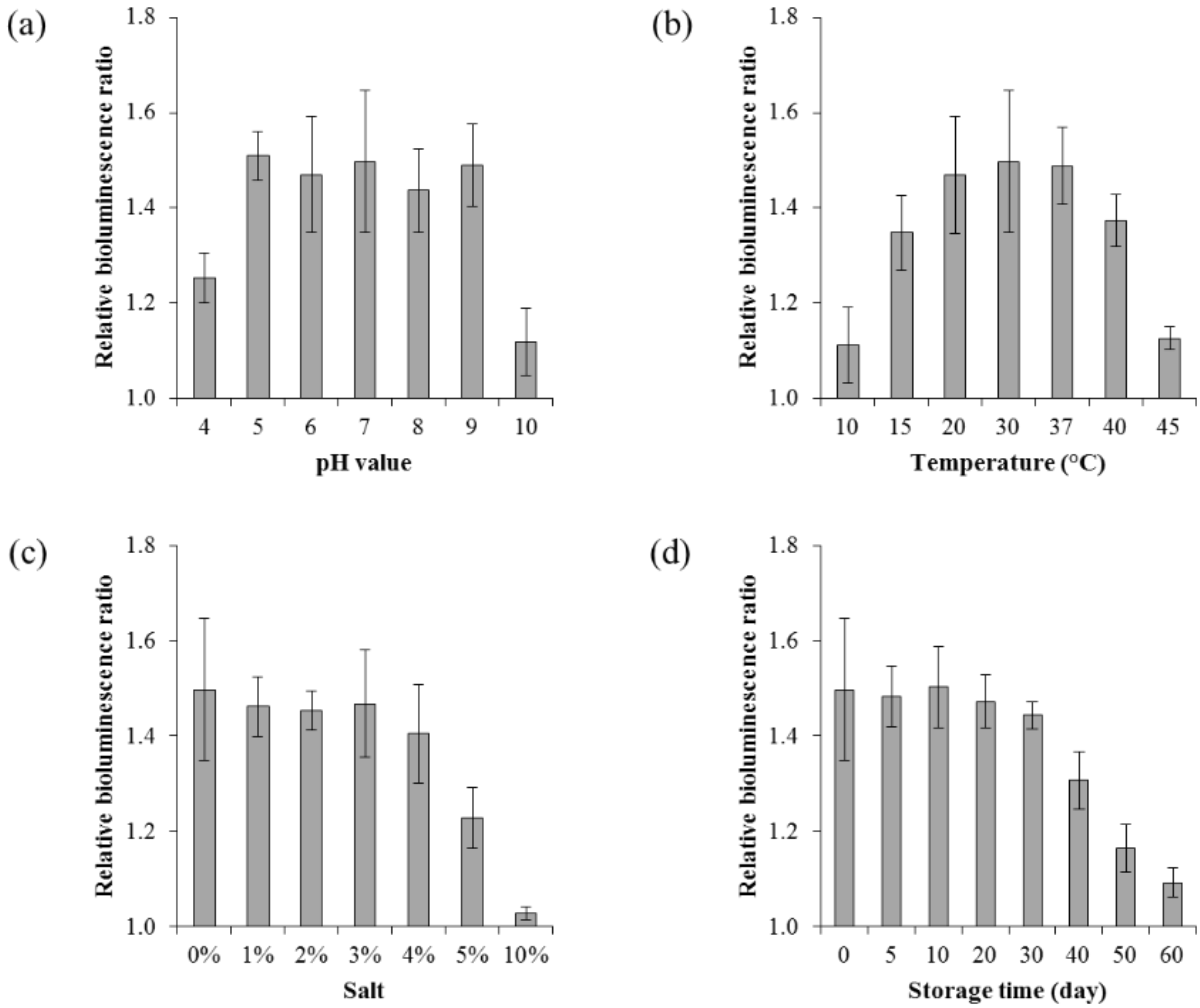



**Figure 3. 4** The impacts of pH (a), temperature (b), salt (c) and storage time (d) on magnetic bioreporter's response to artificial chromium contaminated soils. The chromium concentration was 100 mg/kg soil dry weight.

From the calibration curve of soil/water supernatant (SW-S), soil/water mixture (SW-M) and MNPs functionalized bioreporter (MFB) (Fig.3.5), magnet device had the highest responsive sensitivity and illustrated the chromium bioavailability in contaminated soils. In SW-S and SW-M treatments, ADPWH_recA bioreporter did not show any positive response to the chromium due to the strong light adsorption by soil particles. The negative bioluminescent response was observed when chromium concentration was above 100 mg/kg soil dry weight for both treatments. Significant positive response was only found in

MFB treatment and the limit of detection was 1 mg/kg soil dry weight (Fig. 3.5 and Table 3.1).

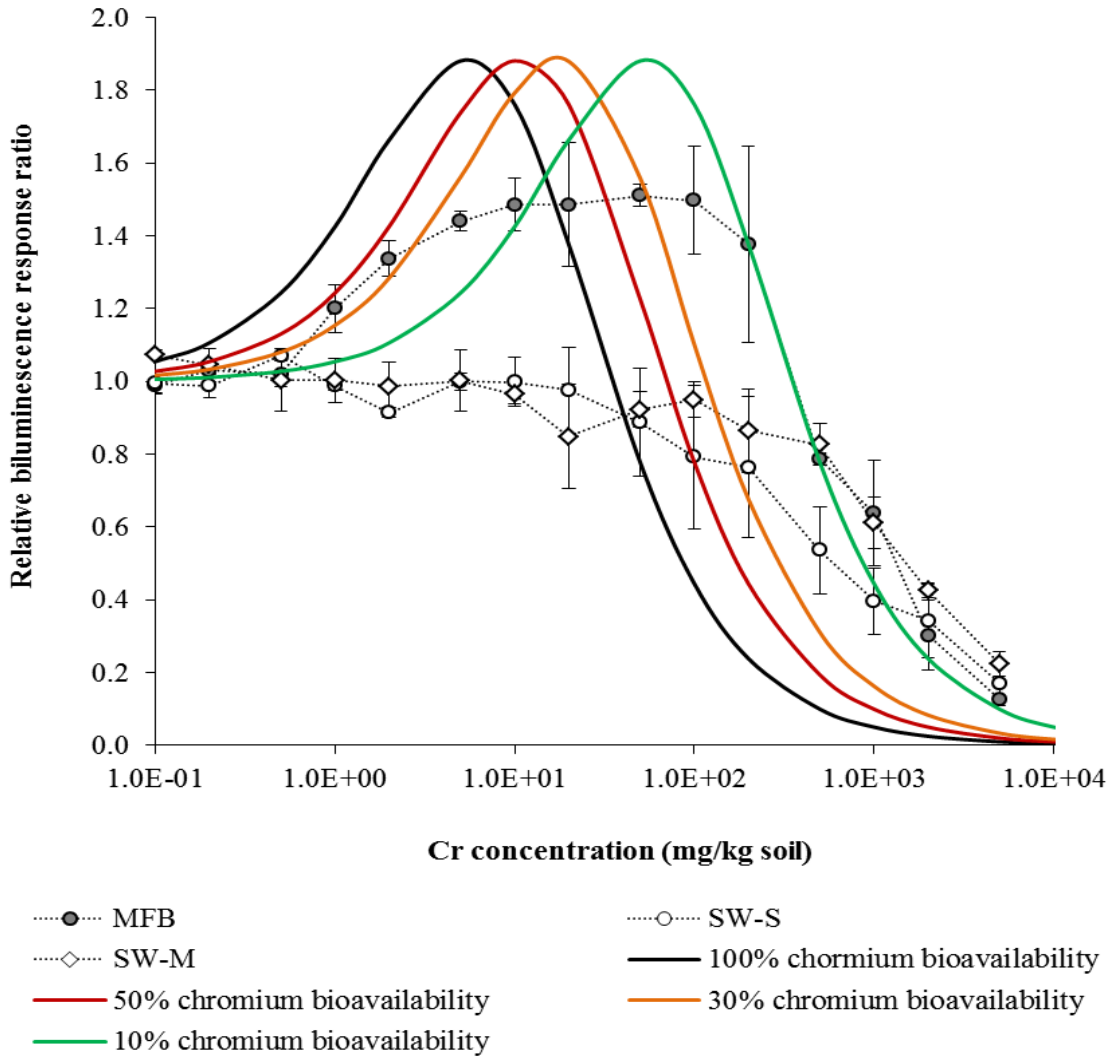



**Figure 3.5** The calibration curve for toxicity assessment on artificial chromium contaminated soils.

Grey circle refers to magnet bioreporter device (MFB); white diamond represents direct measurement of soil/water supernatant (SW-S); white circle is the direct measurement of soil/water mixture (SW-M). The black line represents the simulation of whole-cell bioreporter's response to chromium toxicity with 100% bioavailability, and a significant

bioluminescent response curve shift was found for 50% (red line), 30% (yellow line) and 10% (green line) chromium bioavailability respectively.

From 1 mg/kg to 100 mg/kg chromium contamination in dry soils, the relative bioluminescence response ratio showed a linear relationship to quantify the toxicity and bioavailability of chromium in soil samples, ranging from 1.05 to 1.60. Above 500 mg/kg soil dry weight, chromium predominantly behaved the cytoxicity effects and all the three treatments had similar inhibited bioluminescent signal. From the whole-cell bioreporter growth curve (Fig. 3.6.), there was no significant growth difference when the chromium concentration was less than 500 mg/kg soil dry weight, in which range that the relative bioluminescent response ratio was positively correlated with chromium. It therefore explained the decreasing bioluminescent response ratio at higher chromium level that strong cytoxicity of chromium inhibited bioreporter growth and activities. The Tukey post-hoc test undertaken further supports this argument, as P-values for chromium concentration less than 500mg/kg did not differ significantly ($P > 0.05$), as was the case for chromium concentrations ranging from 500 to 5000 mg/kg. Furthermore, the P-value of 0.000 ($< 0.05$) for the whole-cell bioreporter growth curve ($OD_{600}$) suggests that the bioluminescent response to varying concentration of chromium in the soil samples analyzed over a 6-hour period differ significantly.

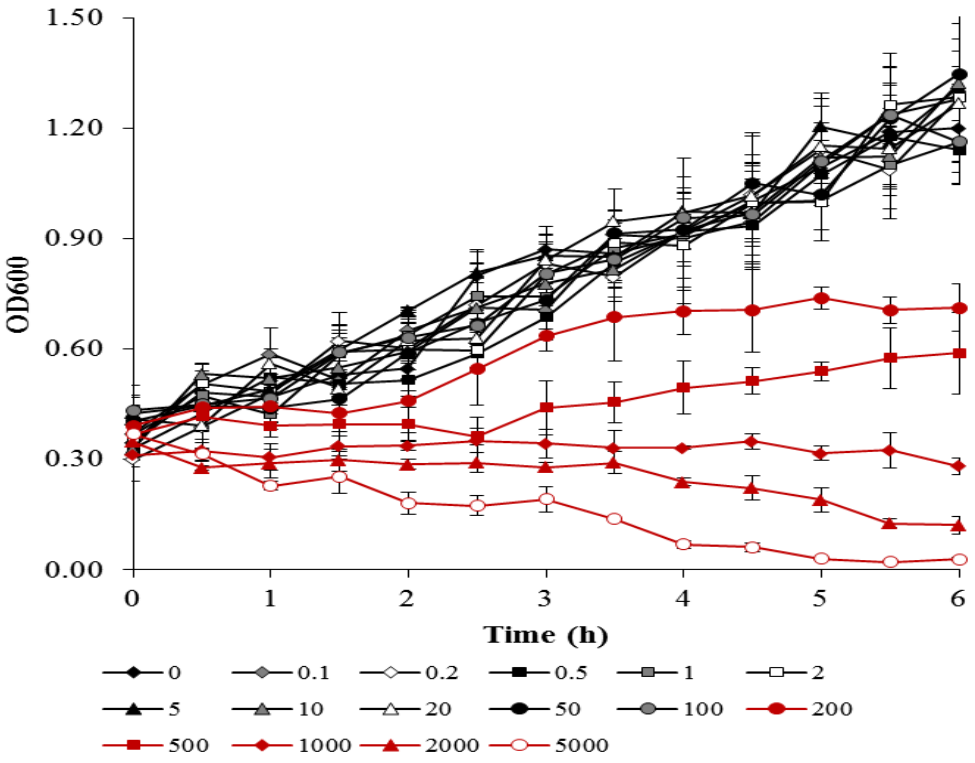



**Figure 3. 6** Whole-cell bioreporter growth curve ($OD_{600}$) against time.

Normal bacterial growth curve was observed when chromium concentration ranged from 0 mg/kg dry soil weight to 100 mg/kg dry soil weight, fitting well with the linear relationship between chromium contamination and bioreporter bioluminescent signal. Above 500 mg/kg soil dry weight, chromium had strong cytoxicity to inhibit bioreporter growth and the $OD_{600}$ was negatively correlated with chromium concentration.

Given the model simulation of bioreporter's response to chromium with different bioavailability in aqueous phase in Fig. 3.5 (Xu et al.), the results further revealed the bacteria-contaminant interaction within the porous soils and its impacts on bioreporter response. With lower chromium bioavailability, the calibration curve shifted towards higher chromium values. The SOS response coefficient ($K_{sLSR} \cdot K_{Cr} \cdot k_{ssDNA} \cdot k_{MO}$, 3.8) and genotoxicity coefficient ($\frac{k_{dsSLR}}{2 \cdot (1+k_{ssDNA})} \cdot [LSR]_{total}$, 1.724 L/mg) kept stable, similar to previous research (Xu et al.). Referring to the synergetic efficiency through the SOS repair process (including genotoxin DNA damage, ssDNA recognition and SOS box activation), the similar SOS response coefficients indicated the same SOS mechanism of bioreporter's

responsive to chromium genotoxicity and cytotoxicity in the soils via the magnet bioreporter device (Zhang et al., 2012d). Since the bioluminescent signal of ADPWH_recA was regulated by the SOS process, all the carcinogens causing DNA damage would activate its response, including mitomycin C, UV light, ethidium bromide and $H_2O_2$ (Song et al., 2014). The bioreporter therefore did not respond to a particular heavy metal (like chromium), but evaluated the synergistic toxicity of all the carcinogens in environmental samples.

From the parabolic curve of MNPs functionalized bioreporter to hexavalent chromium, both the relative bioluminescent response ratio and growth curve (Fig. 3.6.) were considered to evaluate the toxicity of unknown environmental samples. In absence of growth inhibition, the sample had low cytoxicity and its bioluminescent response belonged to the positive relationship range, oppositely in presence of growth inhibition. Only the MFB treatment had the positive bioluminescence response when chromium concentration was less than 200 mg/kg soil dry weight, and the response ratio fitted well with the model prediction of 10% chromium bioavailability when chromium concentration was above 100 mg/kg soil dry weight. At lower chromium contamination level, chromium bioavailability changed due to the complex adsorption effects of soil particles and the irregular bioluminescent response ratio represented the changing bioavailable fraction. Given heavy chromium contamination level (>500 mg/kg soil dry weight), SW-M showed slightly higher response than MFB treatment, both significantly higher than SW-S treatment. Since the whole-cell bioreporter only sensed the water soluble chromium in the supernatant of soil-water mixture in SW-S treatment, it measured the chromium toxicity in the unbound water phase. The dominant fraction of chromium existed in the bound water or was absorbed on the soil particles, and their carcinogenic effects were only assessable by the direct-contact bioreporter assay (Jiang et al., 2015). In MFB and SW-M treatment, both bioreporter cells had the direct contact with chromium absorbed on soil particles and behaved stronger toxicity response. Since it fell in the overtoxicity range and the higher genotoxicity reduced the bioluminescence signal of bioreporter cells, MFB had a higher sensitivity and thus inhibited more than SW-M treatment, showing a relatively lower bioluminescent signals (Michelini et al., 2013).

### *3.3.2 Heavy metal contamination profiles in soils*

The heavy metal profiles of the 16 investigated soil samples and the coal cinder were listed in Table 3.5.

The chromium was 23 times enriched in the rough cinder (from 38.18 mg/kg to 920.82 mg/kg), followed by nickel (5.5 times enrichment). The enrichment of other heavy metals ranged from 1.8 to 3.7 times, except for zinc (no significant change) and mercury (loss due to volatile during the combustion process) (Meij et al., 2002). Similar to previous research on coal combustion residues (Goodarzi et al., 2008, Goodarzi, 2006), chromium and nickel were mostly found concentrated in bottom ash or cinder as the dominant toxic heavy metal residues, due to their low volatility and high stability.

**Table 3.5**. Heavy metal contamination in coal/cinder (mg/kg coal or cinder dry weight) and coal cinder contaminated sites (mg/kg soil dry weight).

| Samples | | Be | Cr | Ni | Cu | Zn | As | Se | Cd | Pb | U | Hg |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Raw coal** | | 0.27 | 38.18 | 4.93 | 6.62 | 8.55 | 2.26 | 0.23 | 0.05 | 3.43 | 0.48 | 0.006 |
| **Rough cinder** | | 0.83 | 920.82 | 26.89 | 22.66 | 9.01 | 4.14 | 0.84 | 0.12 | 7.44 | 1.42 | 0.001 |
| **Background soil** | | 1.33 | 398.94 | 30.99 | 16.22 | 37.91 | 6.36 | 0.87 | 0.26 | 21.62 | 1.20 | 0.008 |
| | **0-20 cm** | 1.07 | 745.15 | 23.11 | 17.92 | 28.78 | 4.71 | 0.75 | 0.25 | 18.19 | 1.29 | 0.003 |
| **0 m** | **20-35 cm** | 1.19 | 552.97 | 17.61 | 10.55 | 27.72 | 3.18 | 0.43 | 0.22 | 20.26 | 0.64 | 0.005 |
| | **35-50 cm** | 1.27 | 505.60 | 14.68 | 10.25 | 20.97 | 3.10 | 0.44 | 0.16 | 20.36 | 0.64 | 0.003 |
| | **0-20 cm** | 1.55 | 802.77 | 29.38 | 21.67 | 23.05 | 5.26 | 1.00 | 0.22 | 18.05 | 1.48 | 0.002 |
| **10 m** | **20-35 cm** | 1.58 | 620.79 | 21.71 | 16.52 | 29.26 | 5.60 | 1.19 | 0.24 | 30.31 | 1.20 | 0.001 |
| | **35-50 cm** | 1.26 | 525.79 | 15.80 | 9.74 | 17.53 | 3.00 | 0.29 | 0.15 | 21.44 | 0.56 | 0.001 |
| | **0-20 cm** | 1.16 | 508.43 | 13.80 | 8.93 | 28.10 | 3.66 | 0.36 | 0.14 | 20.08 | 0.54 | 0.033 |
| **50 m** | **20-35 cm** | 1.12 | 482.68 | 12.29 | 8.09 | 16.19 | 2.62 | 0.41 | 0.13 | 19.88 | 0.45 | 0.001 |
| | **35-50 cm** | 1.12 | 628.89 | 15.03 | 9.35 | 18.22 | 3.08 | 0.46 | 0.14 | 20.17 | 0.53 | 0.001 |
| | **0-20 cm** | 1.15 | 483.48 | 13.26 | 9.03 | 18.61 | 3.22 | 0.30 | 0.16 | 20.01 | 0.53 | 0.001 |
| **80 m** | **20-35 cm** | 1.19 | 502.34 | 13.84 | 9.31 | 19.76 | 3.35 | 0.45 | 0.18 | 21.03 | 0.57 | 0.001 |
| | **35-50 cm** | 1.29 | 613.57 | 19.76 | 15.32 | 29.44 | 4.01 | 0.43 | 0.25 | 21.28 | 0.86 | 0.001 |
| | **0-20 cm** | 1.10 | 494.60 | 13.56 | 8.90 | 19.03 | 3.24 | 0.58 | 0.15 | 19.99 | 0.56 | 0.001 |
| **150 m** | **20-35 cm** | 1.24 | 474.09 | 16.46 | 10.87 | 24.61 | 4.48 | 0.43 | 0.22 | 20.51 | 0.76 | 0.001 |
| | **35-50 cm** | 1.25 | 448.66 | 18.50 | 13.00 | 28.81 | 5.08 | 0.41 | 0.22 | 21.70 | 0.90 | 0.001 |

Note: The analytical instrument is Hg analyzer (DMA-80) for Hg and ICP-MS (X series II) for other elements.

As for heavy metal contamination in soils, the investigated soils only showed significant contamination of chromium from 448.66 mg/kg to 802.77 mg/kg soil dry weight, with the *CF* values from 1.12 to 2.01 (398.94 mg/kg soil dry weight in background). The levels of the other heavy metals were similar to the background soils (*p*-value>0.05), as the *CF* value of beryllium ranged from 0.80 to 1.19, nickel from 0.40 to 0.95, copper from 0.50 to 1.34, zinc from 0.43 to 0.78, arsenic from 0.41 to 0.88, selenium from 0.33 to 1.37, cadmium from 0.50 to 0.96, lead from 0.83 to 1.40, uranium from 0.38 to 1.28 and mercury from 0.13 to 4.13. The results indicated that the key ecological toxicity was attributed to chromium, same as revealed by many previous research on mutagens in heavy metal contaminated soils (White and Claxton, 2004), and its toxicity in soils could be effectively evaluated by various biological assays (Wang, 1999, Codina et al., 1995). The magnet bioreporter device in this study was therefore feasible to enhance the toxicity test sensitive by directly exposing MNPs-functionalized whole-cell bioreporter cells and diagnosing their bioluminescent response signal.

For soils nearer to the coal cinder point (0 m and 10 m), there was higher heavy metal contamination in the upper layer soil. The chromium contamination declined from 745.15 mg/kg (0-20 cm, 0 m) to 505.60 mg/kg (35-50 cm, 0 m), and from 802.77 mg/kg (0-20 cm, 10 m) to 525.79 mg/kg (35-50 cm, 10 m), respectively. Except for Be, Pb and Hg, all the other heavy metal elements (Ni, Cu, Zn, As, Se, Cd, and U) has the same vertical decreasing distribution. Comparing to the heavy metal composition in the cinders, chromium was also the key carcinogenic heavy metal in soils. Given the sequence of the exchangeable fractions of heavy metals as Cd > Zn > Cu > Ni > Pb > Cr (He et al., 2006), chromium was further concentrated in top soils with less mobility. The results further suggested that the main source of heavy metal contamination in soils was the leachates from the cinders and their residues were dependant on the transportation process in soils.

### *3.3.3 Ecological risk profiles of heavy metal contaminated soils*

In the ecological toxicity evaluation by directly applying whole-cell bioreporters to the soil (SW-M, Fig. 3.7) and soil-water supernatant (SW-S, Fig. 3.8), ADPWH_recA only behaved negative (relative bioluminescence response ratio < 1.0) or neutral signal (relative

bioluminescence response ratio = 1.0) and was not suitable to quantify the toxicity impacts of heavy metal contamination *in situ*.

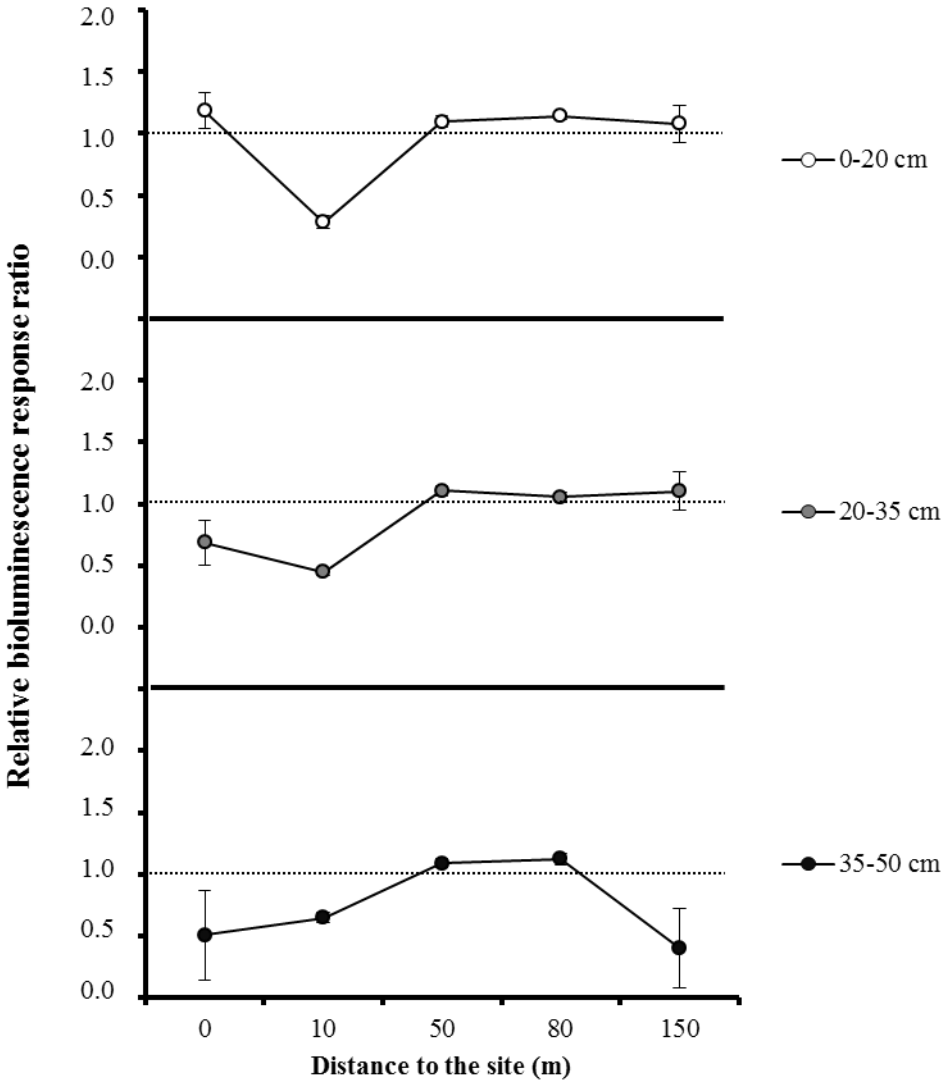



**Figure 3. 7**. Ecological toxicity assessment of heavy metal contaminated soils in SW-M treatment.

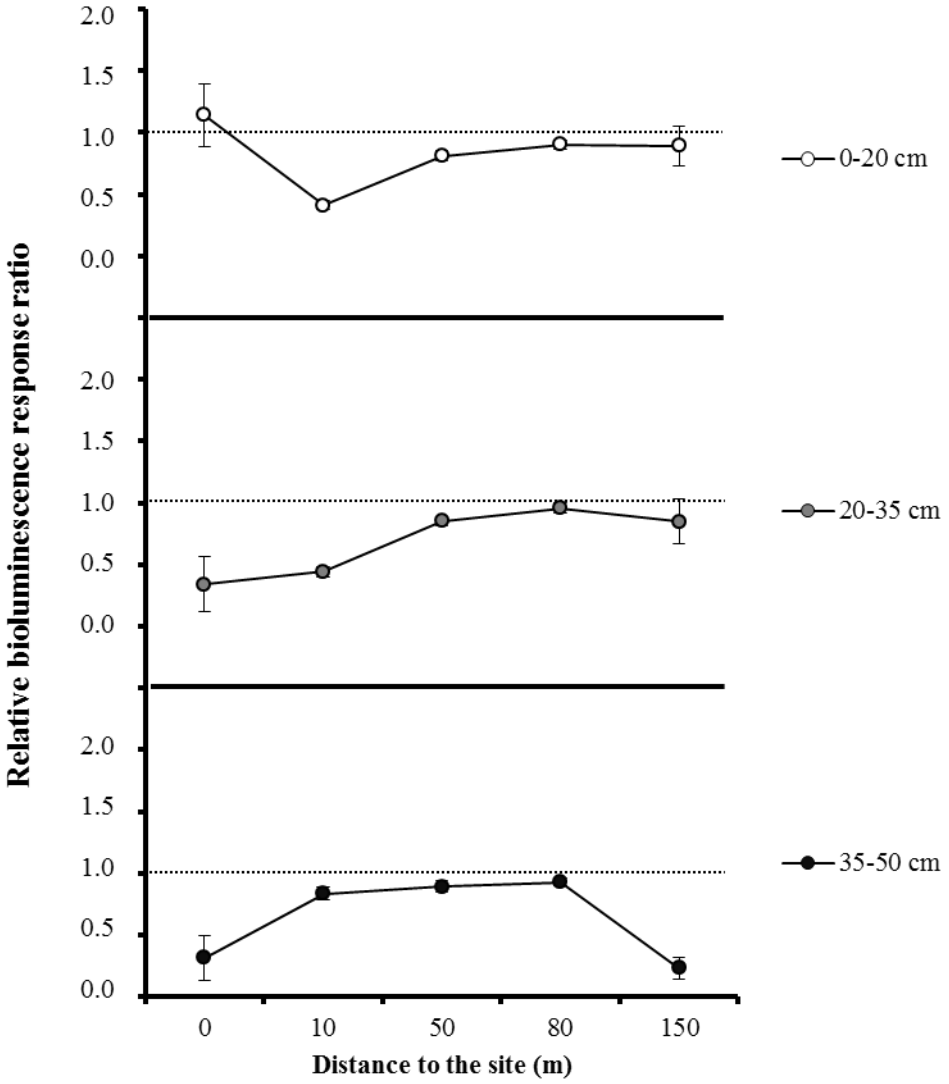



**Figure 3. 8**. Ecological toxicity assessment of heavy metal contaminated soils in SW-S treatment

Fig. 3.9 illustrated the ecological toxicity profiles of the soil samples by the magnetic bioreporter device (MFB treatment), and the toxicity of heavy metals declined with the increasing distance to the coal cinder point.

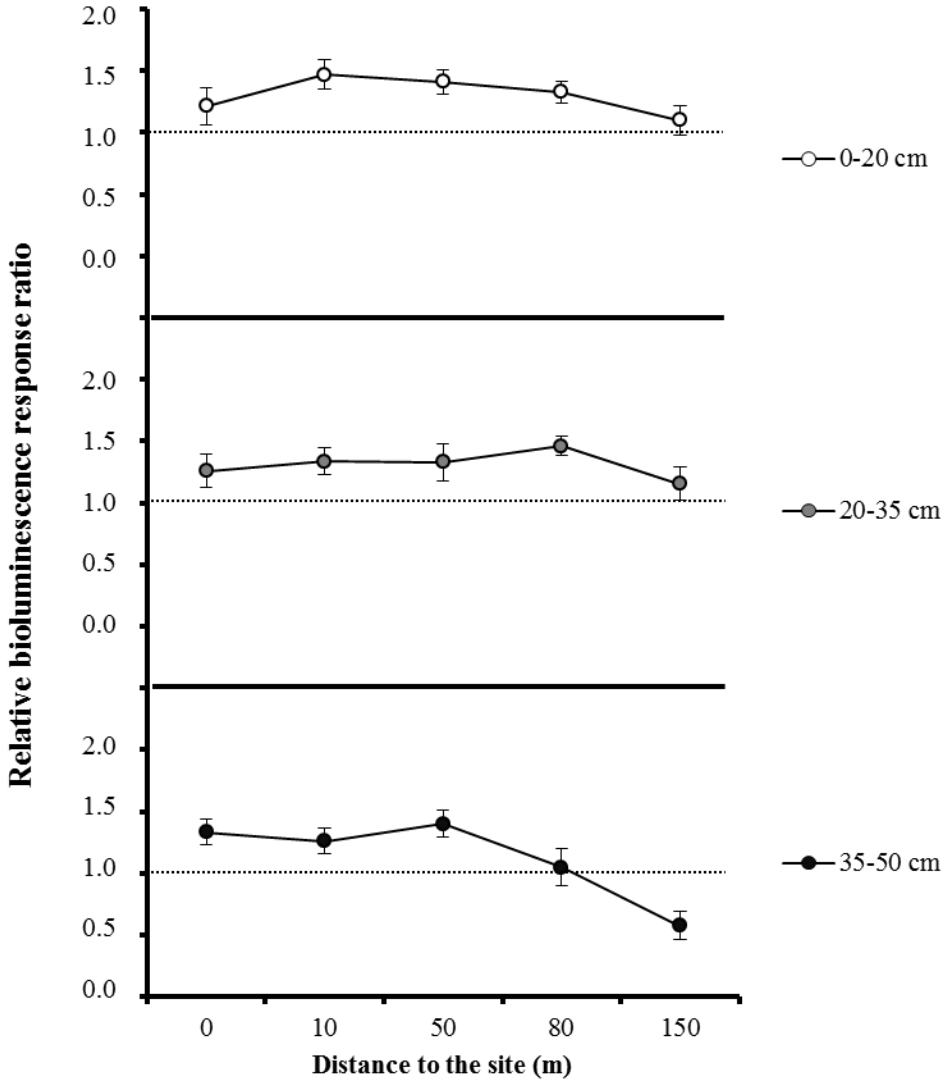



**Figure 3. 9** Ecological toxicity assessment of heavy metal contaminated soils via magnetic bioreporter device.

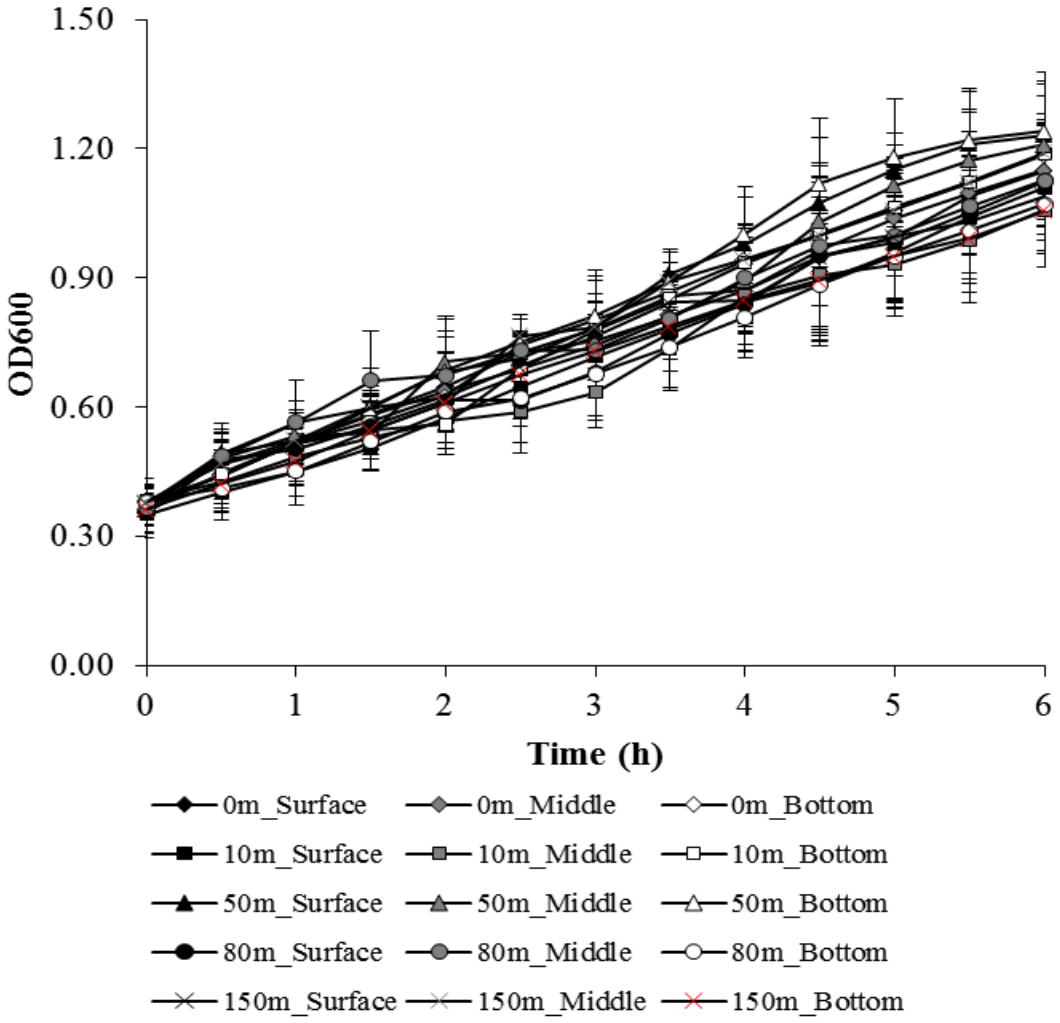



**Figure 3. 10**. Whole-cell bioreporter growth curve (OD600) against time during soil sample detection.

No significant growth inhibition was observed, indicating all the bioluminescent signals of magnetic functionalized bioreporters were located within the linear response range.

From the whole-cell bioreporter growth curve for the soil samples (Fig. 3.10), all the heavy metal contaminated soils did not show inhibition effects on bacterial growth, indicating all the bioluminescent signals were within the linear response range and the relative bioluminescence response ratio had positive relationship with the ecological toxicity in soils. Except for 0 m point, the relative bioluminescence response ratio dropped from 1.47 (10 m) to 1.10 (150 m) in the surface soil, 1.34 (10 m) to 1.16 (150 m) in the middle soil,

and 1.26 (0 m) to 0.58 (150 m) in the bottom soil. At the 0 m point, the low bioluminescence signal of surface soil was caused by the high cytotoxicity effects of chromium (745.15 mg/kg soil dry weigh) and the growth of ADPWH_recA bioreporter was inhibited. The soil sample at 0 m point was therefore characterized with the highest ecological risk.

From the toxicity vertical distribution, the ecological risks had a significant decline in deeper soils. Attributing to the heavy metals leachates from the coal cinders, the ecological risk distribution fitted well with chemical analysis and previous studies. The high ecological risk at the surface soils than bottom soils suggested the leakage and vertical transportation chromium in soils (Ma et al., 2008). Comparing to the horizontal ecological risk distribution, the results further identified the main toxicity sources as the heavy metals from the coal cinders.

### *3.3.4 Correlation between soil heavy metal profiles and ecological risk*

The Principle Component Analysis (PCA) illustrated the main factors causing the ecological risks in soil samples (Figure 3.11).

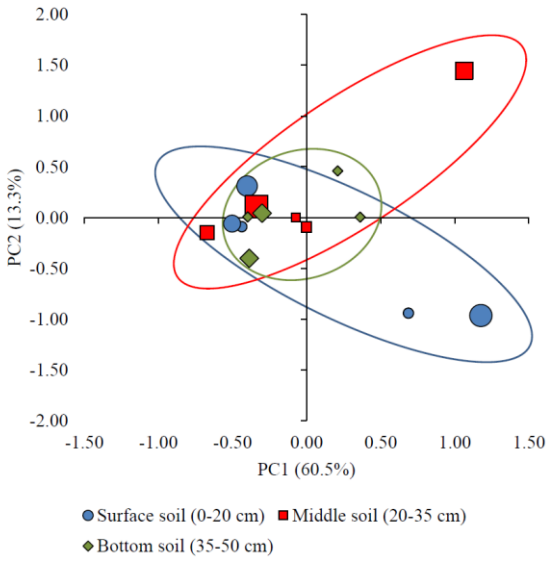



**Figure 3. 11**. The correlation between soil heavy metal profiles and ecological risks.

PCA analysis reveals the two principle components as the heavy metal contamination level (PC1, accounting for 60.5% of variance) and the soil depth (PC2, accounting for 13.3% of

variance). The area of symbol (blue circle for surface soil, red square for middle soil and green diamond for bottom soil) represents the level of heavy metal ecological risk (bioluminescence response ratio).

More precisely, the principle component 1 (PC1) was the heavy metal contamination level, accounting for 60.5% of the total variance. At the sampling points nearer to the coal cinder site (0 m and 10 m), the surface and middle soils were heavily contaminated and therefore recognized as isolated square (red) and circle (blue) to the higher value of PC1-axis. For the rest soils, they gathered due to similar contamination level (*PLI*). PC1 was therefore derived from the external heavy metal sources, leaching from the coal cinder for the surface soil (0-20 cm) and heavy metal vertical transportation for middle soil (20-35 cm). The soil depth was the principle component 2 (PC2), contributing to 13.3% of the total variance). Heavy metals distribution and mobility were reported to depend on soil properties and depth (Hernandez et al., 2003), and their spatial distribution in different depths of soils also affected the mobility and bioavailability (Nemati et al., 2011). Nevertheless, the soil ecological risks (illustrated as the area of each symbol) were associated with neither the load of PC1 nor PC2, suggesting that they were complicatedly affected by both heavy metal profiles and soil features.

There was also no significant correlation between heavy metal pollution load index (*PLI*) and ecological risk (*p*-value>0.05) (Fig. 3.12).

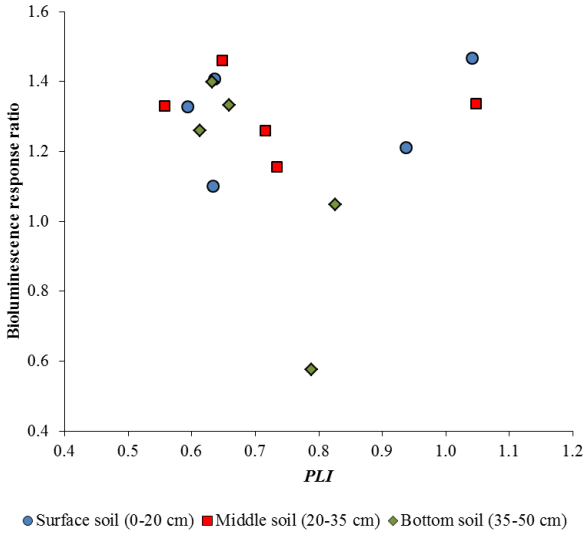


**Figure 3. 12** The correlation between heavy metal pollution load index (PLI) and ecological risk was not significant (p-value>0.05).

Higher *PLI* indicated high heavy metal contamination level, but did not fit with the ecological risk distribution. Previous research had shown the positive correlation between heavy metal content and ecological toxicity at the contaminated sites with individual heavy metal pollutant, like chromium residues (Jiang et al., 2015) or copper contaminated agricultural soils (Brandt et al., 2006). The ecological toxicity was only affected by the individual *CF* value and bioavailability in soil. At the coal cinder contaminated sites, we found the existence of multiple heavy metals and their synergic/antagonistic effects consequently resulted in complicated ecological toxicity (Holmstrup et al., 2010). Many evidences had revealed that the toxicity of individual or multiple heavy metals behaved antagonistic or additive effects, dependent on the composition and soil features, like organic matters or pH value (VanGestel and Hensbergen, 1997, Preston et al., 2000). In this case, *PLI* was an empirical indicator evaluating the multiple heavy metal contamination level, but suffered from identifying and characterizing the interaction between various heavy metal molecules and their association with soil particles. From the mechanisms of ADPWH_recA to sense all the carcinogens activating SOS process, the response of whole-cell bioreporter effectively represented the synergic/antagonistic effects of multiple heavy metals. By directly exposing the living bioreporter cells to the contaminated samples *in situ*, the MNPs functionalized bioreporter had its feasibility as an important approach, supplementary to chemical analysis, in ecological risk assessment and environmental risk management.

## 3.4 Conclusion

This work developed a novel magnet bioreporter device for soil toxicity assessment, via magnetic nanoparticles functionalized whole-cell bioreporters. The living magnetic bioreporter cells could sense the carcinogenic chemicals in the soil and effectively separated from the soil-water slurry in the bioluminescence detection step to avoid the disturbance of soil particles. Comparing to the conventional treatments directly applying bioreporter in soil-water mixture or supernatant, the magnet bioreporter device achieved

high sensitivity and reproducibility under soil pH, salinity and temperature conditions. The dose-toxicity calibration curve revealed the impacts of chromium bioavailability on its ecological risk in soils, where strong genotoxicity was identified when chromium concentration was from 1 mg/kg to 500 mg/kg soil dry weight and the cytotoxic inhibition was found at chromium over 500 mg/kg soil dry weight. For the first time, the ecological toxicity of heavy metal contaminated soils was evaluated by the whole-cell bioreporter at the coal cinder site. Though the existence of heavy metal contamination contributed to the main ecological risks at the site, the pollution load index (*PLI*) had no significantly relationship with the ecological toxicity distribution. The synergic and antagonistic effects of soil multiple heavy metal contamination brought the challenges for environmental risk assessment by chemical analysis. The magnetic bioreporter device behaved as an alternative approach for the high throughput biological measurement and was feasible for *in situ* monitoring.

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# 4 Evaluating oil spill contamination in the niger delta by magnetic whole-cell bioreporter assay

The aim of chapter 4 is to directly apply biosensor on crude oil contaminated Nigerian soil samples and to ascertain the potential of magnetic whole- cell bioreporter for prompt decision making for accident rapid response in water resource management.

The specific objectives are:

- To evaluate crude oil contamination and negative impacts in the Niger Delta with chemical analysis and whole cell biosensor assessment
- To evaluate the toxicity and ecological impacts assessment from crude oil contamination arising from pollution
- To adapt the whole-cell biosensor reporter approach for swift, cheap and robust crude contamination monitoring in the Niger Delta region
- To compare whole-cell biosensor approach against chemical analysis for cross validation in the Niger Delta region of Nigeria

## Abstract

Petroleum is the wealthy natural resources of Nigeria and contributes to its economic development, whereas it also brings severe contamination and threatens to human health and ecology. From 1976, there were over 10,000 crude oil spills accidents occurring in Nigeria and the total oil spillage was 9-13 million barrels, equivalent to 50 times of Exxon Valdez Oil Spill. Considering the high cost and complex operation of chemical analysis which restricts the rapid petroleum monitoring and assessment on the contaminated sites, a new magnetic nanoparticles functionalized bioreporter assay was introduced in this study. From the *in situ* bioreporter data on the four soil and two water samples, this work achieved rapid (less than 4 hours), easy-operation and direct assessment of the crude oil toxicity. The soil contamination ranged from 6250.9 to 55967.6 mg/kg in soil, and the highest water contamination was 248.5 mg/L. The combination of nanoparticles and bioreporter successfully improves the measurement accuracy and sensitivity, showing great application potential in environmental monitoring and risk assessment, particularly in developing countries. It is also a suitable tool for the environmental management crude oil contamination and remediation in Niger Delta.

**Keywords**

crude oil spill, Niger Delta, toxicity, bioreporter, magnetic nanoparticles, monitoring and management

## Abbreviations

- FCT: Federal Capital Territory
- NNPC: Nigerian National Petroleum Development Company
- PHRC: Port Harcourt
- FME: Federal Ministry of Environment
- NCF: Nigeria Conservation Foundation
- WWF: World Wide Fund for Nature
- CEESP-IUCN: Commission on Environmental, Economic and Social Policy of International Union for Conservation of Nature
- TPHs: total petroleum hydrocarbons
- PAHs: polycyclic aromatic hydrocarbons
- LB: Luria-Bertani medium
- ADPWH_recA: *Acinetobacter* bioreporter for toxicity
- ADPWH_alk: *Acinetobacter* bioreporter for n-alkane
- MNPs: magnetic nanoparticles
- $OD_{600}$: optical density at 600 nm

## 4.1. Introduction

Crude oil consists of various toxic compounds and is viewed as an important environmental contaminant. It is therefore a global issue for oil spill and contamination during the crude oil exploration and refinery process. Oil spill accidents have significantly caused real threats to food security, social and environmental deterioration all over the world, especially in Niger Delta where crude oil exploration activities lasted for 50 years.

Nigeria is bounded to the South by the Gulf of Guinea, to the North by Mali, Niger Republic and Chad and to the East by Cameroun and West by Republic of Benin, and has the Federal Capital Territory (FCT) with 36 provincial states. Nigeria was ranked 10th and 7th largest in terms of oil and gas reserves in the world, with the oil and gas reserves as 31.29 billion barrels in 2011. It is one of the seven sedimentary basins in Nigeria. Exploration activities in the country had been concentrated onshore, particularly, in the Niger Delta area which is locate in the Atlantic coast of Southern Nigeria (Ite, 2007) and the region is about 20,000 $km^2$ as it is the largest wetland in Africa and among the third largest in the world (Chinweze and Abiol-Oloke, 2009).

The Nigerian oil exploration began in 1908 and has four refineries owned by the Nigerian National Petroleum Development Company (NNPC). Two are located in Port Harcourt (PHRC), One in Kaduna (KRPC) and another in Warri (WRPC). The cumulative petroleum production of Nigeria from 1958 to 2010 was 29.8 billion barrels with highest annual production of 920 million barrels in 2006. With the long history of crude oil exploration and refinery, Niger Delta has suffered from the severe crude oil contamination (Adelana et al., 2011c), which behaved as accidental oil spillage or operational discharges of petroleum into the environment (Ite et al., 2013b). Studies have indicated that the quantity of oil spilled over 50 years was at least 9-13 million barrels, which is equivalent to 50 times of Exxon Valdez Oil Spill in Alaska in 1989, showing an average oil spill of 0.15 million barrels (FME, *et al*. 2006). From the quantity and number of oil spills incidents from 1976-1990 by the research of Federal Ministry of Environment (FME), Nigeria Conservation Foundation (NCF), WWF and CEESP- IUCN (FME et al., 2006), a total number of 2,796 oil spill occurred and consequently caused significant environmental impacts in the Niger Delta.

As a biological analytical device to sense environmental contaminants via living organisms, whole-cell bioreporter is a complementary tool to evaluate the bioavailability and ecotoxicity of pollutants, especially for the synergetic toxic effects of various components in crude oil (Li et al., 2013, Tecon et al., 2010, Zhang et al., 2013). A novel bioreporter array device was then fabricated in this study for crude oil monitoring and assessment in Nigeria, by the magnetic nanoparticle functionalization of two *Acinetobacter* sp. bioreporters responsive to specific n-alkane and genotoxicity. Further discussion also attempts to reveal the current situation and reasons of crude oil contamination, monitoring, remediation and policies in Nigeria, raising potential solutions for environmental management.

## 4.2 Materials and Methods

### *4.2.1 Crude oil contaminated sites*

Four soil and two water samples were collected from Gokana and Bodo in Rivers State and Jesse in Delta State between 2$^{nd}$ and 4$^{th}$ March 2014. They are all located in the Niger Delta with the geographic coordinates as Soil_1 and Water_2 (E5.903, N5.720), Soil_2 (E5.903, N5.757), Soil_3 and Water_1 (E5.839, N5.893) and Soil_4 (E5.840, N5.640).

### *4.2.2 Chemical analysis*

Without specific statement, all the chemicals in this research were purchased from Sigma Aldrich. The chemical analysis of crude oil was carried out by gravimetric methods as previously described (Zhang et al., 2013). Briefly, 100 mL of water or 10.0 g soil sample was extracted by 50 mL chloroform solvent. The extracts were passed through an anhydrous sodium sulphate column to remove water and separate different oil components. The total petroleum hydrocarbons (TPHs) and polycyclic aromatic hydrocarbons (PAHs) contents were determined gravimetrically after solvent evaporation for 48 hours.

### *4.2.3 Bioreporter strain and cultivation*

Two whole-cell bioreporter strains, ADPWH_alk (Zhang et al., 2012b) and ADPWH_recA (Song et al., 2009a), were applied for rapid evaluation on the n-alkane and toxicity of targeting samples to provide environmental risk assessments at crude oil-contamination sites (Zhang et al., 2013). The 1.0 L of Luria-Bertani (LB) medium contains 10 g Bacto-

Tryptone, 5 g Bacto-yeast extract and 10 g NaCl. After grown in LB medium at 30ºC, the 1.0 mL bioreporter cells (about $10^8$ CFU/mL) were harvested by centrifugation at 3000 rpm for 10 minutes at 4ºC, and subsequently washed and resuspended in 10 mL deionized water as the bioreporter stock solution.

### *4.2.4 Magnetic bioreporter device measurement*

All the soils samples were suspended in aqueous phase with the ratio of 10.0 g soils to 5 mL deionized water. The bioreporter functionalization by magnetic nanoparticles (MNPs) followed previous protocol (Chen et al., 2013). Briefly, the 200 µL of soil-water suspension was directly transferred into the wells without ultrasonic treatment, and mixed with 10 µL magnetic nanoparticles functionalized bioreporter cells. The magnetic bioreporter array was manufactured with MNPs functionalized bioreporter cells and magnet probe assay (Jia et al., 2015). After 1 hour incubation at 30 ºC with soil suspension or water sample, the MNPs functionalized bioreporter was harvested by the magnet probe assay for biological signal measurement to mitigate the disturbance of soil particles. The bioluminescent measurement was applied on FLUOstar Omega Multi-Mode Plate Reader (BMG Biotechnology, UK) for 6 hours. Both the bioluminescence and optical density at 600 nm ($OD_{600}$) were recorded every 10 minutes, and 30 seconds shaking was undertaken for better homogeneity. Both *in-situ* and in laboratory tests were carried out with three replicates to verify the reliability and accuracy of bioreporter methods.

### *4.2.5 Data analysis*

The bioluminescent data of individual bioreporter or bioreporter array were obtained and analysed by MARS software (BMG Biotechnology, UK). The induced bioluminescence was calculated by averaging the monitored bioluminescent data from 200 and 240 minutes. The bioluminescent response ratio was evaluated via dividing the induced bioluminescence by that of negative control (non-induced) samples.

## 4.3 Results and Discussion

### *4.3.1 Crude oil spill monitoring and assessment*

The results of gravimetric analysis were listed in Table 4.1, and the crude oil contamination is significant in all the water and soil samples.

**Table 4. 1** Crude oil contamination by chemical analysis.

| **Sample** | **Crude oil components** | |
|---|---|---|
| | ***Total petroleum hydrocarbon*** | ***Polycyclic aromatic hydrocarbon*** |
| Soil_1 | 86933 mg/kg | 175.81 mg/kg |
| Soil_2 | 46258 mg/kg | 73.27 mg/kg |
| Soil_3 | 9675 mg/kg | 5.39 mg/kg |
| Soil_4 | 9842 mg/kg | 1.43 mg/kg |
| Water_1 | 47.5 mg/L | 0.01 mg/L |
| Water_2 | 186.4 mg/L | 0.11 mg/L |

The soil total petroleum hydrocarbon concentration ranged from 9675 mg/kg to 86933 mg/kg, and the polycyclic aromatic hydrocarbons were 1.43-175.81 mg/kg. The TPHs and PAHs in water samples were 47.5-186.4 mg/L and 0.01-0.11 mg/L, respectively.

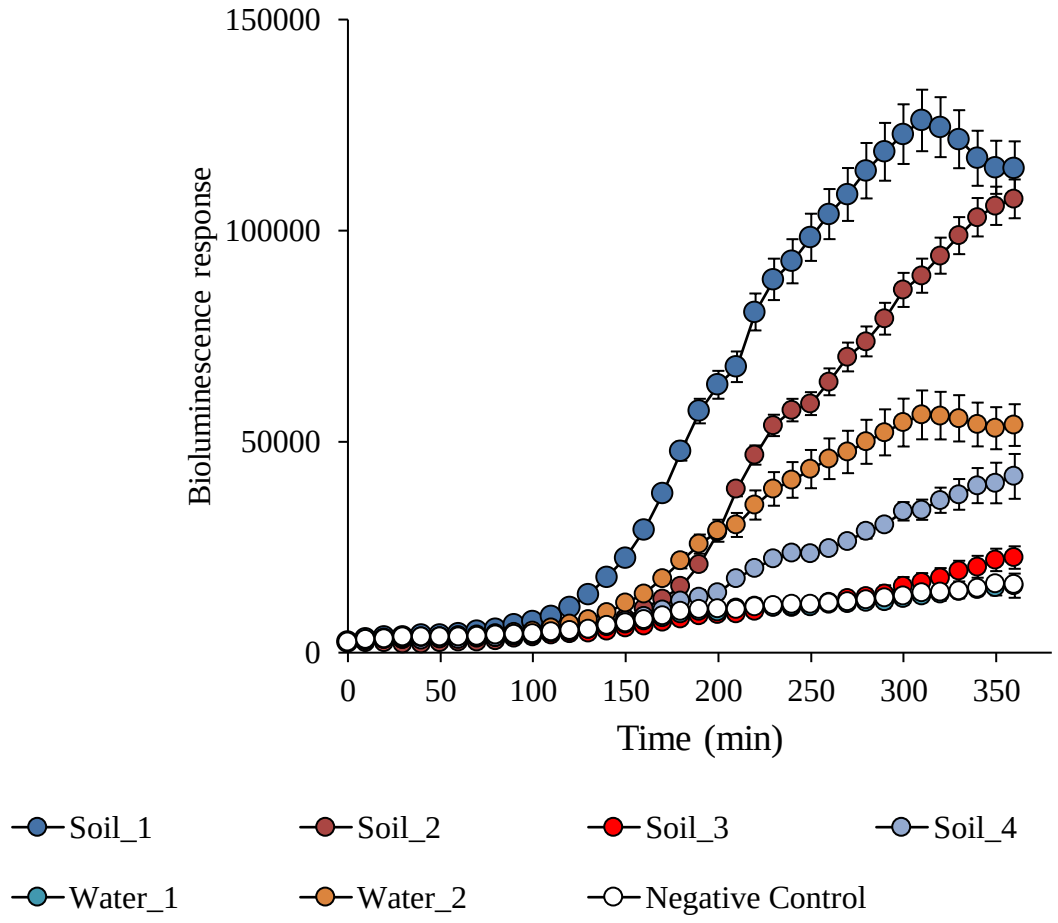


**Figure 4. 1**Whole cell bio-reporter application for rapid detection and evaluation of crude oil spill

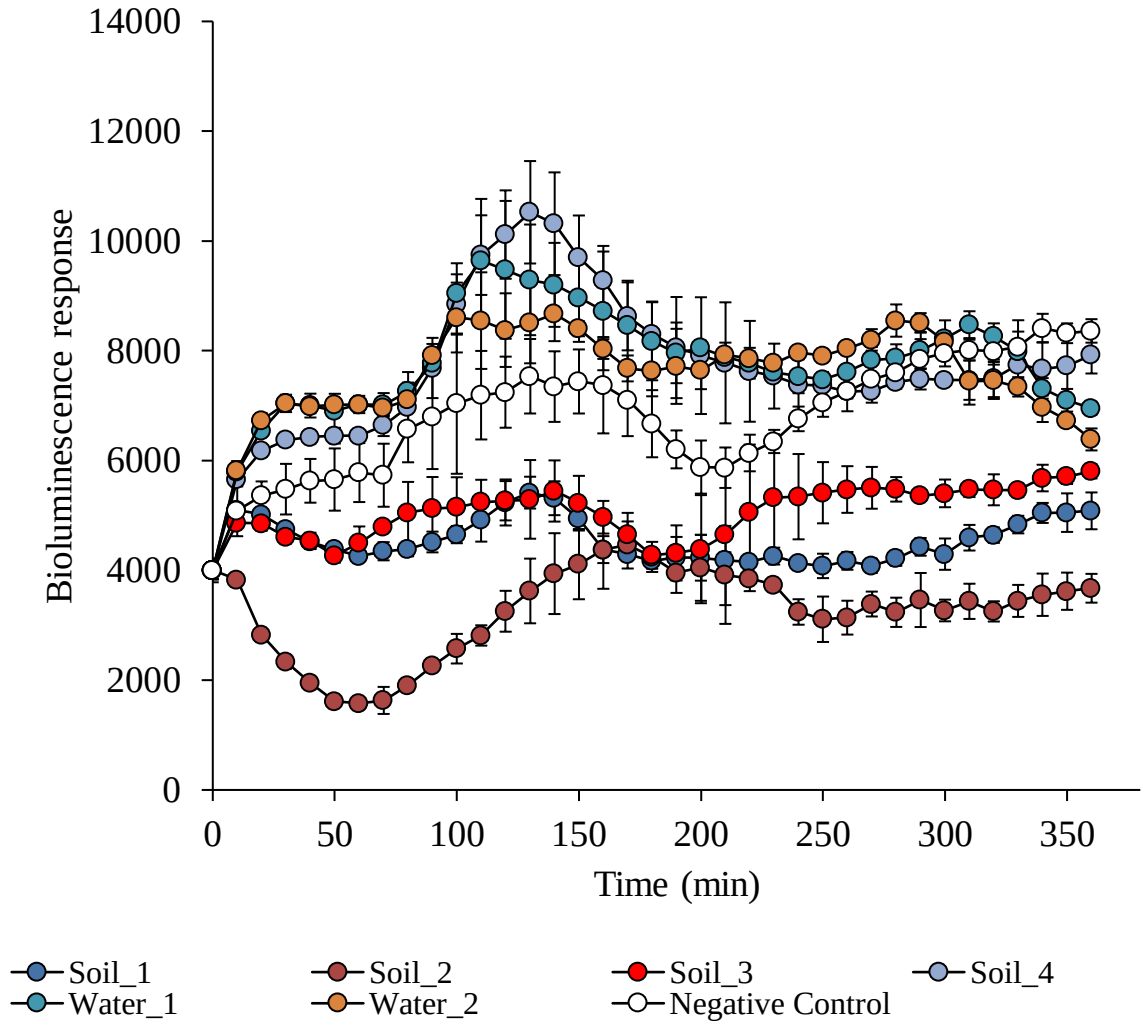


**Figure 4. 2** Bioreporters' response to soil and water samples in Nigeria.

Figure 4.1 for ADPWH_alk's responsive to crude oil content and Figure 4.2 for ADPWH_recA's responsive to toxicity.

It was clear that water and soil samples (Soil_3 and Water_1) without crude oil contamination behaved no bioluminescent response of ADPWH_alk to the oil content (Figure 4.1). For toxicity response, Soil_1, Soil_2 and Soil_3 behaved remarkable lower bioluminescent signal than the non-contaminated samples (Figure 4.2), suggesting strong inhibition effects of heavily contaminated soils on the bacterial bioreporter activities, caused by the acute toxicity of crude oil. Strong positive responsive signal is observed for ADPWH_recA to Soil_4, indicating high genotoxicity is detectable in medium-

contaminated soils. Though the contamination level of crude oil was different for Water_1 and Water_2 samples, their toxicity response (Figure 4. 2) behaved similar, which is further emphasized in the results of the Anova analysis (Table 4.2) and is possibly illustrating other potential carcinogens, such as heavy metal, in the water sample from other industrial activities.

Table 4.2 shows the P-values from the one-way ANOVA performed to assess the statistical significance of the different bioluminescence responses to varying degrees of crude contamination in various soil and water samples analyzed. The results reveal that the bioluminescence response to Alkane and Toxicity for the various samples differ significantly ($P < 0.05$), with the exemption of toxicity in the water samples, where $P = 0.214$.

**Table 4.2** Result from one-way ANOVA analysis of bioluminescence response to soil and water samples Alkane and Toxicity levels.

| | **Alkane (P-value)** | **Toxicity (P-value)** |
|---|---|---|
| Soil | 0.000 | 0.000 |
| Water | 0.000 | 0.214 |

From the calibration curve of n-alkane and toxicity (Zhang et al., 2012b), the n-alkane contents in all the targeting samples were estimated as 55967.6 mg/kg (Soil_1), 38970.8 mg/kg (Soil_2), 6250.9 mg/kg (Soil_3) and 7145.6 mg/kg (Soil_4), as illustrated in Figure 4.3.

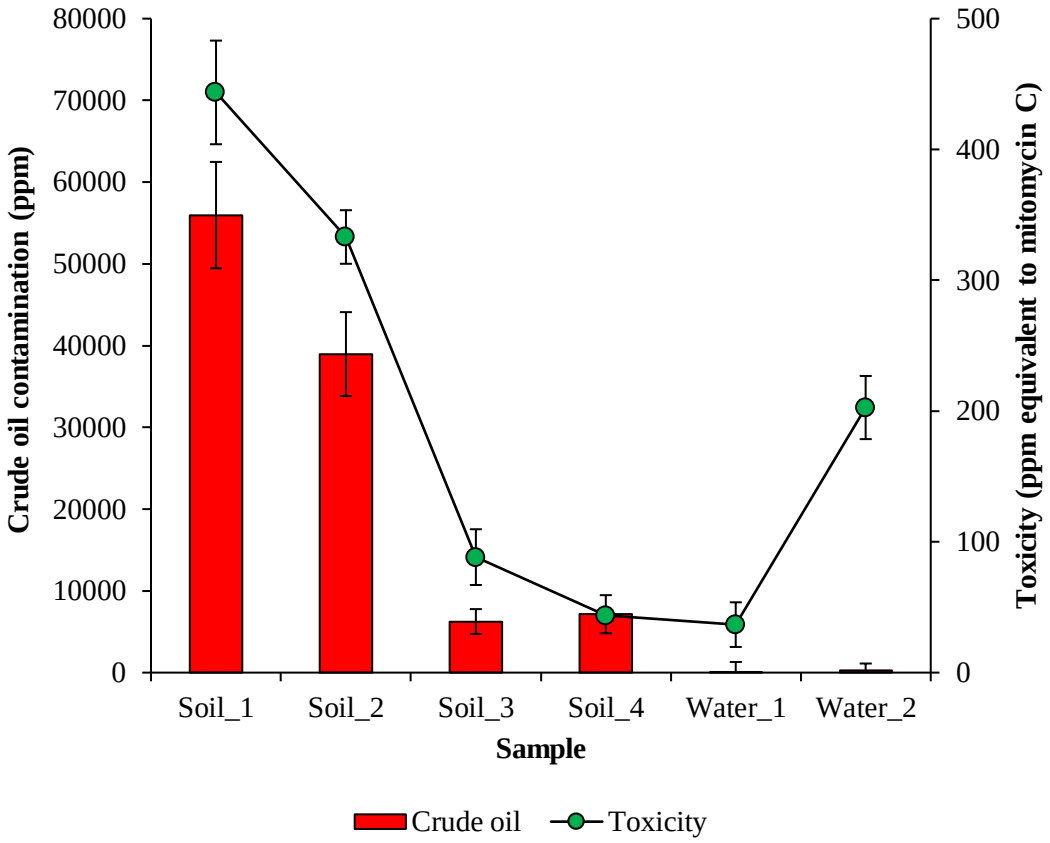


**Figure 4. 3**. Evaluation of crude oil contamination and toxicity in soil and water samples.

The bioreporter results have a positive linear relationship with the gravimetric analysis, proving the feasibility of magnetic bioreporter device for *in situ* crude oil measurement. The highest aqueous n-alkane concentration was found in Water_2 as 248.5 mg/L. Soil_1 has the highest toxicity level, equivalent to 443.6 mg/kg mitomycin C in soils, followed by Soil_2 (333.1 mg/kg), Soil_3 (88.3 mg/kg) and Soil_4 (43.6 mg/kg) samples. The genotoxicity level in water samples are 36.7 mg/L (Water_1) and 202.7 mg/L (Water_2), respectively.

The novel magnetic bioreporter device achieved rapid (<4 hours, from Figure 4.1) and *in-situ* crude oil and toxicity assessment, with the comparable detection limit to chemical analysis at ppm level. This method has the unique advantage of rapid evaluation on toxicity and bioavailability to provide environmental risk assessments at crude oil contamination sites over the physical and chemical methods (Zhang et al., 2013). Previously applied to the detection of alkanes and alkenes in water, seawater and soils (Zhang et al., 2012b), the

bioreporter was regarded as cost-effective and reliable technique for crude oil monitoring. With the recent development of nanotechnology, the combination of nanoparticles and biological process is successful in enhancing measurement accuracy and broadening biochemical application in environmental research (Zhang et al., 2011a). As an imperative approach in case of decision making for accident rapid response in water resource management, whole cell bioreporter array has great potential in engineering application and will technical support for policies and regulations in the Niger Delta.

### *4.3.2 Ecological and health impacts of oil spills*

Evaluated by the posed dangers in oil spills on mangrove ecosystem recovery in the Niger Delta from 1986 to 2008 (Orimoogunje and Ajibola-James, 2013b). It was found that major oil spill incidents in the region represent a significant source of hydrocarbons locally and episodically (Benson and Essien, 2009). Oil spills can last for 15 years or more in the environment without proper clean-up and remediation (Kadafa, 2012c). Oil spills in Nigerian effectively damage both flora and fauna within the environment of the contaminated soils. The terrestrial and aquatic ecosystems are also severely polluted as a result of the oil drill wastes pumped out from the crude oil wells (Bello et al., 2004).

Moreover, concentrations of some organic petroleum chemicals have been investigated, the PAHs in the sediments of the Niger Delta are characterized between 1.99 µg PAHs/g organic carbon to 120.2 µg PAHs/g organic carbon, PAHs in sediments posed real threats to the ecosystem (Sojinu et al., 2010b). There have been a dearth of figures and statistics rates in Nigeria that research carried out by link PAHs to cancer risk among Nigerians as a result of oil pollution (Chukwuma, 2006a). The results proved the posed threats to humans and animals, indicated decreases in sperm count and fertility. From the long term health impacts of crude oil spill in Alesa Eleme near Port Harcourt (Ordinioha, 2013), 60% reduction in household food security was observed and the ascorbic acid content reduction of vegetables was 36%. It resulted in a 24% increase in the prevalence of childhood malnutrition. Animal studies also indicated that crude oil could be hemotoxic and hepatotoxic, causing infertility and cancer in Nigeria (Ordinioha and Brisibe, 2013b). The environmental impact of the 1997 leakage of the high-pressure crude-oil pipeline at Isiokpo in the Niger Delta has also proved the similar mechanisms of oil spill toxicity (Osuji and Adesiyan, 2005b).

## 4.4 Conclusion

The severe crude oil contamination has caused serious environmental disaster in Niger Delta, consequently resulting in significantly ecological and health impacts on local community. As the world's most severely petroleum-impacted ecosystems facing the risk, Nigerian government and local community should take actions for their future sustainable development to mitigate the negative impacts of crude oil contamination. The challenges and difficulties for oil spillage contamination control attribute to both political and technical barriers. Lack of political support and sound legal actions restricts the engineering actions in monitoring, assessment and clean-up for oil spillage, especially for the downstream operations. From technical point of view, novel biological tools are suggested as a sustainable approach for the monitoring, assessment and remediation of crude oil contaminated land. Linking contamination to ecological and health impacts and achieve low-cost bioremediation, biological approach helps the decision making for accident rapid response in contaminated sites management in terms of technical support for policies and regulations.

## Competing interests

The authors declare that they have no competing interest.

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# 5 Simultaneous and online detection of crude oil contamination via biological-phase microextraction and biosensing (BPME-BS) device

This chapter aims to develop innovative passive sampler approach for the simultaneous quantification of alkane concentration using a novel biological-phase micro-extraction and biosensing (BPME-BS) device. The specific objectives are:

- To addressed the challenges of online measurement of crude oil contamination.
- To develop biosensor device for on-field and in-situ measurement of crude oil contamination.
- Explore collaboration with Nigerian companies for the possible commercialization of biosensor for crude oil contamination monitoring.

## Abstract

Oil spills incidents are frequent and show significant threats to ecosystem and human health. Chemical analysis is the widely accepted method for crude oil detection, but requiring sample pre-treatment and not suitable for online monitoring. Given the increasing demands of rapid and online detection of oil spill, we developed a passive sampler for simultaneous quantifying alkane concentrations via biological-phase microextraction and biosensing (BPME-BS) device, introducing alkane-chemotaxis bioreporter ADPWH_alk to seek for and accumulate alkanes from water samples. Immobilizing ADPWH_alk cells in agarose gel, the BPME-BS device achieved high enrichment factor (>4.6) and satisfactory limit of detection (0.05 mg/L) for alkanes. The quantitative response of BPME-BS device to alkane concentrations was comparable to that of gas chromatography flame ionisation detector (GC-FID). The alkane bioreporter ADPWH_alk kept stable sensitivity and limit of detection under a wide range of environmental conditions, like pH between 4.0 and 9.0, temperature from 20°C to 40°C, and salinity from 0% to 3.0%. The response of BPME-BS device was reliable within 30 days when stored at 4°C. More importantly, the BPME-BS device could detect the dynamic concentration of alkanes in water samples, and the 7-day simultaneous measurement proved its feasibility as online alkane detection device. Our work proves the concept that whole-cell bioreporter cells can be immobilized as passive sampler for online diagnosing environmental contaminants. As a successful case, the novel BPME-BS device achieves simultaneous quantification of oil spill, contributing to fast detection of and rapid response to crude oil spill.

Key words: Alkane, bioreporter, *Acinetobacter baylyi*, chemotaxis

## 5.1 Introduction

Crude oil spills have significantly negative impacts on ecosystem and human health, and they frequently occurred due to the rapid industrial development and increasing usage of crude oil. During the exploration, transportation, storage and manufacturing of crude oil, there were over 40 large oil spill incidents worldwide since 1969, including Exxon Valdez Oil Spill in Prince William Sound (Bence et al., 1996, Bragg et al., 1994), Deepwater Horizon Oil Spill in Gulf of Mexico (Camilli et al., 2010) and Dalian Xingang Oil Explosion (Zhang et al., 2013). Large areas of oil-contaminated sites have damaged ecological systems and threatened human health (Peterson et al., 2003, Piatt et al., 1990). Many chemical analytical methods can be used to measure the contents of crude oil, including gravimetric, fluorophotometry (Zhou et al., 2013, Wang et al., 2016a), infrared (IR) absorption (Lay-Ekuakille et al., 2013), gas chromatography flame ionization detector (GC-FID) (Krupcik et al., 2013) or gas chromatography mass spectrometry (GC-MS) methods (Liu et al., 2007). However, those techniques require the solvent extraction or solid-phase microextraction for sample pre-treatment, which restrict their applications in online or real-time detection (D'Auria et al., 2008). Some new tools need to be developed to address such question, for the simultaneous detection of oil spill and rapid response to the disasters.

Different from chemical analytical methods, whole-cell bioreporter is another technique to analyse chemicals via biological cells. It helps our understanding of the bioavailability of pollutants in natural environment by measuring the biological response. Some whole-cell bioreporters have been developed (Zhang et al., 2012b) and even applied in real-world cases (Zhang et al., 2013). Nevertheless, these methods are feasible only in laboratory and suffer from the complicated operation and low reproducibility when the bioreporter strains are reactivated for measurement. Meanwhile, the whole-cell bioreporters need to be pre-treated and mixed with the targeting environmental samples, restricting its potential for online or real-time measurement of contaminants. Such a huge gap shows the importance of device implementation, particularly for the rapid response to oil spill incidents, which is one of the most common environmental contaminants. The combination of biological

sensing and instrumental measurement can provide the real-time and non-destructive features to achieve fast and reliable detection of crude oil contamination.

Naturally, some alkane degrading microorganisms have chemotaxis towards alkanes or can bio-accumulate alkanes (Wang and Shao, 2013). These features enable bacterial access to and utilization of alkanes in different phases (Parales and Harwood, 2002). The *tlpS* gene in *Pseudomonas aeruginosa* PAO1 is located in downstream of the alkane monooxygenase *alkB1* gene and encodes a methyl-accepting chemotaxis protein (MCP) recognizing hexadecane (Smits et al., 2003). Similarly, the *alkN* gene in *P. putida* GPo1 can encode an MCP for alkane chemotaxis (van Beilen et al., 2001). Other alkane chemotactic MCP encoding genes can be also found in other strains, such as *Flavimonas oryzihabitans* (Lanfranconi et al., 2003) and *Alcanivorax dieselolei* (Lai et al., 2012), responding to alkanes of different chain length. *Acinetobacter baylyi* is a bacterial species with the strongest alkane chemotaxis and accumulation (Zhang et al., 2012b), in which the putative gene encoding an MCP is *chpA* with high genetic similarity to *cheY* (fused chemotactic sensory histidine kinase) and *ompR* (two-component response regulator) in *Escherichia coli*, hinting similar chemotaxis machinery. It is reported *A. baylyi* cells have high affinity to alkane droplets (Zhang et al., 2012b), showing the high recognition specificity of MCP to linear alkanes. The alkane chemotactic and accumulation behaviour hints at the possibility allowing them assembled as device to sense and accumulation alkanes from aqueous phase for alkane detection.

This study developed a novel passive sampler (biological-phase microextraction and biosensing, BPME-BS) for simultaneous detection of alkane concentrations in water samples. By immobilizing alkane chemotactic and accumulation bioreporter ADPWH_alk in agarose gel, this BPME-BS device was able to enrich alkanes from water samples and responded to alkane contaminations with high sensitivity. More importantly, it could simultaneously detect alkane concentrations and achieve real-time or online alkane analysis.

## 5.2 Methods

### *5.2.1 Bioreporter strains and cultivation*

Acinetobacter *baylyi* ADPWH_alk (Zhang et al., 2012b) was used as the alkane whole-cell bioreporter in this study. It has been proved to show quantitative response to alkanes in water and can metabolize alkanes. The ADPWH_alk cells were grown in LB medium at 30°C and 150 rpm for 16 h. The 1.0 mL cells were then centrifuged at 4,000 rpm for 5 min, washed three times using minimal medium, and finally resuspended in 1.0 mL minimal medium as the stock solution. The 1.0 litre minimal medium contained 1.0 g $(NH_4)_2SO_4$, 2.5 g $KH_2PO_4$, 0.1 g $MgSO_4{\cdot}7H_2O$, 0.005 g $FeSO_4{\cdot}7H_2O$, 0.25 g nitrilotriacetic acid, 0.55 g NaOH, and 1 mL Bauchop and Elsden solution.

### *5.2.2 BPMS-BS device assembly*

The manufacture of BPMS-BS device followed the 4-step assembly (*gelatification-immobilization-molding-solidification*) as illustrated in Figure 5. 1) *Gelatification*: the agarose gel stock solution (1.1%) was prepared by adding 1.1 g agarose powder into 98.9 mL sterile deionized water, heated up to 100°C for 10 min and cooled down to 50°C. 2) *Immobilization*: 1.0 mL stock solution of bioreporter ADPWH_alk cells were then added into the agarose solution, mixed well by shaking at 300 rpm for 10 seconds. 3) *Molding*: the agarose-bioreporter suspension was subsequently embedded in glass deployment unit with mold (base, 3.0 × 3.0 × 0.2 cm, length×width×thickness, Figure 5.1A; mold, 2.5-cm diameter and 0.2 cm depth, Figure 5.1B). 4) *Solidification*: after solidification with glass cover (3.0 × 3.0 × 0.2 cm, length×width×thickness, Figure 5.1C) for 30 minutes, the cover was removed and the BPME-BS device was ready for alkane detection. During *gelatification* step, the agarose gel was cooled to 100°C, 80°C, 60°C, 50°C and 45°C to test the impacts of immobilization temperature on bioreporter viability.

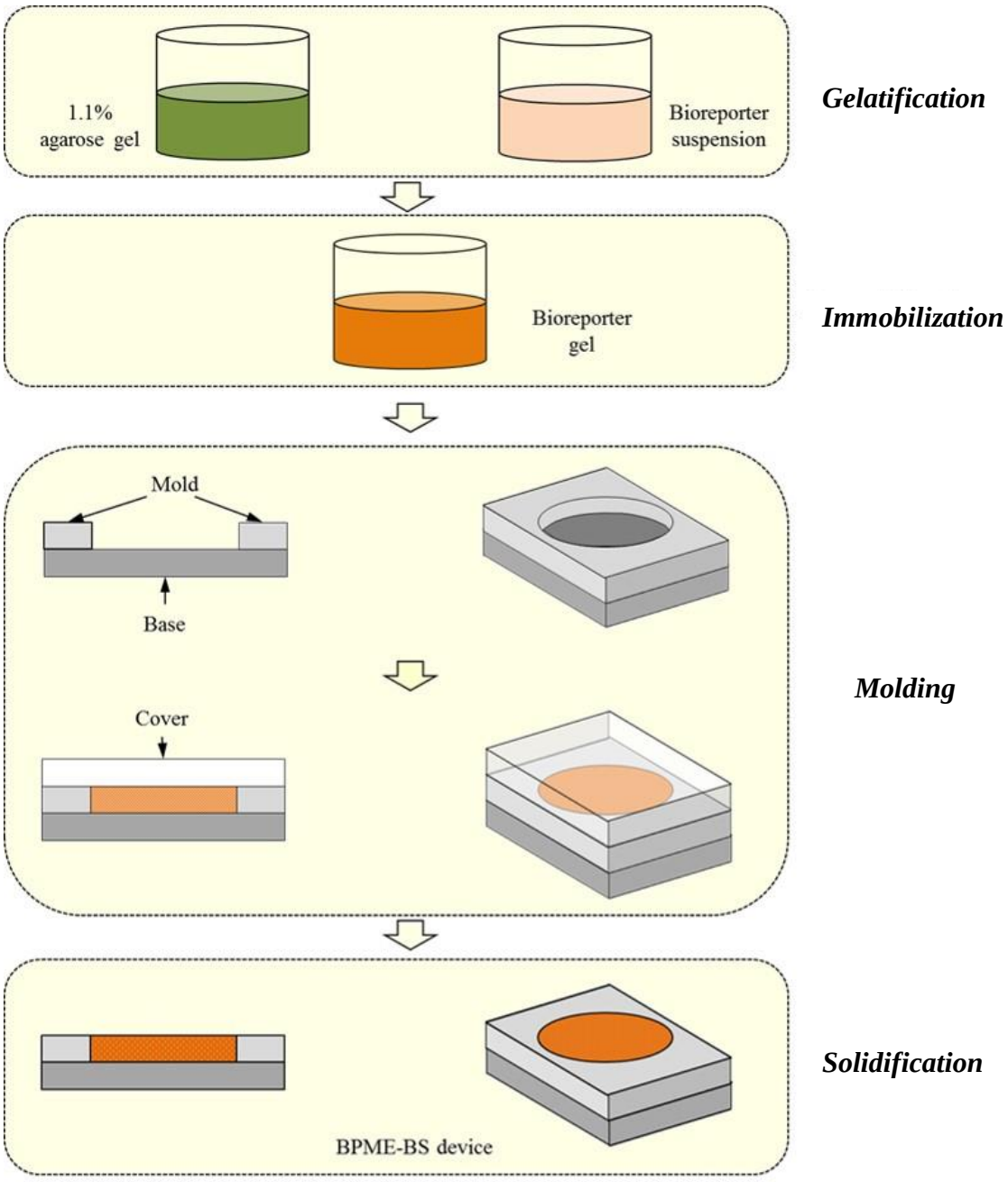



**Figure 5. 1** Schematic assembly of BPMS-BS device, following four steps as *gelatification*, *immobilization*, *molding* and *solidification*.

### *5.2.3 BPMS-BS device deployment and detection*

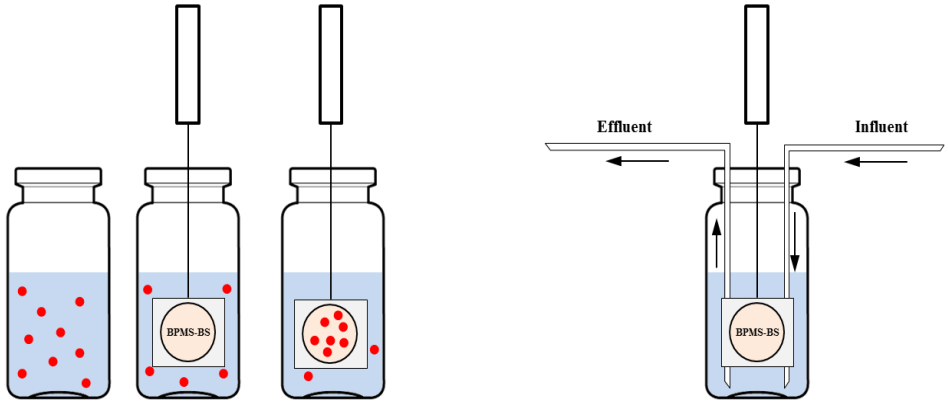
The 1.0 mineral oil (Sigma Aldrich, USA) was dissolved in 100 mL dimethyl sulfoxide (DMSO), and seriously diluted in DMSO to the final concentrations 5,000, 1,000, 500, 100,

50, 10, 5, and 1 mg/L. The alkane stock solutions were prepared by dissolving 1.0 mL oil-DMSO solution in 99 mL deionized water, and the final alkane concentrations were 100, 50, 10, 5, 1, 0.5, 0.1, 0.05 and 0.01 mg/L. The blank control was prepared by adding 1 mL DMSO in 99 mL deionized water.

The alkane enrichment and response was tested in static system (Figure 5.2.A). The BPME-BS device was deployed in 100 mL alkane stock solution with different concentrations. After 1hour deployment, the n-alkane concentrations in suspension before and after deployment were analyzed by GC-FID to calculate the enrichment factor. The BPME-BS devices were then fixed in each well of a 6-well clear-bottom microplate (Corning, USA) for biological signal detection. After adding 10 mL mineral medium with 20 mM sodium succinate as the sole carbon source, the BPME-BS devices were incubated at 30ºC for 6 hours, and the bioluminescent signals (relative luminescent unit, RLU) of each well were measured every 10 min using the microplate reader (FLUOstar Omega, BMG Labtech, UK). To test the uniform of bioluminescent signals of BPME-BS device, the scanning mode was carried out to map the bioluminescence distribution, with 900 (30×30) points detected on each agarose gel. The bioluminescent scanning was applied every 1 hour. For the quantification of alkane concentration and impacts of environmental variants on BPME-BS device performance, a kinetic measurement was employed and bioluminescent signals were collected every 10 minutes. The calculation of bioluminescence response ratio followed out previous methods (Zhang et al., 2013, Zhang et al., 2012b). Briefly, the induced bioluminescence was calculated by the average of bioluminescent measurements between 180 and 210 min. The bioluminescence response ratio was calculated by dividing the induced bioluminescence by the original bioluminescence (time = 0 min), and the relative bioluminescence response ratio was calculated by dividing the induced bioluminescence (samples) by that of the blank control.

In simultaneous system, BPME-BS device was deployed in a chamber with 100 mL deionized water (Figure 5.2.B), continuously injected with artificial water sample at the rate of 1 mL/min. The artificial oil contamination was simulated by pulse addition of alkane stock solution (final concentration 50 mg/L). The whole system was placed within the microplate reader platform and the bioluminescent signals were measured every 1 min for

150 hours and the 5.0 mL of effluent water samples were collected every 4 hours for chemical analysis.




(A) (B)

**Figure 5. 2** Static (A) and simultaneous (B) system for detecting alkane concentrations in water samples via BPME-BS device.

### *5.2.4 Impacts of environmental variants on BPME-BS performance*

To test the impacts environmental variants on the performance and stability of BPME-BS device, we tested the response of BPME-BS device under different pH, temperature and salinity conditions. The pH was adjusted by adding 1.0 M NaOH or HCl solution to the final values of 4.0, 5.0, 6.0, 7.0, 8.0, 9.0 and 10.0. The deployment temperature ranged from 10°C to 50°C. Water samples with different salinity (0%, 1%, 2%, 3%, 4% and 5%) were prepared by adding concentrated NaCl solution in to alkane stock solutions. In all the treatments, the alkane concentrations were 50 mg/L.

The BPME-BS device was stored at 4°C and tested for its response to water samples after long-term storage every 10 days for 60 days. The BPME-BS device was directly deployed in water samples containing 50 mg/L alkanes, and the measurement followed the same procedure in static detection.

### *5.2.5 Chemical analysis*

Determination of alkane content followed the hexane extraction method. One microlitre 5α-cholestane was spiked as a surrogate standard in 1 mL of each water sample. Afterwards, the samples were added with 10 mL hexane and ultrasonically homologized for 2 min (40 kHz). The hexane fraction was further collected and evaporated in 40°C water bath and re-dissolved in 1.0 mL hexane. The internal standard solution was tetracosane ($C_{24}D_{50}$) at 50 mg/L (Fryirs et al., 2014). The analysis was carried out with a gas chromatography flame ionization detector (GC-FID). The 1 μL of sample was injected into a Hewlett Packard gas chromatograph GC 5890 coupled with a flame ionization detector 5971A. The GC was equipped with a capillary column DB 5MS (60 m × 0.2 mm × 0.32 μm, J&W Scientific). The temperature program was set as 1 min at 35ºC, then a progressive increase to 310ºC at a rate of 10ºC/min and finally 10 min at 310ºC.

### *5.2.6 Data analysis*

For each treatment, three individual replicates were carried out. All the statistical analysis was performed by SPSS 17.0. One-way ANOVA and least significant difference (LSD) test were employed in the analysis of statistical significance of differences and variance ($p$-value<0.05) of n-alkane concentrations in different treatments. The quantitative regression between biological signals of BPME-BS device and n-alkane concentration was calculated by the gene expression model as described previously (Zhang et al., 2012d, Al-Anizi et al., 2014b, Zhang et al., 2012b).

## 5.3 Results and Discussions

### *5.3.1 Performance of alkane bioreporter immobilization*

During *immobilization* process, the viability of whole-cell bioreporter ADPWH_alk was significantly affected by the temperature of agarose gel. From Figure 5.1, similar bioluminescent signals of BPME-BS gel were obtained when the immobilization temperature was 45ºC and 50ºC. They were less than 10% lower than the signals of bioreporter cells without immobilization, showing high cell viability in these treatments. With the increasing immobilization temperature, the bioreporter viability significantly

declined. At 100ºC, there was no bioluminescent signal, showing that all the bioreporter cells were dead. As a soil bacterium, *Acinetobacter baylyi* is extremely robust to environmental variation, including temperature. It was reported to maintain high viability and responsive sensitivity within a wide range of temperatures. Immobilization temperature is an important factor for BPME-BS device manufacturing. Higher temperature helps easier homologous mixture of bioreporter suspensions and agarose solution, contributing to better uniformity of the entire gel. However, it significantly reduced the cell viability and damaged the responsive sensitivity from our data. On the contrast, the agarose gel is easier to become solidification at lower temperature. Considering both sides, 50ºC in the present study behaved well in both maintaining bioreporter viability and easier solidification for device manufacture. It was then selected as the optimal immobilization temperature for the following experiment.

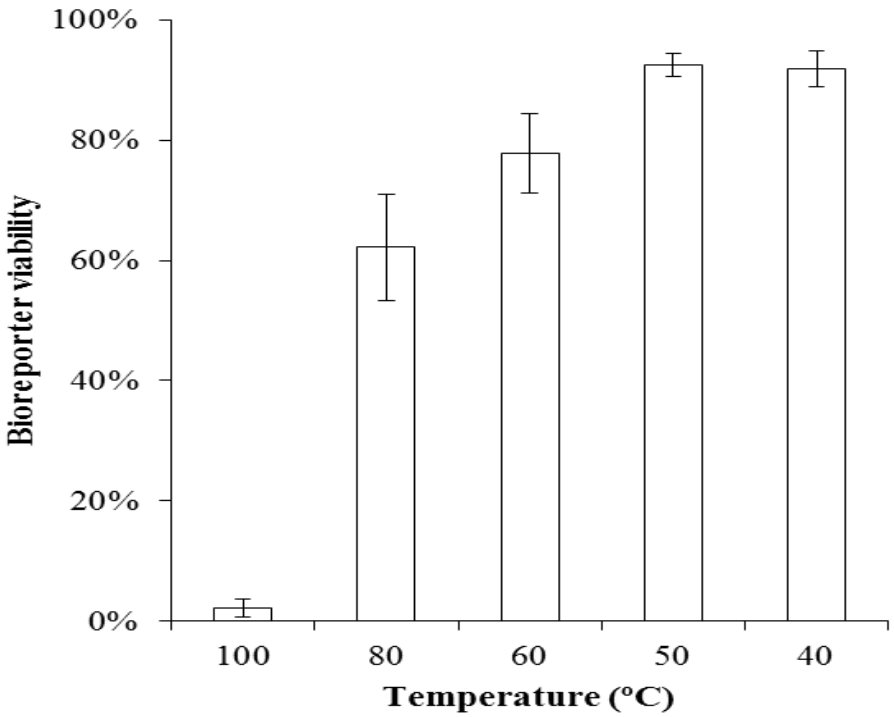



**Figure 5. 3**. Bioreporter viability under different immobilization temperature.

Exposed to alkane solutions, BPME-BS device showed positive bioluminescent response

and the bioluminescent signals were highly uniformly distributed on the gel throughout the induction time (Figure 5.4.). No significant bioluminescent signals were detectable in the negative control (the agarose gel device without bioreporter cells immobilization). The extremely low bioluminescent background ranged from 0 to 300 RLU, similar as the wells without any device and indicating that the bioluminescent signal came from the bioreporter, not the agarose gel. In absence of alkanes (blank treatment), BPME-BS device did not show significant bioluminescent response and the bioluminescent intensity was less than 2,000 RLU throughout the experiment. After exposure to 50 mg/L alkanes, BPME-BS device showed significantly stronger bioluminescence, averagely 23,535±2,620 RLU after 6 hours induction. Compared to the blank, the relative response ratio ranged from 9.8 to 13.6, similar as the response ratio of ADPWH_alk to 50 mg/L alkanes without immobilization.

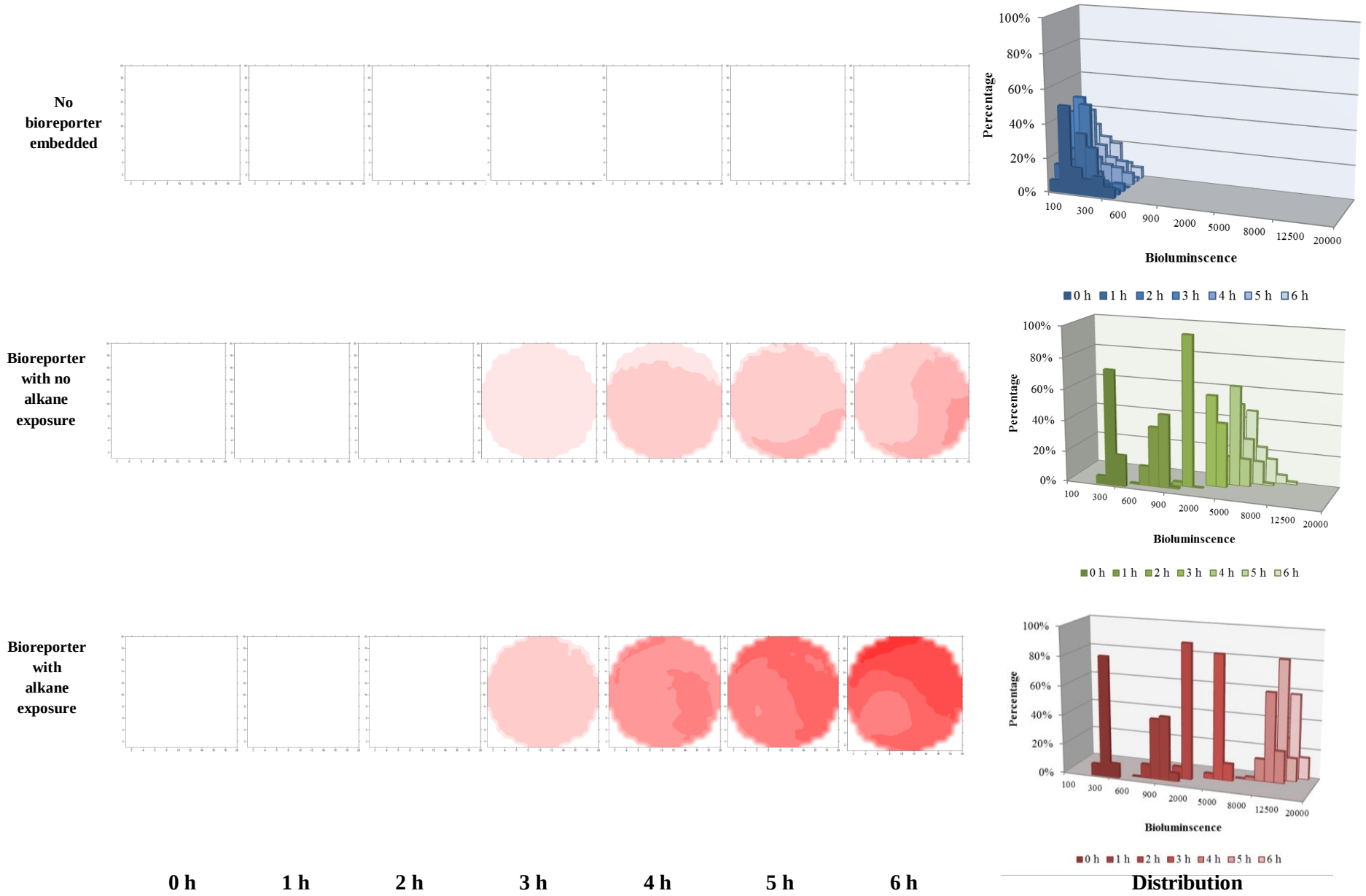


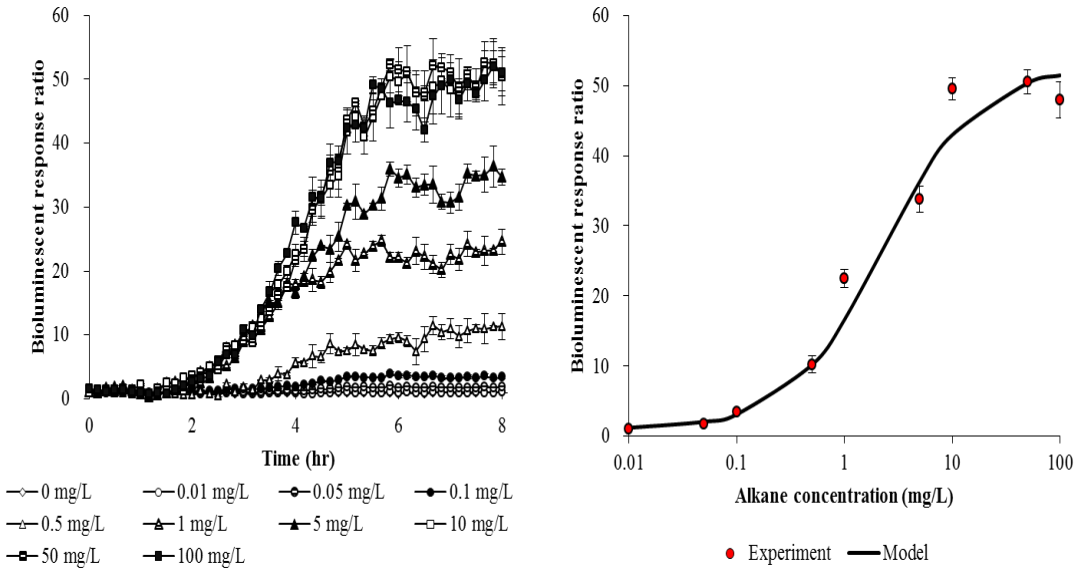

Figure 5. 4. **Bioluminescent signal dynamic and distribution on BPME-BS device**

The device with no bioreporter embedded showed extremely low bioluminescent background (<300 RLU), indicating the biological signal comes from bioreporter. The device with bioreporter did not show significant bioluminescent response in absence of alkanes (<2,000 RLU after 6 hours exposure), whereas a significant response was only observed for the BPME-BS device after exposure to alkanes (>20,000 RLU after 6 hours).

Meanwhile, the bioluminescent signal of all the treatments (negative, blank, and 50 mg/L treatments) followed the normal distribution pattern on the film (Figure 5.4). A higher deviation was observed when exposure to 50 mg/L alkanes, but the total bioluminescence on the BPME-BS device showed relatively even across the film, indicating the bioreporter ADPWH_alk were evenly immobilized within the agarose gel of BPME-BS device.

### *5.3.2 Limit of detection and quantitative response of BPME-BS device*

The real-time bioluminescent curve of BPME-BS device was illustrated in Figure 5.5.A. The bioluminescent signals of blank control and positive treatment were not of significant difference within the first 2 hours. The bioluminescence of BPME-BS ranged from 1,000 RLU to 2,000 RLU in this period, and the response ratio was less than 1.2 for all the treatments. It is reported that ADPWH_alk bioreporter needed about 30 to 60 minutes to active and response to the external alkanes. Thus within the first 2 hours, the *alkB* gene was not activated and the bioluminescent signal represented the baseline and viability of whole-cell bioreporter cells immobilized within the BPME-BS device. The significantly increasing bioluminescence from 2 hours to 6 hours, indicating the activated expression of *alkB* gene which is triggered by the recognition of alkane molecules by AlkM regulator. Meanwhile, the increasing rate of bioluminescent response ratio was positively correlated with the concentration of alkanes, showing the potential of quantitatively measuring alkane concentration in environmental samples. After 6 hours induction, the bioluminescent signals became saturated, indicating the saturated expression of *alkB* gene.




(A) (B)

**Figure 5. 5** (A) Bioluminescent response dynamics of BPME-BS device exposure to different alkane concentrations. Alkane concentrations ranged from 0 mg/L to 100 mg/L. (B) Quantitative response of BPME-BS device to alkanes.

Red dot and black line represent the experimental data and model simulation, respectively.

Attributing to the stable bioluminescent response ratio between 6 to 8 hours, it was used for the regression relationship between response ratio and alkane concentrations. From the gene regulation model (Zhang et al., 2012d, Al-Anizi et al., 2014b, Zhang et al., 2012b), the saturated transcription rate ($\alpha_m$) was 51.6 $s^{-1}$·$cell^{-1}$, and the stimulus binding rate ($K_1$ ) was 0.434. Compared to other gene regulation intensity and specificity (Zhang et al., 2012d, Al-Anizi et al., 2014b, Zhang et al., 2012b), the recognition of alkanes by AlkM regulator is of high specificity and the activation is strong to be a bioreporter sensing alkane molecules. Also based on such gene regulation model, the regression line (Figure 5.5B) illustrated the correlation between bioluminescent response ratio and alkane concentration, which could be used for the calculation of alkane amount in environmental samples.

It was interesting that the significant response was observed when the concentration of mineral oil was 0.05 mg/L or above, comparing to the blank treatment. The limit of detection was significantly improved by comparing to the direct application of

ADPWH_alk bioreporter (0.5 mg/L) (Zhang et al., 2012b). It was explained by the active sensing and accumulation of alkanes by ADPWH_alk cells within the agarose gel.

### *5.3.3 Alkane enrichment factor and limit of detection*

The whole-cell bioreporter ADPWH_alk has the capability to search for and accumulate alkanes attributing to alkane chemotaxis. After immobilized in BPME-BS device, a significant enrichment of alkanes was observed, as illustrated in Figure 5.6 The average enrichment factor for all the alkane with different chain length was 3.8, ranging from 3.4 ($C_{10}$ dodecane) to 5.5 ($C_{12}$, dodecane). It is worth noting that the enrichment factor was dependent on the carbon chain length. It has been reported previously that the alkane chemotaxis of *A. baylyi* to alkanes has high selectivity to the number of carbon chain lengths. The *cheY1* and *cheY2* genes in *Alcanivorax dieselolei* are triggered by $C_8$-$C_{24}$ n-alkanes and alkanes longer than $C_{24}$, respectively(Wang and Shao, 2014). Our results here could be explained by such mechanism that the bioreporter ADPWH_alk has higher chemotaxis towards linear alkanes with carbon chain length from 12 to 21, in which range a higher enrichment was observed.

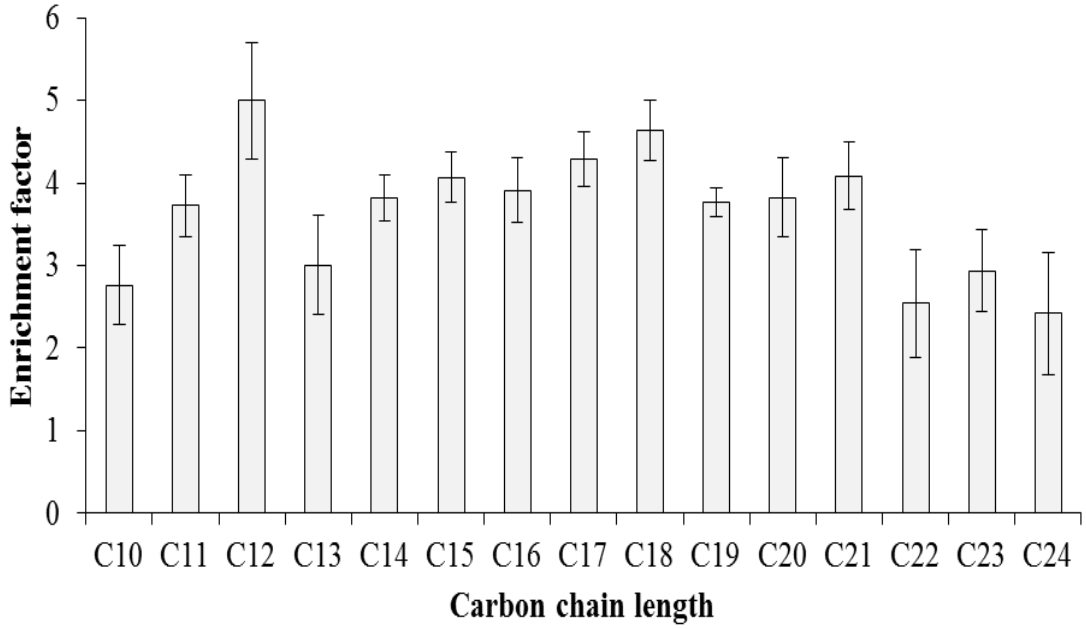



**Figure 5. 6** The enrichment factor of BPME-BS device towards alkanes of different carbon chain length.

### *5.3.4 High tolerance to environmental variants*

As mentioned above, *Acinetobacter baylyi* is a soil bacterium, and ADPWH_alk therefore can tolerate the environmental variations. Accordingly, BPME-BS maintained the detection sensitivity and bioluminescent response ratio under different pH, salinity and temperature condition. Though the bioluminescent responses were weak under extreme low or high pH (pH=4.0 or 10.0), BPME-BS device achieved stable and high response towards 50 mg/L mineral oil when pH values ranged from 5.0 to 9.0 (Figure 5.7A). The results were similar to previous work (Li et al., 2009). Fig. 5.7B also illustrated the good responsive performance of BPME-BS device at 20°C (relative bioluminescent response ratio=20.1), 30°C (relative bioluminescent response ratio=25.8) and 37°C (relative bioluminescent response ratio=23.9). The reduction of bioluminescent response at 10°C and 40°C was attributed to the less bacterial activities at higher or lower temperatures, in accordance with previously reported optimal response range of *A. baylyi* bioreporters. The response was even weaker under extreme temperature conditions (10°C or 40°C). Our data showed that salinity did not significantly affect the response sensitivity of BPME-BS device to alkanes when the salinity was no higher than 3%, and the relative bioluminescent response ratios were above 20.0 (Fig. 5.7C). It gradually declined at higher salinity level, indicating that BPME-BS device had high reproducible response in most of environmental samples, e.g. marine water (salinity around 3.5%). The wide range of optimal response of the BPME-BS device in the present study hinted its potential application in real environmental samples without specific pre-treatment.

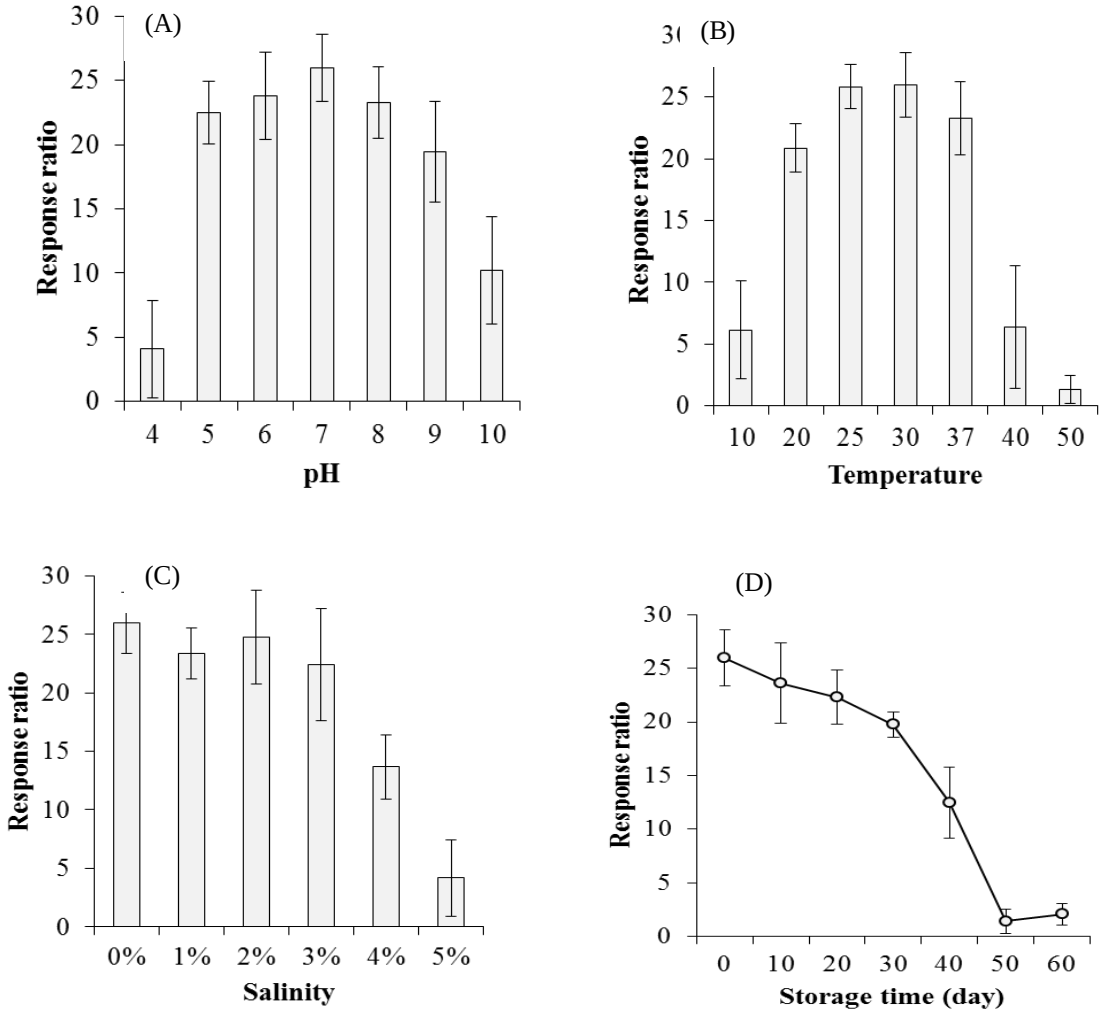



**Figure 5. 7**. The response and performance of BPME-BS device under different pH (A), temperature (B) and salinity (C). The response ratio of BPME-BS device after different storage time (D) showed its feasibility as commercial device.

BPME-BS device also maintained high activity and responsive sensitivity after long-term storage (Fig. 5.7D). Stored in deionized water at 4ºC, the response ratio of BPME-BS device had a slight decreasing trend after the first 30 days and the remaining responsive sensitivity was above 90% of original ones (Figure 5.7D). Afterwards, the bioluminescent response ratio had a dramatic decline, indicating longer storage not suitable for alkane detection. Our results suggested that BPME-BS device could be directly applied for alkane measurement without any pre-treatment, and the relative bioluminescent response ratio was above 20.0 for waters with 50 mg/L mineral oils. The life-time of BPME-BS device in the

present study was the same to the reported ones of original *Acinetobacter* based bioreporters (Zhang et al., 2012a, Song et al., 2009b), indicating that agarose gel immobilization had minimal impacts on the bacterial activities and was an appropriate approach to expand its application in water contamination assessment.

### *5.3.5 Simultaneous detection of alkanes*

BPME-BS device achieved real-time and reliable response to alkanes simultaneously for 150 hours (7 days), as illustrate in Figure 5.8. The bioluminescent signals of BPME-BS device were simultaneously measured by the microplate reader, converted to the continuous alkane concentration in water based on regression curve the in Figure 5.8. The red lines therefore illustrated the real-time alkane content measured by BPME-BS device, and the data fitted well with the results of chemical analysis (GC-FID, blue dot in Figure 5.8). The dramatic increase of bioluminescent response was observed after the injection of water samples with alkane contamination, and gradually declined due to the diluted alkane concentrations in the chamber. A relatively stable peak response after each injection indicated the stability of BPME-BS device response to the same concentration of alkanes, and the continuous measurement lasted for over 7 days, showing the feasibility of applying BPME-BS device for online monitoring. It was worth mentioning that, although GC-FID chemical analysis could also provide accurate data of alkane pulse contamination, the gap between each sampling and measurement missed some contamination, such as injection 3 (30 hours), injection 4 (44 hours), injection 6 (72 hours) and injection 7 (96 hours). The results showed the limitation of chemical analysis, which was determined by the sampling time point and could not provide the high resolution of alkane contamination along with time. Therefore, the BPME-BS device provided a supplementary tool for online monitoring of alkane contaminated water samples, although less accurate but illustrating the alkane dynamics in real time.

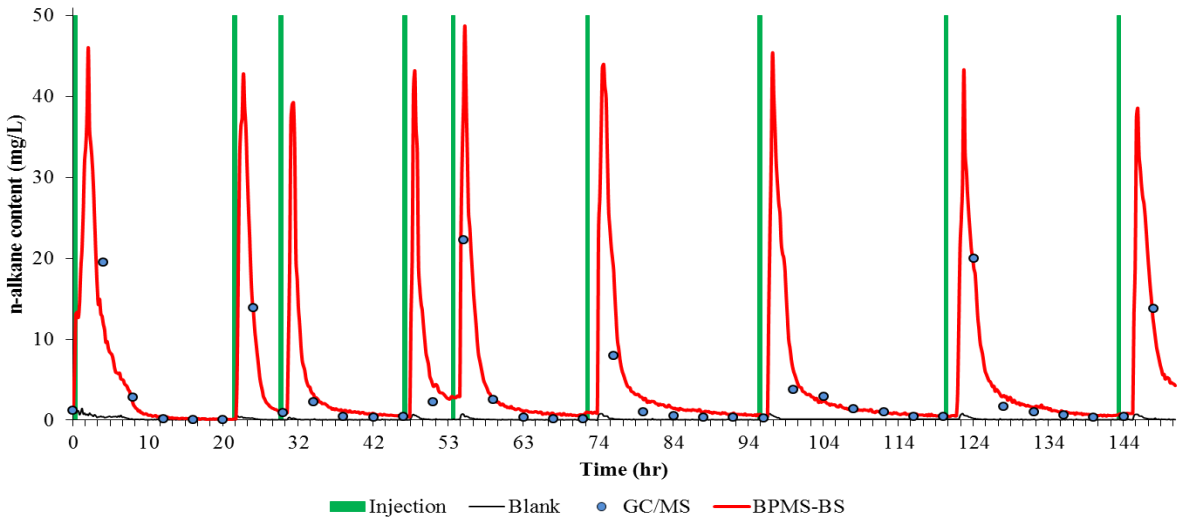



**Figure 5. 8**. Simultaneous response of BPMS-BS device to water samples with pulse alkane contamination.

Blue bar indicated the pulse alkane contamination by injection. Black and red lines represented the alkane concentrations in water samples which were calculated from the bioluminescent response of BPME-BS device. Blue circle referred to the alkane results of GC-FID analysis which was carried out every 4 hours.

## 5.4 Conclusion

In this study, the developed BPME-BS device achieves high enrichment of alkanes, and can effectively respond to the dynamics of influent alkane. Such enrichment has significantly improved the limit of detection from 0.5 mg/L by directly applying alkane bioreporters to 0.05 mg/L in BPME-BS device. We also identified the dose-effect between alkane concentration and bioluminescent response. This BPME-BS device also has high tolerance to environmental variants, including temperature, pH and salinity, and its responsive reproducibility remained satisfied after 30 days storage. All these features indicate that BPME-BS device is reproducible, sensitive and feasible for the measurement of alkanes in water samples. More interestingly, our simultaneous test proves that BPME-BS device can produce real-time bioluminescent signals, representing the dynamic alkane concentrations. It shows the huge potential as low-cost and online device to monitor crude oil spill, suitable for rapid response to management for oil spill incidents.

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# 6 Separating and characterizing functional alkane degraders from crude oil contaminated-sites via magnetic nanoparticle-mediated isolation (MMI)

This chapter aims to develop a new method investigating functional n-alkane degraders in the natural soil microcosm with n-alkane mixtures as carbon sources.

.The specific objectives are:

- Introduce the application of the novel magnetic nanoparticle-mediated isolation (MMI) technology in the Nigerian soil.
- To evaluate the oil degradation process in the soils and access the microbial response for alkane mineralization
- To study the alkane degradation process by bioremediation, and potentially identify indigenous micro-organisms that can aid bioremediation through various carbon source introduction.
- To overcome the challenge of using traditional function and sequence-based approaches to reveal the in situ ecological functions of those uncultivable microorganisms, where the function-based cultivation cannot effectively isolate those microbes and the sequence-based method brings unpredictability from a huge database without accurate allocation of their functions.

## Abstract

Uncultivable microorganisms account for over 99% of all the species on the planet, but their functions are yet not well characterized. Though many cultivable degraders for n-alkanes have been intensively investigated, the roles of functional n-alkane degraders remain hidden in the natural environment. This study introduces the novel magnetic nanoparticle-mediated isolation (MMI) technology in Nigerian soils and successfully separates functional microbes belonging to the families *Oxalobacteraceae* and *Moraxellaceae*, which are dominant and responsible for alkane metabolism *in situ*. The *alkR*-type n- alkane monooxyygenase genes, instead of *alkA*- or *alkP*-type, n-alkane were the key functional genes involved in the n-alkane degradation process. Further physiological investigation via BIOLOG PM plate revealed some carbon (Tween 20, Tween 40 and Tween 80) and nitrogen (tyramine, L-glutamine and D-aspartic acid) sources promoting microbial respiration and n-alkane degradation. With further addition of promoter carbon or nitrogen sources source, the separated functional alkane degraders significantly improved n- alkane biodegradation rates. This suggests that MMI is a promising technology for separating functional microbes from complex microbiota, with deeper insight into their ecological functions and influencing factors. The technique also broadens the application of BIOLOG PM plate for the physiological research on the functional yet uncultivable microorganisms.

**Key words:**

n-Alkane; Biodegradation, Magnetic nanoparticle-mediated isolation (MMI); Uncultivable microorganisms; Functional alkane degraders; BIOLOG PM plate

## 6.1 Introduction

Many environmental hazardous chemicals have been released into the environment through various industrial activities. With the industrial development and urbanization process, increasing use of crude oil has consequently caused numerous oil spill accidents and contaminated sites. Since 1969, there have been over 40 large oil spill accidents throughout the world, such as the Exxon Valdez oil spill in Prince William Sound in 1989 (Bence et al., 1996, Bragg et al., 1994), the Deepwater Horizon oil spill in the Gulf of Mexico (Camilli et al., 2010) and the Xingang oil spill in Dalian in 2010 (Zhang et al., 2013). These resulted in large areas of oil contaminated sites, affected ecological systems and threatened human health (Peterson et al., 2003, Piatt et al., 1990).

Many microbes are involved in the natural n-alkane degradation process, under either aerobic or anaerobic conditions (Van Beilen et al., 1994, Jobson et al., 1972, Becker and Dott, 1995, Berthe-Corti and Fetzner, 2002, Hamamura et al., 2008, Heiss-Blanquet et al., 2005). The identified n-alkane degraders include *Acinetobacter* (Lal and Khanna, 1996, Fondi et al., 2013), *Alcaligenes* (Lal and Khanna, 1996), *Alcanivorax* (Hara et al., 2003, Kasai et al., 2002, Sabirova et al., 2006, Schneiker et al., 2006), *Arthrobacter* (Radwan et al., 1996), *Geobacillus* (Feng et al., 2007), *Bacillus* (Kato et al., 2001, Chaerun et al., 2004), *Brachybacterium* (Yan, 2006), *Burkholderia* (Yuste et al., 2000), *Desulfatibacillum* (Cravo-Laureau et al., 2004, Cravo-Laureau et al., 2005), *Dietzia* (von der Weid et al., 2007, Yumoto et al., 2002), *Geobacillus* (Wang et al., 2006), *Gordonia* (Kotani et al., 2003), *Marinobacter* (Doumenq et al., 2001, Bonin et al., 2004), *Mycobacterium* (Churchill et al., 1999, van Beilen et al., 2002), *Paracoccus* (Chaerun et al., 2004, Zhang et al., 2004), *Planococcus* (Engelhardt et al., 2001), *Pseudomonas* (Koch et al., 1991, Naik and Sakthivel, 2006), *Rhodococcus* (van Beilen et al., 2002, Kunihiro et al., 2005, Andreoni et al., 2000) and *Thermooleophilum* (Zarilla and Perry, 1984). They are widely distributed in hydrocarbon-polluted or non-polluted environments, with essential roles in n- alkane degradation. Meanwhile, alkane monooxygenases encoding genes vary widely among these alkane degraders, although they all share considerable sequence homology (Jurelevicius et al., 2013). One type of *alkB* gene from *Pseudomonas* (Smits et al., 2002, Chaerun et al.,

2004) and *Rhodococcus* (Amouric et al., 2010) encodes alkane monooxygenases metabolizing short- or medium-chain n-alkanes with a carbon chain length from 14 to 20. *Rhodococcus* is capable of degrading $C_7$ to $C_{20}$ n-alkanes, with *alkB1/alkB2* nucleotide sequences sharing high similarity to *alkB* (Razak et al., 1999). In addition, *Acinetobacter* has a different *alkM* gene for utilizing n-alkanes from $C_{13}$ to $C_{44}$ (Pleshakova et al., 2001, DiCello et al., 1997, Lal and Khanna, 1996), and its n-alkane oxidation capacity is higher for medium- and long-chain alkanes (Tanaka et al., 2010, Kennedy et al., 1975) than for short-chain ones (Bajpai et al., 1998). Other research also identified various alkane hydroxylase genes with different sequences identities from in pure cultured strains. Such diverse alkane monooxygenase-encoding genes involved in alkane metabolism therefore cause an underestimation of the alkane biodegradation pathway in the natural environment and are attracting increasing academic attention.

To understand the behavior of n-alkane degradation, both sequence- and function-based approaches have been attempted. Sequence-based techniques include denaturing gradient gel electrophoresis (DGGE), the 16S rRNA clone library and metagenomics high-throughput sequencing (Muyzer et al., 1993). All these molecular tools provide new opportunities for interpreting and diagnosing the characteristics of microcosms in natural environments (Tringe et al., 2005). Lindstrom et al. reported declining microbial diversity with long-term crude oil contamination (Lindstrom et al., 1999), and the relative abundance of n-alkane degraders (*Rhodococcus*, *Sphingomonas* and *Pseudomonas*) was significantly increased (Aislabie et al., 2004). In marine sediment, oil contamination also affects microbial community structure and function, consequently resulting in increased oil metabolizing activities and decreased diversity of the microbial population (Powell et al., 2003, Yakimov et al., 2005). It is also reported that geographic locations determine functional or species diversity within bacterial communities at oil-contaminated sites (Maila et al., 2006, Liang et al., 2011), and contamination type and history significantly affect the community and population of soil microorganisms, leading to less microbial diversity and functions in heavily-contaminated soils than in those with light contamination (Cheung and Kinkle, 2001, Liang et al., 2015). Function-based approaches focus cultivation and physiological behavior of n-alkane degraders, or soil enzymatic activities, to

investigate the ecological functions and responses of soil microbes to n-alkane contamination (Juck et al., 2000). For instance, *Pseudomonas* (Al-Saleh and Akbar, 2015) and *Rhodococcus* (Sorkhoh et al., 1990) are characterized as the most common cultivable n-alkane degraders in soil. The correlation between microbial diversity degradation and their physiological functions in crude-oil-contaminated soils has been successfully explained by BIOLOG phenotype assay (Lindstrom et al., 1999). The dynamics of soil the microbial population, community composition and enzymatic activities also reveal the response of the microbial community to crude oil contamination during the degradation process (Parrish et al., 2005). By directly analyzing the functions and phenotypic behaviour of alkane degraders, bioaugmentation and biostimulation have been applied as cost-effective and environmentally friendly methods to improve biodegradation performance by adding exogenous degrading strains or growth substances (Lin et al., 2016), such as electron acceptors (oxygen supply) and nutrients (nitrogen and phosphorus substrates) (Jackson and Pardue, 1999).

Most microorganisms (>99%) are uncultivable under laboratorial conditions, but functional in natural environments (Kaeberlein et al., 2002). They play key roles in the natural carbon and nitrogen cycle but their physiology is hard to investigate, especially that of n-alkane degraders. It is of great challenge when using traditional function- or sequence-based approaches to reveal the *in situ* ecological functions of uncultivable microorganisms, where function-based cultivation cannot effectively isolate those microbes and the sequence-based method is unpredictable due to a huge database without accurate allocation of their functions. Stable Isotope Probing (SIP) is a promising technique investigating functional-yet-uncultivable microbes (Radajewski et al., 2000). The biomass (DNA, RNA or protein) of functional-yet-uncultivable microbes becomes heavier during the metabolism of stable isotope-labeled ($^{13}C$ or $^{15}N$) and can be further separated by the difference in buoyant density (Zhang et al., 2015b). Numerous degraders of phenolic compounds and polycyclic aromatic hydrocarbon (PAHs) have been identified via SIP at crude- oil -contaminated sites, including *Burkholderia*, *Alcanivorax* and *Cycloclasticus* (Uhlik et al., 2012, Song et al., 2015). Nevertheless, SIP has a challenge, since the $^{13}C$ labeled substrate is very expensive and the dosage is normally single pure chemical instead

of mixtures (Chen and Murrell, 2010). In most environmental degradation cases, multi-contaminants exist at the contaminated sites. Particularly for alkane degradation, the complicity of n-alkane composition in the natural environment strongly restricts the applicable feasibility of SIP. Magnetic-nanoparticles-mediated isolation (MMI) is a recently developed method for separating living functional microbes from complex microbiota (Zhang et al., 2015b). After being functionalized with magnetic nanoparticles (MNPs) and dosed with targeted carbon sources, the living active degraders gradually divide and ultimately lose their magnetic attraction, whereas inert bacteria remain silent and their magnetism is constant. Therefore, functional microbes can be effectively be separated by magnetic field from the whole microbiota. In this way, the MMI technique does not rely on substance labeling and can be used in microcosms with multiple carbon or nitrogen sources. More importantly, the separated bacterial cells are still alive and suitable for further physiological investigation, providing more comprehensive information on microbial diversity and ecological functions.

To address these challenges, this research aims to develop a new method investigating functional n-alkane degraders in the natural soil microcosm with n-alkane mixtures as carbon sources. Via magnetic separation of living n-alkane degraders, the present study focused on their phenotype and n-alkane degradation performance by the BIOLOG PM plate. To the best of our knowledge, this is the first successful identification of functional n-alkane degraders from soils that reveals their phenotypic behavior, and the enhancing of n-alkane degradation efficiency with the additive of extra nitrogen sources.

## 6.2 Materials and methods

### *6.2.1 Site and sample collection*

The crude-oil-contaminated site is located in Delta State, Nigeria (N 7º15’16.9”, E 4º41’23.95”). Five national crude oil drilling wells are distributed within 5 km of the site and there have been intensive oil exploration activities since the 1980s. With a long history of crude oil contamination caused by drilling wells and pipeline spillages, severe cases of crude oil contamination have been observed, and the average n-alkane content in the research area is about 2.0% (w/w). The soil samples were collected on June 14, 2015.

During the collection, the surface soils (0-10 cm) were gently removed to avoid the impacts of human activities and disturbance. A total of 500 g of soils from a depth of 10-20 cm were collected, sieved to remove plant debris and stones, and finally stored at 4 ºC before further analysis.

### *6.2.2 MNPs synthesis and targeting soil functionalization*

The synthesis of MNPs followed previous instructions (Zhang et al., 2011b). One mL $FeCl_2$ (1.0 M) and 2 mL $FeCl_3$ (2.0 M) were gently mixed, with further drop-by-drop addition of 25 mL NaOH (2.0 M). After continuous shaking for 30 min, the synthesized dark nanoparticles were harvested by a magnet for 10 min and washed by 30 mL deionized water several times until neutral pH value (7.0). The synthesized MNPs concentration was 9.1 g/L.

To test the soil magnetic-functionalization efficiency and optimize soil magnetism for effective separation, the 1.0 mL synthesized MNPs were mixed with soils of weights from 0.06 mg to 17,700 mg. After gently shaking for 5 min, the magnetic functionalized soils were harvested by a permanent magnet for 10 min. A quantitative polymerase chain reaction (qPCR) was used to quantify the bacterial concentration in the supernatant (bacterial 16S rRNA copy numbers in magnetic-free fraction, $BC_{MF}$ for short, copies/mL) and magnetic soil pellet ($BC_{MS}$, copies/mL). The soil magnetic functionalization efficiency was calculated as the ratio of the bacterial amount in magnetic soil pellet to the total amount ($BC_{MS}$/ $BC_{MF}$ + $BC_{MS}$). Here, 100% soil magnetic functionalization efficiency indicates that all soil bacteria are successfully magnetically functionalized ($BC_{MF}$ = 0 copies/mL), and 0% refers to no soil bacteria with magnetism ($BC_{MS}$ = 0 copies/mL).

From the curve of soil magnetic functionalization efficiency (Figure 6.1), the MNP-functionalized soil samples were prepared by mixing 500 mg soil (dry weight) and 0.91 mg MNPs as the optimal condition for n-alkane biodegradation treatments.

### *6.2.3 Alkane biodegradation treatments*

For n-alkane biodegradation, the soil samples were spiked with/without 2% (w/w) mineral oil (Sigma Aldrich, UK) and thoroughly mixed well. The five treatments included $HgCl_2$ (0.1%)-treated soils with mineral oil (sterile control), original soils without mineral oil

(CKN), original soils with mineral oil amendment (CKP), MNP-functionalized soils without mineral oil (MNPN) and MNP-functionalized soils with mineral oil amendment (MNPP). All treatments were carried out in biological triplicates and the microcosms were incubated at room temperature for 40 days. Around 2.0 g of soils were collected at Day 5, 10, 20, 30 and 40 for chemical and biological analysis directly in CKN and CKP treatments. To evaluate the in situ phenotype of separated n-alkane degraders in MNPN and MNPP treatments, we prepared the sterile oil extraction solution by adding 1.0 g original soils in 10 mL deionized water and passing through a 0.45 μm filter. The 0.45 μm filter aimed to remove most of the soil particles and living bacterial cells in the soil suspension. Some small bacterial cells might still remain in the aqueous phase, but their impact on oil degradation was minimal from our BIOLOG tests. To separate magnetic-free cells (MFCs), 2.0 g of soil samples from MNPN and MNPP treatments at each sampling time point were further suspended in the sterile soil extraction solution and the MFCs were separated from the inert microbes (magnetic pellets) by a magnet and marked as MFCN for MNPN treatment and MFCP for MNPP treatment.

### *6.2.4 DNA extraction, amplification and sequencing*

The soil and MFCs DNA was extracted via PowerSoil DNA extraction kit (MOBIO, USA) in accordance with manufacturer's instruction. Targeting DNA amplification was amplified by polymerase chain reaction (PCR). The primer pairs and thermos cycling program for 16S rRNA and n-alkane degrading functional genes were listed in Table 6.1 (Herlemann et al., 2011, Smits et al., 1999, Kuhn et al., 2009, Marchant et al., 2006). The three pairs of primers for n-alkane monooxygenase gene (alk_A, alk_P and alk_R) followed previous research to characterize the n-alkane microbial profiles (*Acinetobacter*, *Pseudomonas* and *Rhodococcus*, respectively) in soils following previous protocols (Jurelevicius et al., 2013). These three types of alkB genes shared considerable sequence homology, but varied in different species with phylotypic differences. The 50 μL PCR reaction system contained 2 μL deoxynucleotide triphosphates (dNTPs, 5 mM), 2 μL of each primer (5 mM), 1 μL DNA template, 0.3 μL Dream Taq DNA polymerase (Fermentas, UK), and 37.7 μL, ultrapure water (molecular biology grade, Sigma Aldrich, UK).

**Table 6. 1** Primers and amplification programs.

| Target | Name | 5'-3' | Heating | | | Amplification | | | | | | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | Denaturation | | Annealing | | Extension | | |
| | | | Temp (ºC) | Time (s) | Cycle | Temp (ºC) | Time (s) | Temp (ºC) | Time (s) | Temp (ºC) | Time (s) | |
| **Total bacteria** | alk_RF/alk_RR | CCTACGGGNGGCWGCAG/TACNVGGGTATCTAATCC | 95 | 240 | 30 | 95 | 45 | 40 | 60 | 72 | 300 | (Herlemann et al., 2011) |
| **n-alkane monooxygenase gene** | alk_PF/alk_PR | ATCTGGGCGCGTTGGGATTTGAGCG/CGCATGGTGATCGCTGTGCCGCTGC | 94 | 180 | 30 | 94 | 60 | 45 | 60 | 72 | 60 | (Smits et al., 1999) |
| | alk_AF/alk_AR | GCICAIARITIRKICAYAA/GCITGITGITCISWRTGICGYTG | 94 | 180 | 30 | 94 | 60 | 58.5 | 30 | 72 | 30 | (Kuhn et al., 2009) |
| | alk_RF/alk_RR | GGTACGGSCAYTTCTACRTCGA/CGGRTTCGCGTGRTGRT | 94 | 180 | 34 | 94 | 45 | 52 | 45 | 72 | 45 | (Marchant et al., 2006) |

Quantification of 16S rRNA and n-alkane monooxygenase genes (alk_A, alk_P and alk_R) was determined by qPCR. The 20 μL qPCR system was consisted of 2 μL of each primer, 1 μL DNA template, 5 μL molecular water and 10 μL iTaq™ Universal SYBR® Green Supermix (BioRad, USA). Standard curves were obtained with serial dilutions of quantified plasmid DNA (via nanodrop) containing the fragment of 16S rRNA and alkB genes. The qPCR programs were the same as the above PCR programs, except for the extra fluorescence data acquisition at 80ºC for 15 s in each cycle.

To determine the microbial community structure in soils and MFCs, the extracted DNA were sequenced with PCR amplicon libraries of the hypervariable V3, V4 and V6 region of the 16S rRNA genes (Annoroad Gene Technology Co., Ltd, Beijing, China.).. Pyrosequencing was carried out by an Illumina HiSeq4000 with the average reads length of 450 bp after PEAR alignment (Zhang et al., 2014). All the reads passed the quality filtering, and the reads were discarded if the barcodes were uncorrectable, the bases with Phred Quality Score <19 covered above 30% of the read, or the ambiguous bases were over 5%. Sequences were assigned to operational taxonomic units (OTUs) with a 97% pairwise identity as the threshold, and then classified taxonomically by the Greengenes 16S rRNA reference database. The distance matrices from samples were generated by the Bray-Curtis metric and visualized by principal coordinates analysis (PCoA) by QIIME (Quantitative Insights Into Microbial Ecology) software.

### *6.2.5 Community substrate utilization analyses*

Biolog PM plates (Biolog, USA) were used to examine the carbon and nitrogen metabolism of MFCs in treatments with MNPs-functionalized soils. The 150 μL MFCs were added into each well of the PM1 (95 carbon sources) and PM3 (95 nitrogen sources with additional 500 mg/L mineral oil as the sole carbon source), supplemented with 1.5 μL Biolog Redox Dye Mix A (100×). The plates were incubated at 25ºC for 48 hours and the color development was read every 15 min as absorbance by a multimode microplate reader (FLUOstar Omega, BMG Labtech, UK) at 590 nm wavelength (Hueso et al., 2012). The data were collected and further analyzed by MARS software (BMG Labtech, UK).

### *6.2.6 n-alkane chemical analyses*

Determination of n-alkane content in soils followed the hexane extraction method. All the soil samples were freeze-dried and each 1 gram of soils was spiked with 1 mL 5α-cholestane as a surrogate standard. Added with 10 mL hexane, the soil-hexane mixture was ultrasonically homologized for 2 min (40 kHz) and the supernatant was further fractionalized by column chromatography (Tang et al., 2010). The glass column ($\Phi$10 mm × 100 mm) was consisted of 2 cm anhydrous $Al_2O_3$, and 0.3 cm anhydrous $Na_2SO_4$ from the bottom to the top. Pre-washed with hexane, the column was loaded with soil-hexane supernatants and washed with 20 mL of hexane. The collection was then evaporated in 40 °C water bath and re-dissolved in 1.0 mL hexane. The internal standard solution was tetracosane ($C_{24}D_{50}$) at 50 mg/L (Fryirs et al., 2014).

Analysis of the extracts was carried out using a gas chromatography flame ionization detector GC-FID. The 1 μL of sample was injected into a Hewlett Packard gas chromatograph GC 5890 coupled with a flame ionization detector 5971A. The GC was equipped with a capillary column DB 5MS (60 m × 0.2 mm × 0.32 μm, J&W Scientific). The temperature program was set as 1 min at 35ºC, then a progressive increase to 310ºC at a rate of 10ºC/min and finally 10 min at 310ºC.

The n-alkane residues in Biolog PM assay, there was a technical problem in our lab when applying hexane extraction for high-throughput extracting and analyzing alkanes in a small volume of water sample in each well (150 μL). We therefore used alkane whole-cell bioreporter, ADPWH_alk (Zhang et al., 2012b) to detect n-alkane concentrations after degradation. This alkane bioreporter had a detection range from 0.1 mg/L to 100mg/L. After cultivation in lysogeny broth medium at 30ºC overnight, the ADPWH_alk bioreporter cells were washed by deionized water and resuspended in minimal medium with 20 mM succinate as the sole carbon source (Zhang et al., 2013, Zhang et al., 2012b). The 50 μL solution from each well of Biolog PM3 (95 nitrogen sources) was mixed with 150 μL ADPWH_alk suspension, and added into the wells of 96-well black & clear-bottom microplate (Corning, USA) with three replicates. Incubated at 30ºC for 6 hours, the bioluminescent signal was measured every 10 min using a FLUOstar Omega microplate

reader (BMG Labtech, UK). The induced bioluminescence was calculated by the average of bioluminescent measurements between 180 and 210 min. The bioluminescence response ratio was calculated by dividing induced bioluminescence by the original bioluminescence (time = 0 min), and the relative bioluminescence response ratio was calculated by dividing the induced bioluminescence (samples) by that of the control (non-induced). The residual n-alkane concentration was evaluated by the gene expression model (Zhang et al., 2012d) and the calibration curve (Zhang et al., 2012b) as described previously.

### *6.2.7 Statistical analysis*

All the statistical calculations were performed by SPSS 17.0. One-way ANOVA and least significant difference (LSD) test were employed in the analysis of statistical significance of differences and variance ($p$-value<0.05) of n-alkane residuals and 16S/alkane-monooxygenase gene copies in different treatments. Correlation analysis between microbial respiration level and n-alkane degradation rate was conducted with significant level less than 0.05.

## 6.3 Results and discussion

### *6.3.1 Optimal condition of soil microcosm functionalization with MNPs*

Both soil microorganisms and particles are predominantly negatively charged, which resulted in the strong electrostatic interaction with positively charged MNPs (Xu et al., 2014). This study investigated the optimal weight ratio of soil to MNPs (ranging from 0.066 to 19,500, w/w) to achieve both high magnetic functionalization efficiency and minimal dosages. The residual bacterial counts were quantified by qPCR and Figure 6.1 shows the magnetic functionalization efficiency maintained over 99.5% when the ratio of soil to MNPs suspension was less than 1100 (w/w). Beyond the critical point, the magnetic functionalization efficiency dramatically declined to only 90.88% (soil:MNPs = 5300, w/w) and 16.65% (soil:MNPs = 19,500, w/w), due to the excessive negative soil particles or bacterial cells in the system.

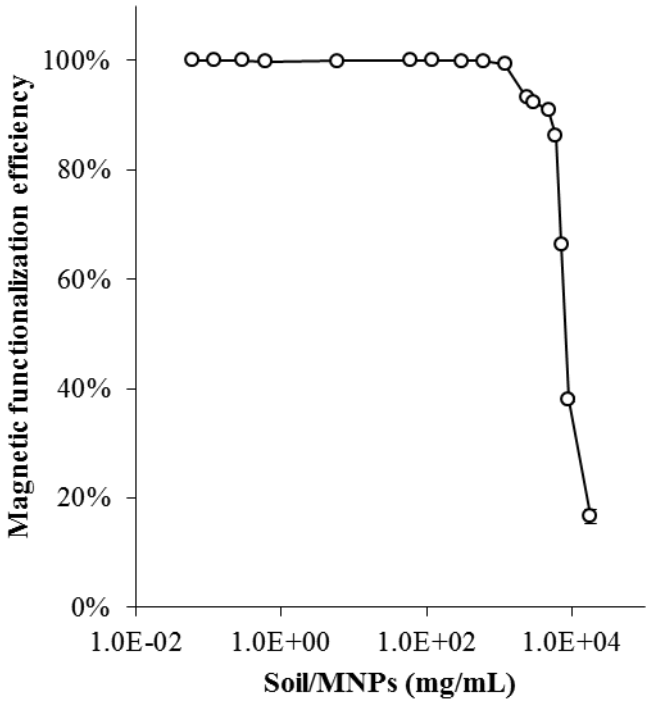
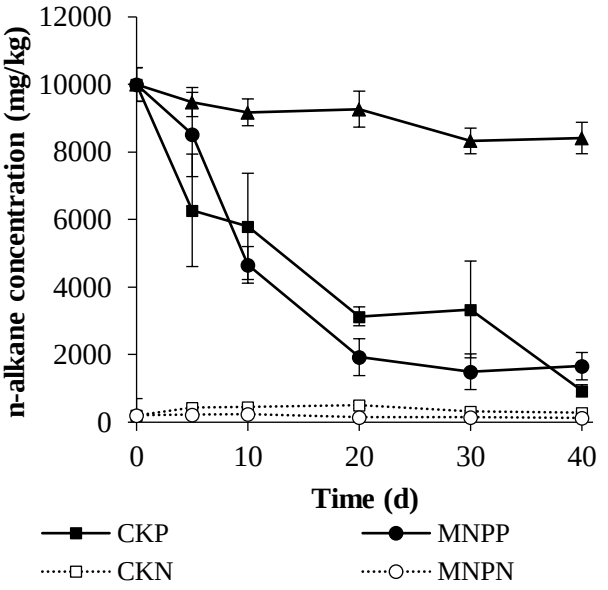



Figure 6. 1 Soil magnetic-functionalization efficiency against the ratio of soil to MNPs suspension (from 0.066 - 19,500, w/w).

The functionalization of bacterial cells by MNPs was attributed to the electrostatic interaction between MNPs and the carboxyl(-COOH)/thiol(-SH)/amine(-$NH_2$) functional groups on bacterial cell membrane (Lin et al., 2015). Since these functional groups are universal for all bacterial cells, the non-selective adhesion ensures that all bacterial species can be effectively functionalized with magnetism. The optimal condition for further n-alkane biodegradation treatment was therefore set as 500 mg soil (dry weight) and 0.91 mg MNPs (0.1 mL suspension).

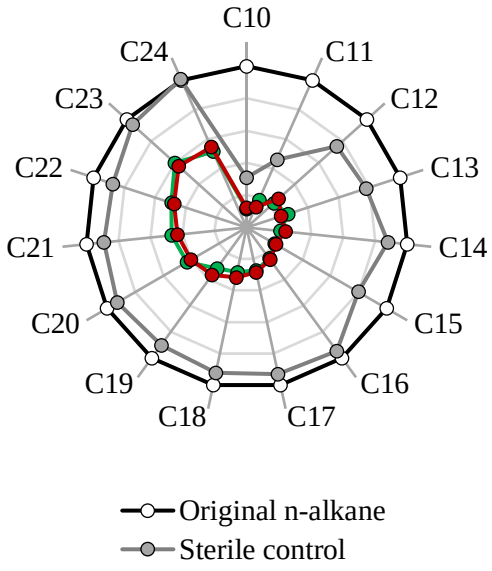
### *6.3.2 The degradation of n-alkanes in soils*

After 40 days of incubation, the n-alkanes were significantly degraded by soil microbes, as illustrated in Figure 6.2.




A

Figure 6. 2A. The n-alkane degradation curve in soils functionalized with/without MNPs. CKN (□) and CKP (■) represent the original soil treatments with/without n-alkane amendment. MNPN (○) and MNPP (●) refer to the treatments of MNPs-functionalized soils without/with n-alkane amendment. Change of individual n-alkanes with specific carbon chain length. B. The abundance of each n-alkane ($C_{10}$ – $C_{24}$) is normalized as 100% for original mineral oil.




B

The concentration of n-alkanes showed a slight decrease (<83%) with time in the sterile control, whereas significantly higher degradation efficiencies were achieved in all n-alkane-amended treatment (CKP and MNPP treatments, *p*-value <0.05). There was no significant difference between the n-alkane degradation rates in soils with/without MNPs functionalization (MNPP and CKP treatments, *p*-value<0.05), indicating MNPs did not affect the bacterial activities or n-alkane degradation performance (Zhang et al., 2015b). Dramatic n-alkane degradation was observed in the first 20 days, when the n-alkane degradation efficiency was 68.6% and 80.7% in CKP and MNPP treatments, respectively. Afterwards, n-alkane degradation was slowed down and n-alkane degradation efficiency achieved 90.7% and 83.4% in CKP and MNPP treatments respectively, after 40 days degradation. The results of GC-FID (Figure 6.3) illustrated the change in individual n-alkanes with specific carbon chain length. In the sterile control, $C_{10}$ and $C_{11}$ alkanes had the lowest residual ratio (30.9% and 46.2%) due to their higher vapour pressure. About 70% - 90% of $C_{12}$ - $C_{15}$ medium-chain alkanes and over 90% of alkanes with carbon chain length > 16 remained in the soil. For both CKP and MNPP treatment, the removal efficiency for short- and medium-chain alkanes have higher solubility and degradation rates than long-chain alkanes and they might favor bacterial metabolism. Therefore, the slower alkane degradation rates after 20 days might possibly be attributed to declining alkane solubility and degradation rates in soils. Our results were similar to previous research on aerobic alkane biodegradation (Chaineau et al., 2005), but significantly higher than anaerobic alkane degradation (Caldwell et al., 1998, Hasinger et al., 2012). From the n-alkane biodegradation curve, the soil DNA was extracted on Day 20 and Day 40, representing the rapid and slow degradation step to address the respective profiles of microbial community structure and ecological functions.

### *6.3.3 Microbial community responsible for n-alkane degradation*

Bar-coded pyrosequencing generated 220,584 quality sequences from the 13 samples, from 13,066 sequences in MFCP_40 to 29,231 reads in MFCN_20. At the 97% similarity level, a total of 2176 phylotypes were defined. The Original soil sample (NC) and samples without n-alkane addition (CKN_20, CKN_40, MNPN_20 and MNPN_40) had the largest number

of phylotypes detected from 1,122 to 1,244. The phylotypes in samples with n-alkane degradation were significantly lower (1,045 in CKP_40 to 739 in MFCP_40). Significant declining alpha diversity was observed during the n-alkane degradation process, wherein the Shannon-index ranges from 7.8-8.2 in original soil samples (NC) or those without n-alkane amendment (CKN_20, CKN_40, MNPN_20 and MNPN_40) to 6.4 in soils with n-alkane degradation after 40 days (CKP_40), and as low as 5.8 in the MFC fractions showing that microbial diversity with n-alkane degradation (MFCP_20 and MFCP_40). Our results fitted well with previous findings showing that microbial diversity and functions declined after n-alkane contamination and during the bioremediation process that followed (Powell et al., 2003, Yakimov et al., 2005, Cheung and Kinkle, 2001).

Cluster analysis of the relative abundance of bacteria at the family level was illustrated in Figure 6.4(A), representing microbial diversity in soil samples amended with/without n-alkane at different time point. Of all the classifiable sequences, 25 phylotypes were the most dominant at the family level and accounted for over 70% of all the sequences. In original soil (NC), the key microbes belonged to the families *Nitrospiraceae* (10.3%), *Ellin515* (7.8%), *Solibacteraceae* (5.6%), *Syntrophobacteraceae* (5.2%) and *Koribacteraceae* (4.8%). They were all soil microorganisms with essential roles in soil carbon and nitrogen cycling. There was no significant difference between CKN and MNPN treatments ($p$-value<0.05), indicating no microbial community change in the soils with or without MNP functionalization. Thus, MNP functionalization did not change soil microbial activities or community structure, consistent with previous findings. In treatments without n-alkane addition (CKN_20, CKN_40, MNPN_20 and MNPN_40), a similar microbial community structure was observed, showing the constant microbial diversity and population throughout the experiment without n-alkane amendment. These five treatments were therefore within close distance in the Bray-Curtis analysis (Figure 6.4(B)). Directly amended with n-alkanes, the bacterial community composition gradually changed and the dominant microbes in CKP_40 (40 days n-alkane degradation) belonged to *Moraxellaceae* (13.5%) and *Bdellovibrionaceae* (6.2%). *Moraxellaceae* is a common cultivable soil microbe family with the capability of n-alkane metabolism. *Bdellovibrionaceae* is also previously reported with the *alkB* alkane monooxygenase after the oil spill in the Mexico

Gulf (Smith et al., 2013). Our results indicated that they were the cultivable n-alkane degraders in the targeted soils.

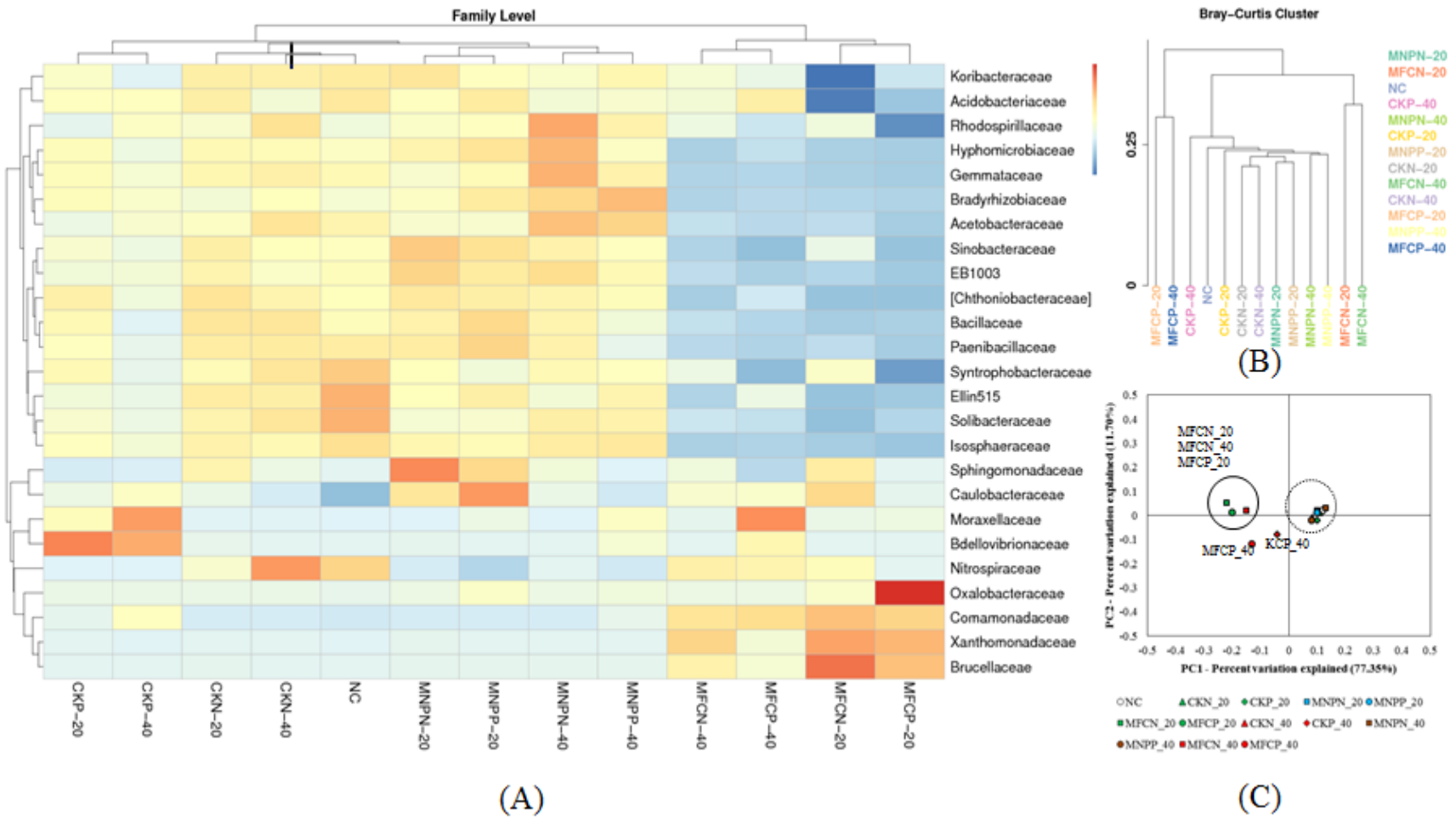



Figure 6. 3 Relative abundance of microbial taxonomic at the family level in the soil samples amended with/without n-alkane at different time point.

(A). Taxonomic assignment was carried out with the Greengenes 16S rRNA database. NC refers to the original soils (Day 0); for all the samples, "_20" and "_40" mean DNA collected on day 20 and 40 respectively; CKN and MNPN represent the soil DNA of the treatments without n-alkane additives; CKP and MNPP the soil DNA of the treatments with n-alkane additives; MFCN and MFCP represent the DNA in magnetic-free cells (MFCs) fraction of the treatments without/with n-alkane additives. No significant difference between CKN, MNPN and MNPP treatments (p-value<0.05), indicating MNPs functionalization did not change microbial activities of n-alkane degradation. Bray-Curtis cluster analysis (B) and PCoA (C) showed that MFCN_20, MFCN_40 and MFCP_20 had similar community structure, all with far distance to MFCP_40, CKP_40 and other soil microcosms. It indicated that the magnetic free fraction microbes kept stable without n-alkane addition, and the increasing abundance of OTUs in MFCP_20 and MFCP_40 was attributed to the utilization of n-alkane. The bacteria belonging to the families Oxalobacteraceae and Moraxellaceae were dominant in MFCs with n-alkane additive (MNPP treatment) on Day 20 and Day 40 respectively, suggesting they were the key soil n-alkane degraders in situ.

It is quite interesting that the microbial diversity of magnetic microbes in soils with MNP functionalization and n-alkane amendment (MNPP_20 and MNPP_40) were similar to CKN_20 and CKN_40 (Figure 6.4(A)). Meanwhile, an entirely different microcosm structure was identified in MFCs, which contained phylotypes belonging to the families *Oxalobacteraceae* (47.6%), *Xanthomonadaceae* (8.6%), *Comamonadaceae* (5.8%) and *Brucellaceae* (5.2%) in MFCP_20 treatment, and *Moraxellaceae* (28.6%) and *Comamonadaceae* (14.6%) in MFCP_40 treatment. All these microbes have been previously reported to have the capacity of metabolizing n-alkanes from the diversity analysis or direct cultivation of soil communities (Yang et al., 2014, Alonso-Gutierrez et al., 2009, Mattes et al., 2008). For the first time in this study, we successfully isolated these living functional n-alkane degraders using a cultivation-independent approach. Our results show that active n-alkane degraders gradually lost their magnetism due to division and remained in MFC fractions. Meanwhile, the remaining microbes in soil microcosm (MNPP_20 and MNPP_40) could not metabolize n-alkanes and maintained magnetism, and

they were therefore effectively captured by permanent magnet and separated from MFC fractions. Their community diversity therefore remained stable and similar to the control treatment. Based on the difference between MFCP_20 and MFCP_40, it is suggested that, during the first 20 days of the fast degradation process, identified *Oxalobacteraceae* were the key functional degraders, followed by the metabolisms of *Moraxellaceae* from Day 20 to Day 40. Considering the change in individual n-alkanes with specific carbon chain length (Figure 6.4(B) *Oxalobacteraceae* hypothetically had preferential utilization of short- and medium-chain alkanes, whereas *Moraxellaceae* might be capable of metabolizing long-chain alkanes. PCoA results in Figure 6.4C provide further evidence that MFCP_40 and CKP_40 were of different community structure, both separated from the other MFC fractions (MFCN_20, MFCN_40 and MFCP_20) and the inert soil samples (NC, CKN_20, CKN_40, MNPN_20, MNPN40, MNPP_20, MNPP_40 and CKP_40).

### *6.3.4 Dynamics of 16S rRNA and n-alkane monooxygenase genes*

The copy numbers of 16S rRNA and n-alkane monooxygenase encoding genes were estimated by qPCR and illustrated in Figure 6.4.

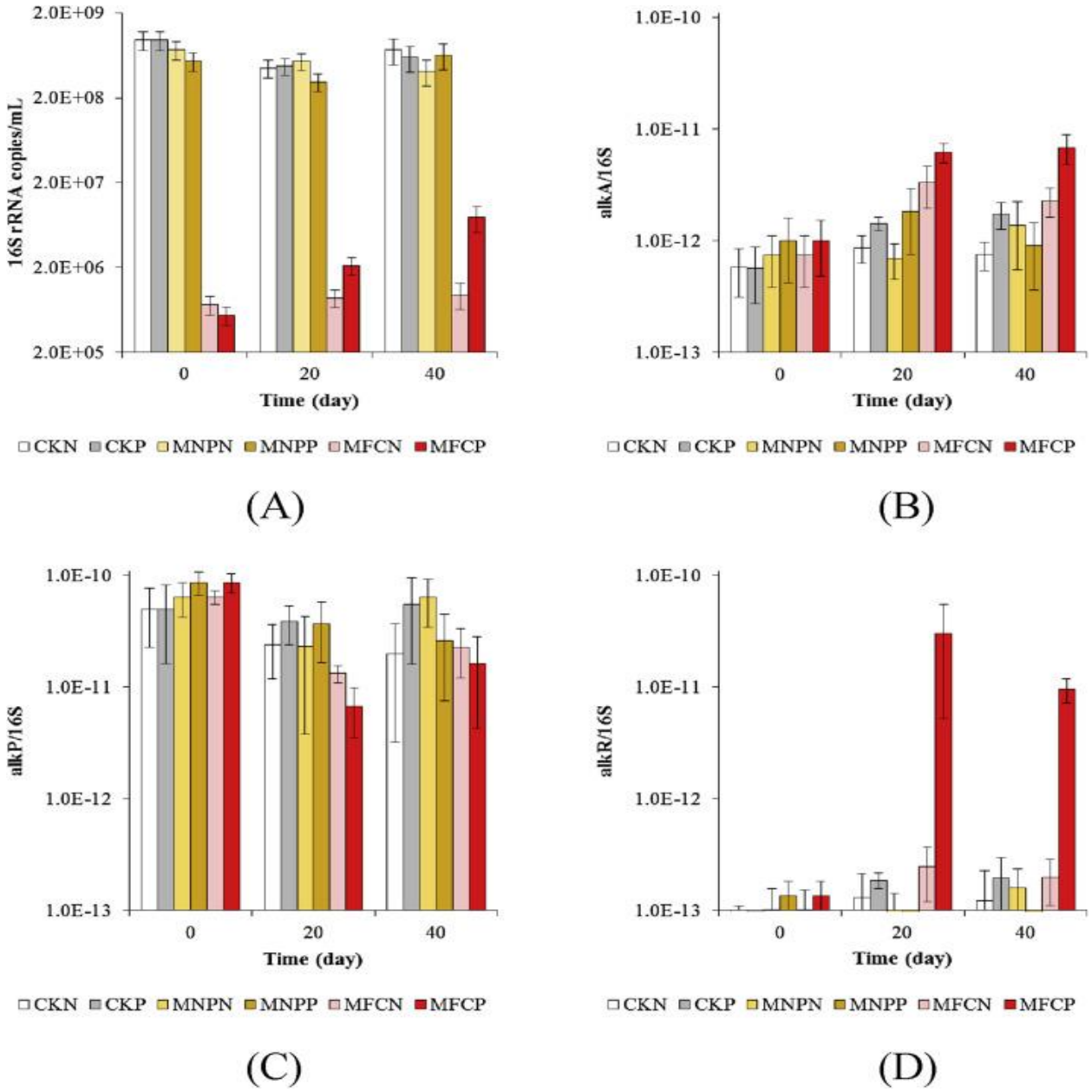



Figure 6. 4 Quantification of 16S rRNA and n-alkane monooxygenase encoding gene level in different treatments. (A): 16S rRNA abundance against cultivation time, where y-axis represents the 16S rRNA copies per mL. (B), (C) and (D): relative abundance of n-alkane monooxygenase encoding gene (alkA/16S, alkP/16S and alkR/16S) against cultivation time.

Throughout the n-alkane degradation process, the relative abundance of 16S rRNA in CKN, CKP, MNPN and MNPP samples were identical and remained at the same level without significant difference (Figure 6.4A, $4.48\times10^{8}$ - $7.40\times10^{8}$ copies/mL, *p*-value>0.05). The

16S rRNA copy numbers of MFCs from MNPN and MNPP treatments were similar on Day 0, ranging from $5.47\times10^5$ - $7.41\times10^5$ copies/mL, accounting for less than 1/1000 of total soil microorganisms. In MFCs from MNPN treatment, there was no significant difference in the abundance of 16S rRNA during cultivation without n-alkane ($7.41\times10^5$ – $9.64\times10^5$ copies/mL, *p*-value>0.05). Results indicated that only limited number of microorganisms could utilize soil residual carbon sources, divide and lose magnetism. With n-alkane additives in MNPP treatments, 16S rRNA abundance increased to $2.11\times10^6$ copies/mL on Day 20 and $7.89\times10^6$ copies/mL on Day 40, showing the growth and dominance functional n-alkanes of n-alkane degraders in MFC fractions.

The relative abundance of three n-alkane monooxygenase encoding gene (*alkA*-, *alkP*- and *alkR*-type) behaved differently during n-alkane degradation process. On Day 20 and Day 40, *alkA*-type genes were significantly higher in CKP treatment than those in CKN treatment (Figure 6.4B, *p*-value<0.05). Compared to MFCN fraction, they also increased in MFCP fraction but only 0.88 (Day 20) and 2.0 (Day 40) times higher, showing their limited roles in n-alkane metabolism. Throughout n-alkane biodegradation, there was no significant difference in the *alkP*-type alkane monooxygenase genes in any of the treatments (*p*-value>0.05 (Figure 6.4C). The results indicated that the microbes with *alkP*-type genes had minimal impacts on n-alkane degradation and they were not the key functional n-alkane degraders in the microcosm. Interestingly, *alkR*-type n-alkane monooxygenase increased significantly and became more predominant in MFC fraction from MNPP treatment (MFCP), as illustrated in Figure 6.4D. Their relative abundance was 123 and 48 times higher in MFCP on Day 20 and Day 40 than those in MFCN. The addition of n-alkane as the sole carbon source clearly encouraged the growth of microbes with *alkR*-type genes and they therefore participated in the n-alkane biodegradation process. In contrast, the relative abundance of *alkR*-type genes was not significantly increased in CKP and MNPP treatments, compared to CKN and MNPN treatments accordingly. This was explained by the rare abundance of functional n-alkane degraders with *alkR*-type genes (around $1.0\times10^{-13}$ copy per 16S rRNA copy) in the original soil microcosms. Their abundance change was not as significant as that in MFCs where only functional n-alkane degraders were enriched and separated.

Most of research on n-alkane degraders in the soil microbial community has addressed the cultivation of n-alkane degraders (Chaerun et al., 2004) or the direct pyrosequencing and qPCR, to analyze the change in community structure and functional gene abundance. The cultivable n-alkane degraders can only effectively metabolize n-alkane under artificial conditions, whereas true functional n-alkane degraders have rare abundance in the microbial community and their change is barely distinguished by a normal pyrosequencing approach. In the present study, *Oxalobacteraceae* and *Moraxellaceae* were identified as the dominant microbes in the MFC fraction with n-alkane as the sole carbon source, and their alkane monooxygenase-encoding genes had high similarity to those of *alkA*- and *alkR*-types (Jurelevicius et al., 2013), respectively. Thus, the significant increase in *alkA*-type genes in CKP and MFCP treatments fitted well with our microbial community analysis, and their enrichment was attributed to the dominance of *Moraxellaceae*. However, the functional *alkR*-type n-alkane monooxygenase genes (belonging to *Oxalobacteraceae*) were only enriched in the MFC fraction, but not the CKP treatment. Results suggested that direct pyrosequencing and qPCR of alkane monooxygenase genes might be misleading us to conclude that only microbes with *alkA*-type genes are key n-alkane degraders *in situ*. Our separation provided more details on the alkane oxidation functional gene dynamics and the MFCs fractions had a higher resolution of quantifying both *alkA*- and *alkR*-type genes due to the enrichment of functional microbes. The unexpected high abundance of *alkR*-type, particularly in MFCP_40 treatment, was not consistent with the relative abundance of *Oxalobacteraceae*. Phylogenetically widespread and genetic mobility of the *alkB* gene is supported by previous studies (van Beilen et al., 2001, Giebler et al., 2013). Here, we make a similar hypothesis that horizontal gene transfer occurred and that the *alkR*-type n-alkane monooxygenase genes were widespread within the soil community.

### *6.3.5 Phenotype analysis of isolated n-alkane degradation microbes*

The sequence-based approach only identifies genetic information of n-alkane degraders, with lack of phenotypic evidence to directly link microbial functions to their identity or solutions providing more information on practical implementation of n-alkane biodegradation. In contrast to direct pyrosequencing of microbial community structure in the soils, our MMI technique has an attractive advantage in that separated functional n-

alkane degraders are still alive and suitable for further ecophysiological analysis. Both BIOLOG high-throughput phenotypic PM01 (carbon sources) and PM03 (nitrogen sources) plates were employed in this study to characterize the phenotypes of separated functional n-alkane degraders and identify key nitrogen sources that might encourage n-alkane biodegradation performance.

MFCs from MNPN and MNPP treatments showed different phenotypic patterns for carbon or nitrogen metabolism (Figure 6.5).


Negative Control
L-Arabinose
N-Acetyl-D-Glucosamine
D-Saccharic acid
Succinic acid
D-Galactose
L-Aspartic acid
L-Proline
D-Alanine
D-Trehalose
D-Mannose
Dulcitol
D-Serine
D-Sorbitol
Glycerol
L-Fucose
D-Glucuronic acid
D-Gluconic acid
DL-a-Glycerol Phosphate
D-Xylose
L-Lactic acid
Formic acid
D-Mannitol
L-Glutamic acid
D-Glucose-6-Phosphate
D-Galactonic acid-g-Lactone
DL-Malic acid
D-Ribose
Tween 20
L-Rhamnose
D-Fructose
Acetic acid
a-D-Glucose
Maltose
D-Melibiose
Thymidine
L-Asparagine
D-Aspartic acid
D-Glucosaminic acid
1,2-Propanediol
Tween 40
a-Ketoglutaric acid
a-Ketobutyric acid
a-Methyl-D-Galactoside
a-D-Lactose
Lactulose
Sucrose
Uridine
L-Glutamine
m-Tartaric acid
D-Glucose-1-Phosphate
D-Fructose-6-Phosphate
Tween 80
a-Hydroxyglutaric acid-g-Lactone
a-Hydroxybutyric acid
b-Methyl-D-Glucoside
Adonitol
Maltotriose
2`-Deoxyadenosine
Adenosine
Gly-Asp
Citric acid
m-Inositol
D-Threonine
Fumaric acid
Bromosuccinic acid
Propionic acid
Mucic acid
Glycolic acid
Glyoxylic acid
D-Cellobiose
Inosine
Gly-Glu
Tricarballylic acid
L-Serine
L-Threonine
L-Alanine
Ala-Gly
Acetoacetic acid
N-Acetyl-D-Mannosamine
Mono-Methylsuccinate
Methylpyruvate
D-Malic acid
L-Malic acid
Gly-Pro
p-Hydroxyphenyl Acetic acid
m-Hydroxyphenyl Acetic acid
Tyramine
D-Psicose
L-Lyxose
Glucuronamide
Pyruvic acid
L-Galactonic acid-g-Lactone
D-Galacturonic acid
Phenylethylamine
2-Aminoethanol
Time (h)
0
10
20
30
40
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0



(A)


Negative Control
L-Arabinose
N-Acetyl-D-Glucosamine
D-Saccharic acid
Succinic acid
D-Galactose
L-Aspartic acid
L-Proline
D-Alanine
D-Trehalose
D-Mannose
Dulcitol
D-Serine
D-Sorbitol
Glycerol
L-Fucose
D-Glucuronic acid
D-Gluconic acid
DL-a-Glycerol Phosphate
D-Xylose
L-Lactic acid
Formic acid
D-Mannitol
L-Glutamic acid
D-Glucose-6-Phosphate
D-Galactonic acid-g-Lactone
DL-Malic acid
D-Ribose
Tween 20
L-Rhamnose
D-Fructose
Acetic acid
a-D-Glucose
Maltose
D-Melibiose
Thymidine
L-Asparagine
D-Aspartic acid
D-Glucosaminic acid
1,2-Propanediol
Tween 40
a-Ketoglutaric acid
a-Ketobutyric acid
a-Methyl-D-Galactoside
a-D-Lactose
Lactulose
Sucrose
Uridine
L-Glutamine
m-Tartaric acid
D-Glucose-1-Phosphate
D-Fructose-6-Phosphate
Tween 80
a-Hydroxyglutaric acid-g-Lactone
a-Hydroxybutyric acid
b-Methyl-D-Glucoside
Adonitol
Maltotriose
2`-Deoxyadenosine
Adenosine
Gly-Asp
Citric acid
m-Inositol
D-Threonine
Fumaric acid
Bromosuccinic acid
Propionic acid
Mucic acid
Glycolic acid
Glyoxylic acid
D-Cellobiose
Inosine
Gly-Glu
Tricarballylic acid
L-Serine
L-Threonine
L-Alanine
Ala-Gly
Acetoacetic acid
N-Acetyl-D-Mannosamine
Mono-Methylsuccinate
Methylpyruvate
D-Malic acid
L-Malic acid
Gly-Pro
p-Hydroxyphenyl Acetic acid
m-Hydroxyphenyl Acetic acid
Tyramine
D-Psicose
L-Lyxose
Glucuronamide
Pyruvic acid
L-Galactonic acid-g-Lactone
D-Galacturonic acid
Phenylethylamine
2-Aminoethanol
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3.0
2.5
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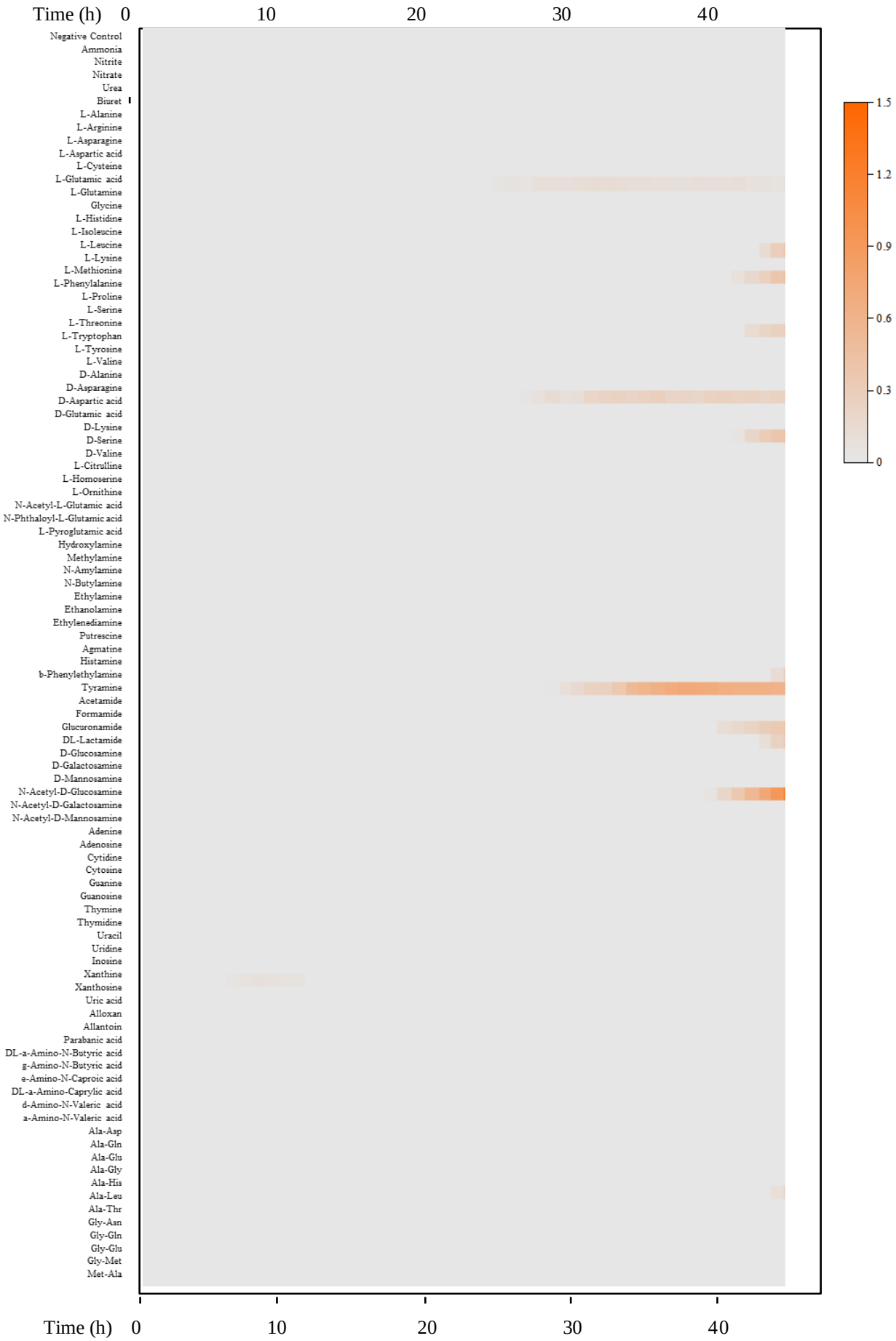
1.5
1.0
0.5
0.0

(B)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Negative Control
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Nitrite
Nitrate
Urea
Biuret
L-Alanine
L-Arginine
L-Asparagine
L-Aspartic acid
L-Cysteine
L-Glutamic acid
L-Glutamine
Glycine
L-Histidine
L-Isoleucine
L-Leucine
L-Lysine
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine
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D-Alanine
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D-Glutamic acid
D-Lysine
D-Serine
D-Valine
L-Citrulline
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N-Phthaloyl-L-Glutamic acid
L-Pyroglutamic acid
Hydroxylamine
Methylamine
N-Amylamine
N-Butylamine
Ethylamine
Ethanolamine
Ethylenediamine
Putrescine
Agmatine
Histamine
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Tyramine
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Xanthine
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Allantoin
Parabanic acid
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g-Amino-N-Butyric acid
e-Amino-N-Caproic acid
DL-a-Amino-Caprylic acid
d-Amino-N-Valeric acid
a-Amino-N-Valeric acid
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Ala-Gln
Ala-Glu
Ala-Gly
Ala-His
Ala-Leu
Ala-Thr
Gly-Asn
Gly-Gln
Gly-Glu
Gly-Met
Met-Ala
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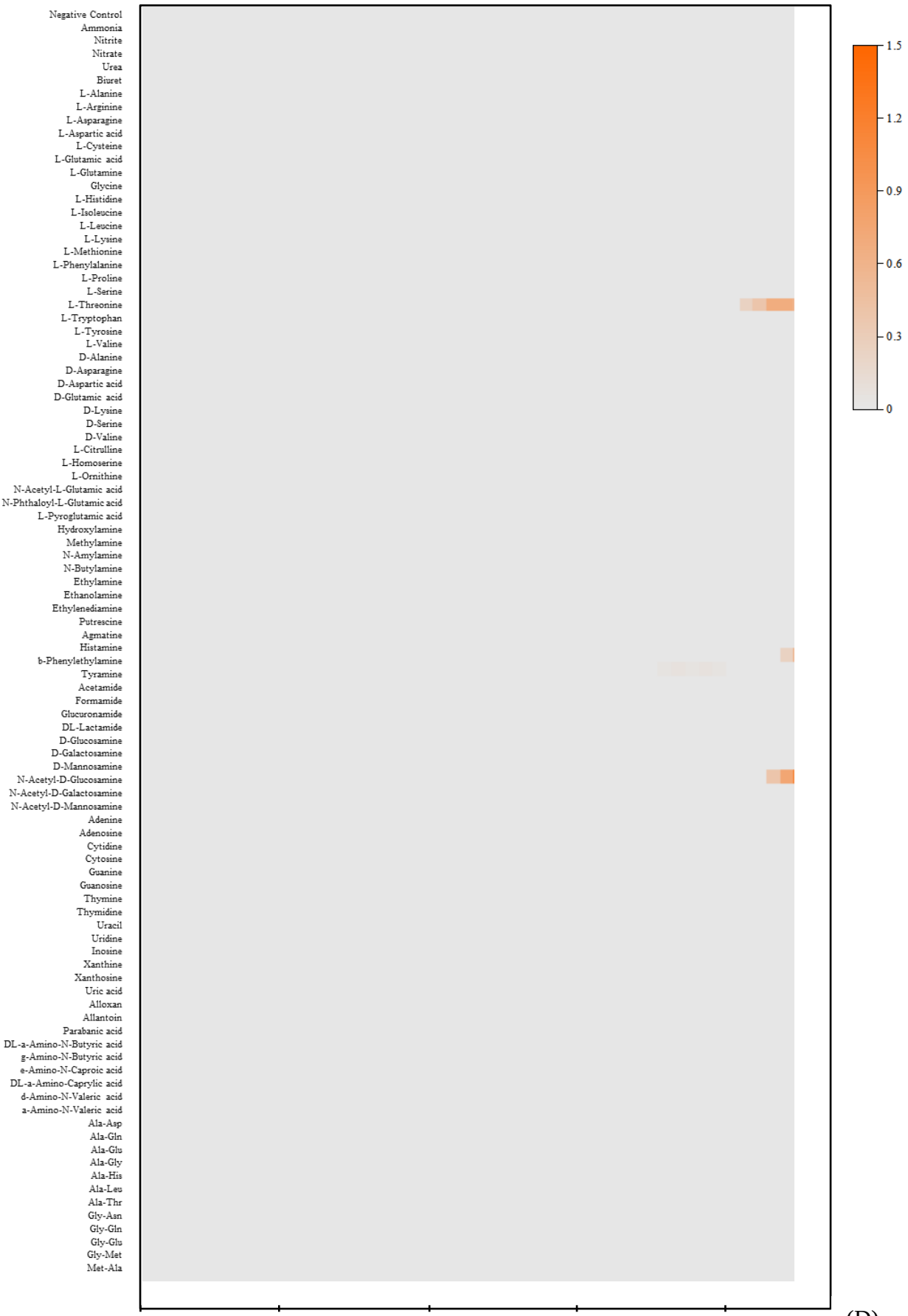
1.2
0.9
0.6
0.3
0
Time (h) 0 10 20 30 40



(C)


Negative Control
Ammonia
Nitrite
Nitrate
Urea
Biuret
L-Alanine
L-Arginine
L-Asparagine
L-Aspartic acid
L-Cysteine
L-Glutamic acid
L-Glutamine
Glycine
L-Histidine
L-Isoleucine
L-Leucine
L-Lysine
L-Methionine
L-Phenylalanine
L-Proline
L-Serine
L-Threonine
L-Tryptophan
L-Tyrosine
L-Valine
D-Alanine
D-Asparagine
D-Aspartic acid
D-Glutamic acid
D-Lysine
D-Serine
D-Valine
L-Citrulline
L-Homoserine
L-Ornithine
N-Acetyl-L-Glutamic acid
N-Phthaloyl-L-Glutamic acid
L-Pyroglutamic acid
Hydroxylamine
Methylamine
N-Amylamine
N-Butylamine
Ethylamine
Ethanolamine
Ethylenediamine
Putrescine
Agmatine
Histamine
b-Phenylethylamine
Tyramine
Acetamide
Formamide
Glucuronamide
DL-Lactamide
D-Glucosamine
D-Galactosamine
D-Mannosamine
N-Acetyl-D-Glucosamine
N-Acetyl-D-Galactosamine
N-Acetyl-D-Mannosamine
Adenine
Adenosine
Cytidine
Cytosine
Guanine
Guanosine
Thymine
Thymidine
Uracil
Uridine
Inosine
Xanthine
Xanthosine
Uric acid
Alloxan
Allantoin
Parabanic acid
DL-a-Amino-N-Butyric acid
g-Amino-N-Butyric acid
e-Amino-N-Caproic acid
DL-a-Amino-Caprylic acid
d-Amino-N-Valeric acid
a-Amino-N-Valeric acid
Ala-Asp
Ala-Gln
Ala-Glu
Ala-Gly
Ala-His
Ala-Leu
Ala-Thr
Gly-Asn
Gly-Gln
Gly-Glu
Gly-Met
Met-Ala
1.5
1.2
0.9
0.6
0.3
0
Time (h)
0
10
20
30
40

(D)

Figure 6. 5 Phenotypic microarray profiling of magnetic free cells (MFCs). Respiration level of the PM01 (carbon sources) plates for MFCs in MNPN (A) and MNPP (B) treatments. espiration level and n-alkane degradation rate of the PM03 (nitrogen sources) plates for MFCs in MNPN (C) and MNPP (D) treatments with n-alkane mixtures as the sole carbon source.

Here, the y-axis represents the 95 carbon or nitrogen sources and the x-axis represents the cultivation time (hours). Shading colour changes from light dark to purple, responsive to the respiration level from 0.0 to 3.5 (PM01 plate) and 0.0 to 1.5 (PM03 plate).

The results of carbon metabolism provided evidence that microbes separated via the MMI technique from MNPN and MNPP treatments were not identical, and this was explained by the addition of n-alkane in MNPP treatments and the enrichment of n-alkane degraders in the MFC fraction. The MFCs from MNPN treatments could effectively utilize 32 carbon sources (Figure 6.5A), 21 of which were able to be utilized by MFCs from MNPP treatment as well (Figure 6.5B). In addition to fumaric acid and mucic acid, MFCs from MNPP treatment gave a stronger metabolism performance on Tween 20, Tween 40 and Tween 80. The three carbon sources have a similar structure of polyoxyethylene sorbitan, but consist of different hydrophobes of laurate (Tween 20), palmitate (Tween 40) and oleate (Tween 80). It was therefore stronly hypothetical that the separated functional n-alkane degraders could possess active lipases and their activities will be further investigated in our future work.

To examine the effects of various nitrogen sources on the n-alkane degradation rate, the sterile soil extraction solution with 500 mg/L n-alkane was used in the PM03 plate for the MFCs from MNPN and MNPP treatments. Figure 6.5C and 6.5D illustrated their different microbial respiration profiles. It was evident that only three nitrogen sources could promote microbial respiration in MFCN, i.e. b-phenylethylamine, tyramine and n-acetyl-D-glucosamine, whereas their n-alkane degradation rate was less than 5%. Without n-alkane addition, the separated MFCN had minimal bacterial cell numbers from qPCR results, and they were not responsible for n-alkane degradation. Microbial respiration might result from the metabolism of residual soil carbon sources or cell debris, instead of utilizing n-alkanes. For the MFCP, the seven nitrogen sources improved respiration levels included L-

phenylalanine, D-serine, b-phenylethylamine, tyramine, glucuronamide, DL-lactamide and n-acetyl-D-glucosamine. With these nitrogen sources, the n-alkane degradation rates were all above 10%. Accordingly, there were ten nitrogen sources that promoting n-alkane degradation with the degradation rate over 20% within 48 h, including L-glutamine, L-histidine, L-phenylalanine, L-proline, D-aspartic acid, tyramine, glucuronamide, n-Acetyl-D-glucosamine, thymine and xanthine. In particularly, the highest n-alkane degradation rate was achieved with the addition of tyramine (43.6%), L-glutamine (42.2%) and D-aspartic acid (38.2%). Based on increasing microbial respiration and the n-alkane degradation rate, tyramine was suggested to be the best promoting nitrogen source to encourage *in situ* n-alkane biodegradation.

Further correlation analysis between microbial respiration and the n-alkane degradation rate helped further our understanding of the roles of nitrogen sources in the n-alkane metabolism of functional alkane degraders. The Pearson correlation coefficient was 0.781 ($p$-value<0.001) between microbial respiration and n-alkane degradation rates in MFCs from MNPP treatment (red circle in Figure 6.6).

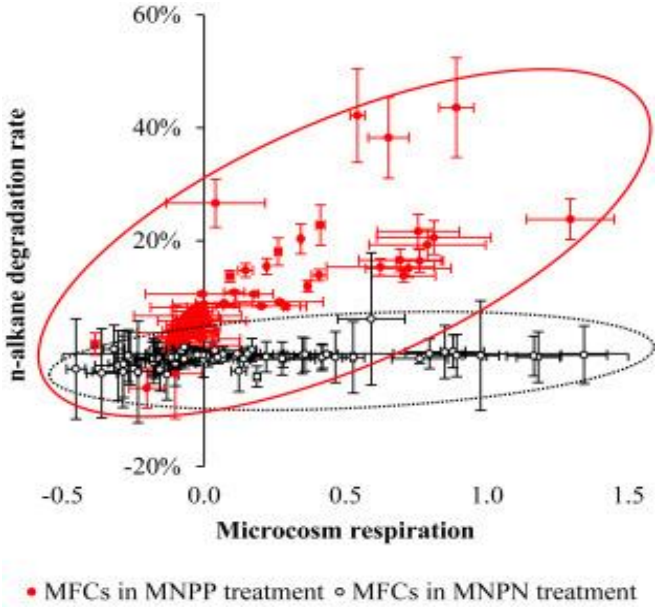



Figure 6. 6 Correlation analysis of microbial respiration level and n-alkane degradation rate in phenotypic microarray. Red and white circles represent the data of MFCs in MNPP and MNPN treatment respectively.

Results showed that separated living microorganisms in MFCs after n-alkane addition were indeed functional n-alkane degraders in situ. There was only weak relationship (Pearson correlation coefficient = 0.335, *p*-value<0.001) between the n-alkane degradation rate and the microbial respiration level in MFCs from MNPN treatment (white circle in Figure 6.6) indicating that they were not predominantly alkane degraders.

Numerous previous researches has attempted to improve alkane biodegradation by adding exogenous degrading strains, and some of them have achieved good alkane degradation performances in liquid culture (Byers, 2002) and in situ. However, additive exogenous strains might compete with indigenous microbes or be affected by soil properties, resulting in the fact that the performance of bioaugumentation or biostimulation is not always satisfied in the complex soil matrix. The risk of species invasion also requires attention due to micohabitat alterations in the soil environment. Meanwhile, the amendment of growth-promoting substrates for stimulating indigenous alkane degraders mainly addresses simple inorganic/organic nitrogen sources, such as $NH_4NO_3$, $NaNO_3$, $(NH_4)_2SO4$, Urea (Chaineau et al., 2005) yeast extract (Sajna et al., 2015) and lipophilic fertilizers. In the present study, it was interesting to note that these commonly used nitrogen sources, like nitrate (A4) and Urea (A5) in the PM03 plate could encourage microbial respiration or the n-alkane degradation rate, indicating that traditional nutrient additives in bioremediation process cannot effectively accelerate n-alkane degradation. A high-throughput nutrient screening method is therefore recommended for improving bioremediation performance at alkane and crude –oil- contaminated sites, relying on the effective separation of functional n-alkane degraders and phenotypic characterization.

In conclusion, we developed a modified magnetic nanoparticle-mediated isolation (MMI) method in this study. For the first time, this work successfully revealed both genetic information and phonotypic behavior of functional n-alkane degraders in soil microcosms. The consistency of phylotypes and n-alkane monooxygenase genes proved that the separated *Oxalobacteraceae* and *Moraxellaceae* were the true functional n-alkane degraders in situ at different metabolism steps. From the physiological study of the functional n-alkane degraders via BIOLOG PM plate, we suggest tyramine as being the

promoting nitrogen source to stimulate indigenous n-alkane degraders and accelerate the bioremediation process. This novel technique opens a new pathway to characterizing the mechanisms of n-alkane attenuation and influencing factors in the biodegradation process, with great potential in crude oil bioremediation enhancement and organic contaminated site management.

## Conflict of interest

The authors have declared no conflict of interest.

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# 7 Suggestions and recommendations on crude oil spill and management in Niger Delta

The chapter 7 aims to highlight technical and social suggestions and recommendations for crude oil spill monitoring and management in the Niger Delta.

The specific objectives are:

- To provide to the Nigerian government recommendations on how to improve oil contamination management using advanced techniques and implementation regimes.
- Explore opportunities and collaboration with the Nigerian government to scale-up the outcome of this study for large scale crude contamination monitoring and effective management using bioreporter devices developed and applied in this study for real time and in situ measurement.
- Similarly, the bioremediation of crude oil contaminated sites needs practical engineering work, which should be carried out on the field.

Considering the crucial importance of managing and cleaning waters and soils contaminated by crude oil in the Niger Delta, Nigerian government needs to improve the scheme of policies and regulations and achieve the goals of national sustainable development. The Nigerian petroleum industrial regulations and policies should also institute proper integration of social development strategies to ensure the protection of the social and human rights of inhabitants, or the Niger Delta might remain in a calamitous position. From the findings and discussions in this research, some recommendations are suggested with specific focus on the social, political and technical improvement regarding crude oil spill and contaminated sites.

## 7.1 Social Development Challenges and Improvement Options for the Niger Delta

Threats on human health is becoming more aggravated in the Niger Delta owing to the industrial development, particularly the oil industry. The organic pollutants released into the environment arise mainly from the industrial activities (Mozaffarian and Rimm, 2006). Causing many social development challenges in the Niger Delta and restricting the space for sustainable growth or future development (Chukwuemeka and Aghara, 2010), there is an urgent need for rehabilitation for the complex interlinkages between environmental pollution and social development which will bring about transparency and accountability in Nigerian government agencies responsible to the local communities (Idemudia, 2012). Since Nigerian petroleum industry, which is operational in the Niger Delta with vast network of petroleum resource development paraphernalia, have severely impacted the region and the local economy, the colossal level of oil contamination in the Niger Delta has significantly negative impacts and resulted in an unrelenting increase in poverty levels, deteriorating health, vice and violence in the region. Poverty incidence for example jumped from 13.3 % to 58.2% between 1980 to 1997 (Adeyemo, 2008). Thus, governmental regulations and policies require amendments to accommodate efforts for the increasing public sensitization and awareness regarding the urgent necessity of monitoring and remediating oil contaminated soils and water. The regulatory framework should be efficient, effective, clearly separated roles of government agencies, purpose driven,

transparent, accountable in administration, conducive for business environment and ensure best national and international standards.

## 7.2 Technical improvement

### *7.2.1 Crude oil contamination monitoring*

Both chemical and biological approaches can be used for monitoring crude oil spill and evaluating its environmental impacts. In the Niger Delta, it is questionable which approach is more suitable, considering lack of stable power supply and operational cost. Meanwhile, crude oil monitoring is also required for the fast decision making to save cost for the operators in drilling activities, which entail an average cost of about $520,000/day in 2015 for a deep-water well. Such situations ultimately create room for the low-cost, robust and fast monitoring methods, such as whole-cell bioreporter, to be applied as a cost-effective and operational tool for detecting oil spill on site and mitigating cost during industrial and further clean-up activities. Whole-cell bioreporter takes only 4 hours for the quantification of crude oil in environmental samples, and such advantage makes it feasible to be recommended by DPR in monitoring wells and pipeline leaching, for appropriate decision making within several hours without suspending the industrial activities for days to avoid triggering additional costs for the operators (Middleditch, 1984).

Environmental risk management of crude oil spill also involves information on toxicity level, which is directly linked to risks and hazards posed to human health (Farré and Barceló, 2003). Such ecological impacts can be also evaluated by whole-cell bioreporters, which help in decision making for accident rapid response in terms of technical support for policies and regulations. Some genotoxicity bioreporters responds to the environmental toxins through detecting SOS response. By linking contamination to ecological and health impacts, the results of whole-cell bioreporter can influence governmental action for sound legal actions in monitoring, assessment and clean-up of oil spillage, further benefiting the strategies of low-cost bioremediation (Ejenavi et al., 2016).

The importance of policies and governance on crude oil spill monitoring needs to be emphasized by Nigerian government. Besides the advantages mentioned above, favor of the whole-cell bioreporter is also anchored owing to other shining sights as cost-effectiveness,

portability, specificity and reproducibility (Ron, 2007). However, compared to chemical analysis, the whole-cell bioreporter is not of the same accuracy (Li et al., 2013), and needs further development to shorten its distance between applications in the laboratory and field. It can serve as a quick supplementary solution to conventional chemical methods for crude oil contamination and ecological assessment in Nigeria. It actually has great potential for development and can be further modified for purposes of encouraging technical support and expediting regulation and policy measures in the Niger Delta.

### *7.2.2 In situ remediating crude oil contamination*

The Nigerian government needs to protect the environment from crude oil spill for benefiting both the present and succeeding generations as a matter of urgency. The report of the World Commission for Environment and Development by the United Nations requires enforcement and implementation measures (Brundtland et al., 1987), the mandate and provision of which will improve crude oil remediation *in situ* and will overcome the current challenges for effective oil field remediation. Only in this way, the debilitating effect on the people in the Niger Delta and the crushing effect on the economy and livelihoods (ranging from basic farming, fishing and hunting) on the oil producing communities which need immediate attention will be ultimately resolved.

The environmental sustainability programme should be a collaborative effort of all the government, public and private stakeholders to prevent further deterioration of the ecosystem. The case study of cleaning-up Ogoni-land, which has been contaminated by crude oil for 30 years (UNEP, 2011), is a good example. Ogoniland inhabitants from at least 10 communities are drinking water from wells contaminated with benzene, a known carcinogen from oil spill, at a level over 900 times above World Health Organization (WHO), as the site is close to the Nigerian national petroleum pipeline (Bassey, 2012, Lindén and Pålsson, 2013). It is estimated the clean-up in Ogoniland will require an investment of over $ 1 billion (Sam et al., 2016). From several comprehensive reviews on various approaches of bioremediation for cleaning-up crude oil contaminated (Bhatnagar and Kumari, 2013), a sustainable method need to be safe, clean, applicable, cost-effective and environmentally-friendly, as well as with high public acceptance (Amadi and Bari,

1992, Song et al., 1990). Some conventional or chemical methods already applied in the Niger Delta have relatively high cost and potentially negative effects on the environment (JC and Mbogu, 2013, Gerard, 2012, Zamani et al., 2014), and they might even need further remediation process in the enriched crude oil extracts during the evaporation and elution treatment (Nkeng et al., 2012). Our work thus raises an alternative way of enhancing in situ soil bioremediation by encouraging indigenous uncultivated-oil-degraders at crude oil contamination sites via magnetic nanoparticle-mediated isolation (MMI) method (Wang et al., 2016c). Targeting the uncultivated petroleum hydrocarbon degraders, which hide within the over 99% of unknown microorganisms in the natural environment (Whitman et al., 1998), the results successfully revealed both genetic information and phonotypic behaviour of these key players who have an important role in hydrocarbon degradation in the natural ecosystems habitats. Since species invasion should not be introduced into the Nigeria soils, the new technology will be a great potential in enhancing crude oil bioremediation with ingenious bacteria and the influencing factors during biodegradation process can be uncovered to further improve the degradation performance (Zhang et al., 2011a, Zhang et al., 2015a, Wang et al., 2016c). Our results suggest the feasibility of applying MMI on crude oil contaminated soils and wetlands in the Niger Delta, and recommend a pilot project study for further practical field work to validate the standard strategic operation, which removes the risk of species invasion and minimizes operation cost in comparison with the conventional chemical method for ecological restoration and the management of crude oil contaminated sites.

## 7.3 Regime Implementation and Solutions to Social Concerns

### 7.3.1 New PIB framework for effective management

Effective environmental management approaches have become illusory in Nigeria (Ogri, 2001) and need improvement in regime framework (Ingelson and Nwapi, 2014). The Petroleum Industry Bill 2015 (PIB 2015) has strengthened petroleum laws and regulations from its inception since 1956-2006 (Frynas, 2000) and resolved some issues of multiple agencies with duplicated functions in the oil and gas industry (Elenwo and Akankali, 2014). It would also eliminate challenges caused by non-experts lacking in technical know-how administering policies and management regimes in the Nigerian oil industry (Sam et al.,

2016). This will eventually reduce the problems of oil spills management because multiple functions and duplication of functions by the agencies/bodies working against purpose of goals would be eliminated (Ambituuni et al., 2014). The Nigeria Petroleum Regulatory Commission shall subsume all functions of the existing upstream and downstream sectors to maintain the sole roles as the regulatory entity like other oil producing states in the world.

A new PIB is suggested for the regulatory authorities to maximize their functional capacity without duplication in the control and enforcement of environmental regulations on petroleum industry governance and institutional framework (PIB, 2015). First of all, the new PIB needs to organize efficient and effective government institutions with clear and separated roles for the petroleum industry. As mentioned in previous discussions, one of the hiccups of crude oil contamination management is lack of clear-cuts functions and roles of the government institutions which PIB seeks to address. Secondly, the new PIB needs to establish a framework for creating commercially-oriented and profit-driven petroleum entities that ensure value addition and internationalization of the petroleum industry. The framework will be enshrined in the policy of crude oil contamination monitoring. Additionally, the new PIB will also promote transparency and accountability in the administration of petroleum resources in Nigeria. The hallmark of global practices in the petroleum industry is transparency and accountability and the Nigeria industry must not be seen to be different. Finally, a conducive business environment for petroleum industry operations needs to be created by the new PIB, which further seeks to have ONE regulatory Commission called Nigeria Petroleum Regulatory Commission as contained in Part 3,4 (1-5) with various functions of 5(a-k), and 6[1(a-k)] and with a strong emphasis on the adherence to environmental standards which is to ensure adherence to applicable national and international environment and other technical standards by all persons involved in petroleum operations; and also to establish, monitor, regulate and enforce health and safety measures relating to all aspects of petroleum operations with strong penalties for offences committed by the IOCs.

The proposed strategies are multi-participatory to ensure that the governments that superintend the regulatory bodies ensure compliance with regulatory obligations, meet

stakeholder expectations and maintain effective cost controls. It must comply with global practices and ensure compliance to HSSE standards, which can been achieved through HSSE management training ((Ming, 2010). The training will help poor environment planning and lack of commitment to existing environmental policies which will embrace a policy framework. Although, many challenges are associated with contaminated site management and water management. The policy framework is therefore necessary for contaminated site management. This entails a policy drive for cleaning the contaminated environment especially in situations where the contaminated environment is classified as either slightly or seriously contaminated (Swartjes et al., 2012).

### *7.3.2 Investment in Efficient Technological Innovation and Social Concern*

A primary recommendation for the improvement of the Nigerian oil industry relates to governmental regulatory and financial investment in efficient and innovative technologies (Grant, 2013) and knowledge acquired to protect the environment and people of the oil producing region. This is a crucial means of achieving a green petroleum industry as more efficient means of oil exploitation with minimal impact or adverse ecological footprint are thus employed. Knowledge exchange mechanisms between the academics and the industries are thus possible and opportunities for technological advancements are made available (Eren et al., 2013). Government's policy when driven by academics or researchers has shown a strong collaboration for effective system performance in area of collaborative research to be funded by both government parties and IOCs, as research expertise helps to assist the industry with essential innovation in the area of environmental protection which at the same time accommodate people oriented approaches. This will eventually result in strategic advantages for the people and overall company (Tahmooresnejad et al., 2011). Knowledge-based mechanisms will increase the technical ability of industry staff (Kim, 1980) and furthermore reduce the recurring problem areas which aggravate spills such as: accidental discharges due to operational errors, dumping, oil theft, transportation of products mode, sabotage by communities claiming for compensation, aging and corrosion of pipelines can be minimized by such collaboration. This will help developing countries like Nigeria to overcome the use of obsolete equipment that is internationally non-standard (Kadafa, 2012b). Poor equipment handling could also be traceable to lack of technical

skills, minimal innovation and dearth of modern trends in the Nigerian petroleum industry. Environmental experts should be consulted for training and innovative approaches to environmental protection as the central driver of long-term growth is traceable to technology and innovation to imbibe the EU2020 innovation policy known as "Smart Growth" (Borowik, 2014).

Nigerian petroleum regulations are not quite specific about technological innovations; as a result, the policy implementation of international standards regarding environmental toxicity levels are therefore absent and unenforceable. This impacts negatively on the people and environment. Implicitly the regulators rely on the multinational companies for virtually all forms of technical assistance, and such reliance is indefensible for sustainable development as it promotes undue dependence on external forces for efficient petroleum resource exploitation. This also creates a major monitoring and enforcement challenge as the government officials like the earlier cited NOSDRA lack the technical capacity to quickly detect and respond to spills in accordance with their regulatory mandates and at the same time ensure that multinationals do not operate short of international guidelines and standards. A good approach to environmental toxicity testing is based on the combination or integration of chemical analysis with toxicity bioassay and biosensor (Farré and Barceló, 2003) as in the case of Europe (DIRECTIVE, 2003). Also, the use of microorganism as *Pseudomonas* cell multiplication inhibition test based on growth inhibition (ISO, 1995., ISO, 1999) which is built on easy manipulation and high level of reproducibility. Another example for water quality is based on algae (*Selenistrum capricornutum, Denaliella tertiolacta* are indicator species (ISO, 1995., Miller and Greene, 1978) in the US and most commonly toxicity test is based on inhibition of bioluminescence of luminescent bacteria *Visbrio fischeri or photobacterium phosphoreum*. These problems relating to quick and easy detection of contaminants in land and aquatic spaces can be easily resolved by regulatory and policy adjustments (NOSDRA, 2014). This is because the whole-cell bio-reporter is undoubtedly one of the simplest and cost effective technological innovations that would remedy this situation among other techniques. Thus, for an instrument of rapid accident response, the biosensor tools can be used to empower organisations and governments to take prompt action to protect the environment. Social development

objectives like poverty eradication, health, human development, respect for and enforcement of human rights, sustainable agriculture, access to potable water and sanitation, shelter, decent jobs, public participation, access to justice, to mention a few, form the crux of the sustainable development debate relating to the Niger-delta, as it serves to eliminate human rights challenges.

### *7.3.3 Provision of Quality Education, Health Care and Public Awareness*

Another paramount recommendation to achieve social development and progress in the Niger-delta as it relates to government is the need to encourage education, health care and public awareness which can be achieved through collaboration of institutions with relevant technologies and novelty. This awareness can reduce crude oil contamination in the Niger Delta regions. A serious awareness campaign can also help to stop the continued rise of oil pipelines vandalization, oil theft and sabotage of the oil facilities which have been evident in recent times. This also includes illegal bunkering activities in the region (Okolo and Etekpe, 2010).

Raising public awareness involves creating a specific messaging campaign about a particular issue, in the case of crude oil contamination prevention and reduction. Awareness can be important part of developing community support for changes in raising social development. It has been shown to change knowledge and attitudes of the implication of oil contamination. Campaign should be focused on tangible issues that are relevant to the lines of community members to understand the adverse impact of crude oil contamination vis-à-vis recuperating the ecosystem.

Message should be clear and simple and awareness campaigns may include events, poster campaigns, websites, documentaries, newspapers articles, radio and television adverts on crude oil contamination. The real solution for solving oil contamination challenges is for government to accelerate the pace of development, that is, an economy with relevant social, economic and physical infrastructure for curtailment of oil contamination areas for business operations industrial growth. Also, innovative and environmental friendly remediation strategies should be adopted or carried out on contaminated soils that have been polluted. Therefore, further strategies medium that can be used to improve crude oil contamination

clean-up includes educational development, prevention and respond based on science and technologies, environmental legislation on prevention and response, stakeholders engagement committee and host community and operators interface on the day to day exploration and production monitoring as it relates to crude oil contamination of the environment. The current technologies that should be used in monitoring crude oil spillages in the Niger Delta should be a tested technologies used elsewhere in the world are the prevention based on safety culture and the best response based on science and engineering which includes the synthetic aperture radar (SAR), Artificial neural networks (ANNs), Visible satellite sensors of improved SAR. These are called Windows-of-opportunity technology which is oil spill response management system and bioremediation technologies which has become an accepted approach to remediating the contaminated sites (Ivshina et al., 2015).

Statistics from NNPC from 2003-2012 shows increase in the number of vandalization against equipment failures and rupture. This therefore called for serious enlightenment or awareness programmes in the Niger Delta communities as a medium or campaign to stop the activities of the vandals or sabotage of the pipelines and also issued warnings regarding the health hazards posed and livelihoods compromised (Subi and Amodu, 2014). The government should organize institutional conferences both local and international using educational and cultural media to salvage the situation. Furthermore, the World Bank 1995 report identified the region to have as its main occupation fishing and farming, with the population depending on the immediate ecosystem for their survival. The Niger Delta States which comprises of Abia, Akwa Ibom, Bayelsa, Cross Rivers, Delta, Edo, Imo, Ondo and Rivers does not have access to health care and social services. The strong neglect of the regions in the area of socio-developmental structures has heightened civil unrests in the region. Nigeria was ranked as 158$^{th}$ out of 177 nations on Human Development Index with over 3.5 million living with HIV and average life expectancy of 45 years. The work or report also placed Nigerian health care as under-resourced with the Niger Delta emerging as the worst hit as most of the ill-health is caused by crude oil contamination and pollution (Anger, 2010). Since the Niger Delta is a malaria endemic region, standard health care facilities could be built by the Nigerian government and multinationals to mitigate the health and environmental hazards. This can boost trust to the communities where the crude

oil exploration and production are carried out and undoubtedly, trust must therefore be restored through several means of education, culture-based approaches and corporate social responsibility (CSR).

Corporate social responsibility (CSR) should be encouraged in the Niger Delta by the oil companies for technical development, tackling the challenges of the crude oil contamination monitoring and remediation to secure the ecosystem and sustainable development. CSR is a business model adopted by some oil multinationals with the aim of earning community trust, relationship, stakeholders trust and at the same time to increase long term profits (Ajayi, 2016). It has been used as an initiative by some major oil companies to access the people and the environment in communities of the rich oil region and also to make the companies take effective responsibilities of the environmental and social wellbeing of the local communities. Examples of CSR of oil spill management used by Royal Dutch Shell in the Niger Delta, American and Latin America etc thus reviewed or highlights the importance of risk reduction if the OICs want to do business in a more sustainable and socially accountable way (Spence, 2011). Basically CSR is primarily to focus on the corporate responsibility of the environment which is being viewed as a socio-cultural framework, and is defined or referred to as the patterns of human activity which comprise codes of manners, dress, language, religion, rituals and norms of behavior such as law and mortality and systems of beliefs (Helg, 2007). Nevertheless, CSR is a relatively new concept in Nigeria in comparison to the developed countries with CSR foundations as far back as in the 1950s. Therefore, CSR can be used to address the menace following oil activities through education and public awareness which can be built into the overall objectives of the operating companies with the company business strategies, objectives and its social responsibilities (Rwabizambuga, 2008). In Nigeria, the drivers of CSR have been the multinational companies operating in the country with international non-governmental organizations (NGOs) and its practices can also be used to address the environmental and social issues or both. The dimensions of CSR which include: Social, Environment, and Economic aims can be used as linkages to revive the Niger Delta being a diverse ethnic-cultural region needs this methodological approach. However, since in Nigeria CSR practice is still an ad hoc initiative with developmental links to cultural roots management, new management tactics should be developed to spread it to a large framework either by

better communication which may be formal or informal to be able to solve the myriad problems plaguing the environment and its ecosystem. Different bodies and IOCs have implemented CSR to help local communities in the past and it can further be used to improve and help to solve the social challenges in the region by way of interrelationship between the local communities and the oil exploration operated companies. Thus, CSR targets can be channeled into adopting code of conduct policy and strategies that will stop the continuing rise of oil spills via sabotage or other factors of oil accidents and this can be achieved majorly by technical development that should be geared towards research and development funding by the multinationals. These strategies should have a vision for sustainability of the environment and can thus be institutionalized.

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# 8 Conclusion

This chapter concludes on the finding of the thesis, and addresses the policy implication of the research for improved crude oil contamination management in Nigeria.

The severity of crude oil contamination as a result of oil and gas exploratory activity in the Niger-delta has been the cause for major concern to the inhabitants of the region (Ohimain, 2003). The catastrophic level of oil pollution in the Niger-delta region has also been accountable for the global and local outcry for immediate restorative or rehabilitative measures in favour of the dilapidated Niger-delta environment (Akhakpe, 2012). The scope of oil pollution invariably intersects all areas of the environmental media relating to the: atmosphere, land, water and biodiversity, with accompanying damaging ecological and health impacts on the inhabitants (Romanelli et al., 2015). Thus to achieve the millennium development goals in the area of poverty eradication, food security, stable or sustainable livelihoods, health and the relevant yardsticks for environmental protection and social development in the Niger-delta; immediate policy, regulatory and administrative reforms are required to halt the looming crises in the region (Sachs and McArthur, 2005). Consequently, to prevent all forms of carcinogenic or other cumulative effects from hydrocarbon compounds such as Poly aromatic hydrocarbons (PAH) which are major contaminants from oil spills affecting land, aquatic areas, wetlands, fresh water sources as well as biodiversity, terrestrial and aquatic ecosystem pollution, this research proposes the bioremediation approach as an effective means of oil pollution control and rehabilitation in the Niger-delta (Zhang et al., 2015a),(Zhang et al., 2011b).

However such a remarkable and innovative approach to prevent oil pollution damage and to promote ecosystem recovery, environmental protection and green economic growth in Nigeria cannot be possible without proactive government approaches in the area of technological innovations and enforcement of international best standards in the oil industry (Okafor, 2011). There is also a crucial need for regulatory improvements and policy adjustments to ensure precautionary measures for curbing oil pollution and safety measures for preventing the spread of oil spills (Iyalomhc, 1998). This can mostly be achieved via efficient spills detection and emergency response procedures which are possible through the Bioremediation techniques proposed by this research. Unfortunately, the lack of political support and sufficiently protective environmental and legislative safeguards restrict enforcement actions in crude oil monitoring to achieve environmental protection and social development imperatives. The political or governmental input is essential and requires commitment to the improvement of environmentally protective and

social development regulations in the Nigerian oil and gas laws. Without these regulatory and legislative reforms there cannot be efficient control or elimination of the factors accountable for these oil spills (Adelana et al., 2011a). These factors as identified in this thesis include oil thefts, accidents, worn or ageing oil industry infrastructure, pipelines rupture, criminal bunkering activities or pipelines vandalism including negligence. Moreover, political, regulatory and legislative measures to ensure the incorporation of technological innovations for optimizing international best practices and standards in the oil industry are also essential precautionary strategies for pollution control in the Niger-delta. This also positively impacts on monitoring, site assessment, prevention and clean-up of oil spills, both in the upstream and crucial downstream areas. Basically, such adoption of biological approaches as identified in this thesis relating to Bioremediation and typified by the bio-sensor and acinetobacter methods expedite the aims of emergency response to spills detection and control to preempt oil pollution damage (Zhang et al., 2013). It also provides realistic data for quality decision making on environmental policies affecting oil contamination or spills reporting as the combination of nanoparticles and bio-reporter considerably improve accuracy and sensitivity for enhanced spills detection (Zhang et al., 2012c). Furthermore, bioremediation identifies the immediate and protracted oil spill impacts as well as the extent of remediation required, which can also be scaled to manageable or optimal levels. It is also recommended as a highly sustainable approach for the monitoring, assessment and remediation of crude oil contaminated areas because it is affordable with very low-cost implications in comparison to the highly damaging potential of hydrocarbon spills on the environment and surrounding populations (Zhang et al., 2015a).

Moreover, the Biological method is preferred to physical and chemical methods to clean up the Niger Delta. It is however submitted that pollution prevention which operates as precautionary actions in favour of environmental protection is better at minimizing or avoiding pollutants creation and wastes. This expedites holistic or beneficial measures in favor of the environment as opposed to post pollution restoration efforts or treatments and clean-ups. Its benefits include:

1. Reduced or pollutants elimination

2. Health risks minimization
3. Technological Innovation and development has been promoted
4. The efficient use of energy, material and resources
5. Enforcement costs are reduced and /or minimized
6. Limitation of future liability with greater certainty and
7. Costly clean up in the future will be avoided

This is the approach that involves governments, communities, stakeholders and industries alike. Similarly, amendments of environmental regulations need to be made by the National Assembly incorporating contributions from environmental experts, Niger Delta Development Commission (NDCC), NOSDRA, and other stakeholders. A critical look at the EU environmental policies that lasted over 30 years with or without prejudice to EGASPIN (Hey, 2005). Environmental policy integration and sustainable development should therefore become key elements for influencing the desired changes in the Niger-delta and optimize a green petroleum industry. Nevertheless, the possibilities or plethora of opportunities afforded by the bio-remediation approach adopted by this research is a step in the right direction for the Niger-delta. The other equally important governmental input in terms of policy and regulations to trigger and promptly implement the aims of environmental protection via biological rehabilitative methods is what is required to maintain a peaceful, socially developed and economically viable Niger-delta.

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