DEVELOPMENT OF TILAPIA PCR ARRAYS FOR POLLUTANT EXPOSURE MONITORING IN THE TROPICAL FRESHWATERS
(A case study in Nigeria)

A Thesis Submitted for the Degree of

Master of Philosophy

By

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Declaration

I hereby certify that, this thesis is the work of myself alone, and has not been submitted previously in part or whole for the award of any academic degree. The entire acknowledgement has been duly made for the information from different sources.

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Isah Lawal Date

Dedication

Dedicated to my parents, family, friends, relatives and the entire community of Umaru Musa Yar'adua University, Katsina State, Nigeria.

Acknowledgement

I would like to give a special thanks to my Supervisors Dr Michael J. Leaver and Prof. Trevor C. Telfer for their invaluable advice, guidance, encouragement and tireless laboratory assistance. Thank you for your valuable time taken in reading through my chapters and your constructive inputs and criticisms, which helped, shaped the thesis into good document. Another special thanks go to Umaru Musa Yar'adua University, Katsina State, Nigeria for funding this project. A thank you message to Keith Ranson also for providing me with prefeeding tilapia larvae during the course of my exposure experiment in the institute tropical aquarium, Jacquie Ireland, John Taggart for their immense contribution in providing me with guidance and reagents during my laboratory sessions, Charlie Harrower and Jane Lewis in the institute stores are also acknowledged.

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Finally, my greatest gratitude to my parent, wife and children for their prayer, support and patience during this project; I say thank you.

General Abstract

Pollutants contaminate both marine and freshwater systems and are known to regulate animals' metabolic pathways. Chemicals are discharged through industrial waste, sewage, agricultural and urban run offs, which, reach aquatic ecosystems and impact organisms including fish. This research investigated the effects of xenobiotics on selected genes using QPCR arrays. Six tissues were chosen from adult Nile tilapia and validated using 28 genes already optimised by Real transcriptase quantitative polymerase chain reaction (RT-qPCR). Nile tilapia (*Oreochromis niloticus*) prefeeding larvae (6-7 dpf) was used as a model species and exposed to different sub-lethal concentrations of ten environmental relevant chemicals in the laboratory using RT- qPCR. Primers sequences of the already selected and optimised genes that passed the quality criteria were used in the assay (*AHR 2, CYP 1A, DIABLO 1 and 2, GST01LA, GSTA2L, GSTMA, GSTR2, MT AND VTG*). Wild tilapia samples were caught at both the polluted Dan Agundi and Daberam sites and the clean Jibia site and the length-weight relationship and condition factor established.

The results indicated that ten of the twenty-eight assays results were within the target amplification efficiencies and coefficient of correlation ranges of 0.90-1.10% and 0.9-0.99 respectively. Most of the genes showed expression in the liver. In the laboratory exposure, different chemicals induced significant mRNA gene expressions, including aryl hydrocarbon receptor and estrogen receptor pathways, phase II biotransformation, apoptosis and metal toxicity in the larvae. While in the field, significant statistical changes in the expression of genes involved in different physiological and metabolic signalling pathways were observed. Different length-weight relationship and the condition factor were also observed in both the polluted and non-polluted sites. The use of biomarkers can provide valuable knowledge of the effects of pollutant mixtures on the aquatic ecosystem and the risks to exposed organisms, especially fish.

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Abbreviations Lists

18S RNA	Ribosomal RNA 18S
AAS	Atomic Absorption Spectrometry
AHR	Aryl Hydrocarbon Receptors
AHREs	Aryl Hydrocarbon Response Elements
AHRr	Aryl Hydrocarbon Receptor Repressor
AOX	Adsorbable oxygen halide
ARE	Antioxidant Response Element
ARNT	Aryl Hydrocarbon Nuclear Translocator
AWERB	Animal welfare and ethical review bodies
BaP	Benzo[alpha]pyrene
BHLH	Basic- Helix- Loop
bHLH/PAS	Basic- Helix- Loop- helix /Per-Arnt-Sim
BPDE	Electrophilic Diol epoxide
CASPASE	Cysteine-Aspartic- Proteases
CAT	Catalase
cDNA	Complementary DNA
CF	Condition Factor
CP	Crossing point
CQ	Quantification Cycle
CT	Threshold Cycle
CYP	Cytochrome superfamily
CYP1A	Cytochrome P4501a
DD	Death Domain
DDT	Dichlorodiphenyltrichloroethane
DEHP	Di(2-Ethylhexyl) Phthalate)
DIABLO	Directly IAP binding protein with low pI
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
DNase	an enzyme to remove DNA
dNTP	Deoxyribonucleosides triphosphate
DREs	Dioxin Response Elements
E2	Estradiol

EDCs	Endocrine Disrupting Chemicals
EE2	17α Ethinyl-Estradiol
EpRE	electrophile response element
ER	Estrogen Receptor
EROD	Ethoxyresorufin O-Deethylase
EtBr	Ethidium bromide
EU	European Union
EU.WFD	European Union Water Framework Directives
FADD	Fas-associated protein with a Death Domain
FEPA	Federal Environmental Protection Agency
GC-ECD	electron captor detector
GC-MS	Gas chromatography mass spectrometry
GC/MS/MS	Gas Chromatography/Mass Spectrometry
GI	Growth index
GPX	Glutathione Peroxidase
GR	Glutathione reductase
GS	Glutathione radical
GSH	Reduced Glutathione
GSI	Gonado Somatic Index
GSSG	Oxidised glutathione
GST	Glutathione-S- Transferases
GST01LA	Glutathione S- transferase 01LA
GST01LB	Glutathione S- transferase 01LB
GST01LC	Glutathione S-transferase01LC
GSTA	Glutathione S-transferase alpha
GSTA2L	Glutathione S-transferaseA2L
GSTK	Glutathione S- transferase Kappa
GSTMA	Glutathione S- transferase Mu (a)
GSTMB	Glutathione S- transferase Mu (b)
GSTR 2	Glutathione S- transferase Rho 2
GSTR 3	Glutathione S- transferase Rho 3
GSTR 4	Glutathione S- transferase Rho 4
GSTR 5	Glutathione S- transferase Rho 5

GSTR1	Glutathione S- transferase Rho 1
GSTT 2	Glutathione S- transferase Theta 2
GSTT1	Glutathione S- transferase Theta 1
GUP	General Use Pesticide
H_2O_2	Hydrogen Peroxide
HAH	Halogenated Aromatic Hydrocarbons
HLH	Helix-Loop-Helix
HPI	Hepatic Somatic Index
HPLC-UV	High-pressure liquid chromatography with UV detectors
HSI	Hepatic Somatic Index
HSP	Heat Shock Proteins
IAPs	Inhibitors of Apoptosis Protein
LC-MS/MS	Liquid Chromatography-Tandem Mass Spectrometry
LOD	Limit of Detections
LSI	Liver Somatic Index
LWR	Length-Weight Relationship
MGST	Microsomal Glutathione S- transferase
MIQE	Minimum information for publication of qPCR experiment
MOA	Mode of action
MRES	Metal Regulatory Elements
mRNA	Messenger Ribonucleic acid
MS	Mass Spectrometry
MSFD	Marine Strategy Framework Directives
MT	Metallothioneins
MTF-1	Metal transcription factor-1
NCBI	National Centre for Biotechnology Information
NESREA Agency	National Environmental Standard and Regulation Enforcement
NTC	No Template Control
OCPS	Organochlorine Pesticides
OECD	Organization of Economic Cooperation and Development
OPs	Organophosphorus
OSPAR	Oslo/Paris (for the protection of Marine Environment of the North-
PAE	Phthalic acid esters

PAH	Polycyclic Aromatic Hydrocarbons
PAS	Per-Arnt-Sim
PBDE	Polybrominated Diphenyl ethers
PCB	Polychlorinated Biphenyl
PCR	Polymerase chain reaction
PFCA	Perflourinated carboxylic acid
PFCS	Perflourinated Compounds
PFOA	Perfluorooctanoic Acid
PFSA	Perflourinated Sulfonic acid
PPAR	Peroxisome Proliferator-activated receptor
PSD	Passive Samplers' Devices
PTBs	Persistent bioaccumulative toxicants
PVC	Polyvinyl Chloride
QPCR	Quantitative Polymerase Chain Reaction
REACH	Registration Evaluations Authorisation and Restrictions of Chemicals
RNA	Ribonucleic acid
RNase	an enzyme to remove RNA
RNAseq	next generation RNA sequencing
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
RPL 3	Ribosomal protein L3
RPS5	Ribosomal protein S5
RPS7	Ribosomal protein S7
RT-qPCR	Real time Quantitative Polymerase Chain Reaction
SIAH 2	*Seven in Absentia Homolog 2*
SINA	Seven in Absentia
SMAC	Second Mitochondria Derived Activator of Caspase
SOD	Superoxide Dismutase
SSC	Secondary Sexual Characters
SSI	Spleen somatic index
STP	Sewage Treatment Plant
SYBR	Fluorescent dye
TL	Total Length

T_M	Melting Temperature
TP	Transformed Products
TRE	Thyroid response element
U. S	United State
U.S.EPA	United States Environmental Protections Agency
UDP-GT	*Uridine Diphosphate Glucuronosyltransferase*
UDPGA	Glucuronic Acid
UN	United Nation
VSI	Visceral somatic index
VTG	Vitellogenin
WFD	Water Framework Directives
XREs	Xenobiotic Response Element
ZP	*Zona Pellucida*
ZPC	Zona Pellucida C
ZRP	Zona Radiata Protein
ΔCT	Delta Ct
ΔΔCT	Delta Delta Ct
µg	Microgram
µL	Microlitre

1.0 CHAPTER ONE

1.1 GENERAL INTRODUCTION

Aquatic ecosystems are the ultimate basins of chemical contaminants from agricultural runoff, domestic and industrial waste effluents. They are detrimental to the health of the organisms living in the polluted environment, especially fish. These effluents often contain mixtures of xenobiotics in high concentrations and their effects on organisms in these waters is difficult to predict from chemical data alone. However, monitoring of the effects on individual organisms can be achieved through an analysis of the biological responses in sentinel species such as fish (Bae et al., 2020). In order to achieve the task of monitoring the biological effects of pollutants, various biomarkers indicating the harmful effects of exposure of organisms to such pollutants have been employed, including histopathological, molecular, morphological and biochemical parameters (Adeogun et al., 2019). Amongst the biomarkers deployed, gene expression is gaining traction as a means of comprehensively evaluating pollutant exposure based on data from acute laboratory exposure to various forms of chemical pollutants such as PCBs, PAHs, metal, estrogenic and other organic chemicals (Adeogun et al., 2019). Comprehensive gene expression studies offer the possibility of extending well known responses such as the induction of Cytochrome P4501A (*CYP1A*) to PAHs, *Vitellogenin (VTG*) to estrogen and heavy metal toxicity to *Metallothionein (MT)* (Kumari and Maiti, 2019). Nevertheless, in field studies, variations of these parameters are still being investigated (e.g., Ibor et al., 2019; Menillo et al., 2020).

The development of such comprehensive gene expression methods for biomonitoring in sentinel organisms may provide a turning point to in-depth identification and an absolute analysis of individual health conditions (Ibor et al., 2019). The application of gene expression is still at an early stage due to a poor representation in protein and gene sequences in the databases of some bioindicator species, as well as a lack of knowledge of the functions of many genes (Louis et al., 2017). A variety of different approaches has been applied, including variation in DNA (genomics), evaluation of cell or tissue protein expression (proteomics), general evaluation of metabolite concentrations (Metabolomics) and measurement of mRNAs expressions for genome-wide evaluation of genes (Transcriptomics) (Ma et al., 2020). Collectively, in the context of chemical exposure, these approaches have been termed toxicogenomic. Amongst these approaches, the transcriptomic approach for the simultaneous measurement of numerous thousands of genes expressions can offer a means to evaluate the mechanism of actions of chemical contaminants through

different biological pathways. In turn, this information can be related, from knowledge of the genes and pathways involved, to the effects in the tissues of an organism (Huang et al., 2017). Therefore, different toxicogenomic approaches in model species of environmental monitoring relevance have been shown to have much potential (Wang et al., 2018).

Whilst comprehensive gene expression profiling, using toxicogenomic technologies, is extremely powerful, their application to a large sample size and to a variety of species is economically not practical for most monitoring programmes. However, these toxicogenomic studies can indicate subsets of genes, whose expression would be informative and who could be assessed by a less expensive and demanding technique such as PCR (Mishra et al., 2020). PCR arrays, consisting of multiple simultaneous quantitative PCR reactions for targeting specific subsets of informative gene expressions, have been used to analyse field collected fish from polluted sites, and can indicate early warning signals of a stress response (Ibor et al., 2019). The prerequisite for developing such PCR arrays is the availability of sequence data for informative genes from target species such as availability of Tilapia genome. Using global PCR arrays, a comparison using Flounder and Zebrafish transcript alterations between an environment contaminated with PAH and a reference site, was documented (Kim et al., 2018). Similarly, in a comparison between an estrogen effluent collecting site and a clean site, an acute estrogen exposure signal was detected in hornyhead turbot (*Pleuronichthys verticallis*) and Black jaw tilapia (*Sarotherodon melanotheron*) (Baker et al., 2009; Adeogun et al., 2019).

PCR arrays are mainly centred on messenger RNA (mRNA) molecules, indicating that a gene expression is quantified. This quantification of genes expression is in the form of either upregulation or downregulation during exposure to environmental contaminants in the aquatic environment. PCR arrays furnish an opportunity to adequately understand how fish respond to changes in the events of alterations in environmental conditions (Chen et al., 2020). PCR assays can be employed to target any given cells, specific organs/tissues or even the whole fish. The gene expression profiling using a PCR array can give an insight into the changes of the genes responsible for immunity, biotransformation, detoxification, estrogenicity, genotoxicity, and other physiological processes in fish.

1.2 Concerns about chemicals in a global society

Our daily lives are surrounded by chemicals, due to excessive demands and use in our society. This paved the way for their increased production globally. The global chemical production in 2017 was estimated to be around 2.3 billion tons, almost doubled from 1.2

billion in 2000, excluding pharmaceuticals. The estimated worldwide sale of chemicals, including pharmaceuticals, was around 5.68 trillion U.S dollars, indicating the pace at which chemical industries are increasing, making them the second largest manufacturing industry in the world (UN global chemicals outlook II, 2019). However, there is concern for the estimated increase of chemical productions in different regions of the world, as sales are projected to double from 2017 to 2030. By 2030, China is predicted to be responsible for almost 50% of global sales. In emerging economies and developing countries, annual growth rates are highest, especially in the Middle East, Asia-Pacific and Africa, where most countries have none or weak regulatory legislation (UN global chemicals outlook II, 2019). Under the European Union's chemical regulations REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), more than 143,000 chemical substances are pre-registered, and almost 13,400 substances with more than 100 tons per annum are put on the European market (European Chemical Agency, 2018).

To address these problems, data gaps should be identified from the previous regulatory framework that will curtail these problems before they become overwhelming. Therefore, a practicable measure in the management of chemicals and waste is of paramount importance to reduce the costs to national economies, as well as be practical and suitable. Moreover, most of the risk assessments on chemical experiments in different countries are made by individuals as chemical exposure assessments in the laboratory. Meanwhile, the legislation does not consider the exposure ramifications of the mixture of chemicals under normal circumstances, let alone the potential for collective consequences (Thrupp et al., 2018). However, most of these chemicals end up in the aquatic environment as a mixture chemical. It is therefore important to develop vast chemical assessment protocols to take care of chemicals previously not highlighted in the monitoring protocols to integrate the legacy of individual chemicals with a cocktail of chemical mixtures.

1.3 Organic chemicals in the aquatic environment

Due to the industrialisation and urbanisation in many parts of the globe, the majority of anthropogenic (man-made) chemicals in regular use today find their way into the aquatic environment. This has become a global concern, as these chemicals are a threat to the aquatic environment and the biota. These chemical mixtures are made up of different classes of natural and man-made contaminants ranging from pesticides, personal-care products, polycyclic aromatic hydrocarbons, pharmaceuticals, polychlorinated biphenyls, phthalates, dioxins, endocrine disruptive chemicals, surfactants and metals to name but a few. They are

discharged through different sources that eventually enter the aquatic environment (Gwenzi and Chaukura, 2018). They do not only exist as individual chemicals, but also as a combine mixture (Altenburger et al., 2013). Discharges of these chemicals from point sources, including municipal sewage effluents, industrial effluents, resource extractions (such as oil explorations and mining), spills, and land waste disposal sites directly result in their release into the aquatic environment. Non-point sources, such as agricultural activities, urban runoff and atmospheric deposition, ultimately find their way into the aquatic environment as well (Babayemi et al, 2016). In this thesis, attention is given to the exposure of different classes of chemicals with global environmental relevance to fish, including Polycyclic aromatic hydrocarbon, Polychlorinated Biphenyls, Pesticides, Pharmaceuticals, Perflourinated compounds, Phthalate esters and heavy metals.

1.3.1 Polycyclic aromatic hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons are high molecular, ubiquitous and hydrophobic organic compounds. They consist of more fused rings in angular, linear and clustered arrangements containing hydrogen and carbon (Verma et al., 2012). They are cytotoxic, carcinogenic and mutagenic and have the ability to accumulate in different organs due to their genotoxic nature. They have become ideal real model compounds for eco-toxicological studies (Aderinola et al., 2018). PAHs leak into the aquatic environment due to natural and human activities like domestic heating sources, or the incomplete combustion of materials (pyrogenic PAHs) including petroleum products, oil and coal, and wood combustion (Ravindra et al., 2008). Other sources include productions of fossil fuel (petrogenic PAHs), coal tar, asphalt, and runoff from roads (Hood et al., 2011). Natural sources of PAHs include fire and volcanic eruptions and diagenesis of organic matters (Meng et al., 2019). Individual PAHs may have a contrasting mechanism of actions and on some occasions antagonistic and synergistic effects have been observed, rather than a combined additive effect (Hernandez et al., 2019). Thus, the secondary source and transient storage compartment of PAHs is the sediment. Due to external environmental variations or bioturbation, PAHs might be re-released into water from the sediment (Yao et al., 2016). The exposure of tilapia fish to PAH chemicals in a mixture and as an individual substance is investigated in this thesis on biotransformation mechanisms and biomarker response.

1.3.2 Polychlorinated biphenyls (PCBs)

Polychlorinated biphenyls (PCBs) belong to the family of coplanar polycyclic halogenated hydrocarbons produced as a result of the chlorination of biphenyls with 10 substituent

positions (2-6 and 2'-6'). The chemical formula for PCB is $C_{12}H_{(10-n)}$ $Cl_{n,}$ where n= the number of chlorine atoms 1-10. Even though the USA in 1979 and the Stockholm Convention on Persistent Organic Chemicals in 2001 banned the use of PCBs, various types of polymeric products, such as plastics, PCBs polyethylene, rubber and pesticides, form PCBs as a by-product. Despite their ban, PCBs are still found in the environment, particularly in sediment, water, air and soil, and can be transported over a long distance, based on the number of chlorine atoms that are able to substitute for the hydrogen atom on the biphenyl structure and at their positions (ortho, para, meta). There are about 209 possible congeners (individual) of PCBs, depending on the distinct number of chlorine atoms and their positions (Ododo and Wabalo, 2019). Each of these has a biphenyl structure (two linked benzene ring), containing from 1 to 10 chlorine atoms. However, only 209 individual PCB compounds are possible in theory, and in industrial mixtures only 130 have been discovered at concentrations > 0.05% (Ododo and Wabalo, 2019).

In this research on the biotransformation mechanisms in fish, the exposure of PCBs congener Aroclor 1254 will be investigated both in the laboratory and in the field. Due to their persistence, bioaccumulation and toxicity, OSPAR list PCBs as a priority substance, even though PCBs are not listed as priority substances in Water Framework Directives.

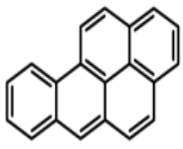


Figure 1.1: Benzo [a] pyrene as an example of PAHs relevant in this study

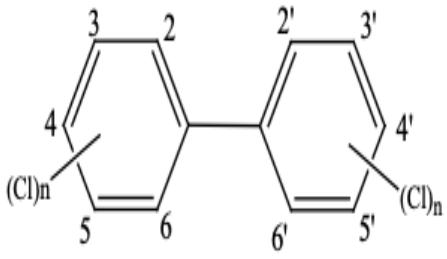


Figure 1.2a: General structure of PCBs with the standard numbering of the substituent's positions: 2-6 and 2'-6' = 10 likely chlorination sites of the biphenyls: Site C2 and C6 = Ortho site; C4 = Para site; and C3 and C5 = Meta site. (ATSDR, 2000; Ododo and Wabalo, 2019).

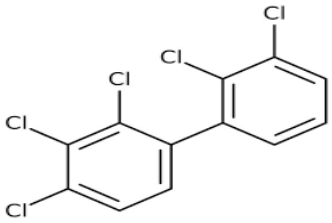


Figure 1.2b: Chemical structure of Aroclor 1254, an example of PCBs applicable in this study.

1.3.3 Pharmaceuticals and personal care products

Pharmaceuticals are produced and used at an increasing pace and their presence in the aquatic environment has multiplied with detrimental consequences to aquatic organisms. Pharmaceuticals are sourced from natural organic or inorganic compounds for the treatment and prevention of different pathologies, infant mortality decrease, feeding efficiency improvement, growth rate acceleration, life expectancy increase (Ojomaye and Petrik, 2019), as well as improvement of the wellbeing of humans and other animals Due to the global increase of pharmaceutical production, they have become ubiquitous in the natural ecosystem (Ebele et al., 2017).

More accurate detections of several pharmaceuticals, even at low level concentrations, ranging from ng/l to µg/l in the aquatic environment, have been documented due to advancement in analytical techniques (Cecilia and Petrik, 2019). More recently, other scientists have documented the negative impact of different pharmaceuticals on the aquatic environment, on surface water (Lacey et al., 2012), in seawater (Lolic et al., 2015), in groundwater (Wen et al., 2014), in sediments (Grabicova et al., 2015), in wastewater treatment plant effluents (Comber et al., 2018), and in organisms (Miller et al., 2015). The constant discharge into the aquatic ecosystem could grant these compounds a permanent presence (pseudo-persistence) (Cecilia and Petrik, 2019). In this thesis, pharmaceutical drugs, especially17-alpha-Ethinylestradiol (EE2) exposure in the laboratory alone and in field mixtures, are investigated on the biomarker response in fish.

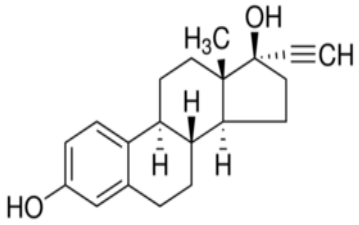
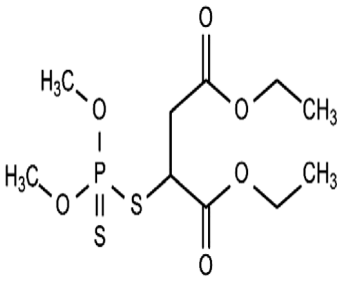


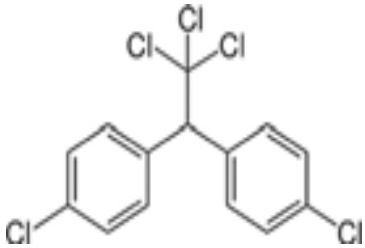
Figure 1.3: General structure of EE2 relevant in this study

1.3.4 Pesticides

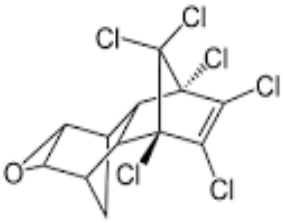
In agricultural production, the extensive application of pesticides as natural or synthetic compounds, was believed to increase the quality and yield of the produce, repel, prevent, reduce and destroy pest infestations and other unforeseen circumstances that could bring losses to the farmers (Dehghani et al., 2017). Some of the common pesticides are insecticides, fungicides and herbicides. Their usage is believed to play a role in the management of pests, be more economical and ease the labour of farmers (Sharma et al., 2014). But due to the presence of pesticide residues in various sections of the aquatic environment, their negative impact might exceed their importance (Ali et al., 2014). Their chemical toxicity to the aquatic biota is due to their properties such as chemical structures, functional group, nature of preparation, long distance transport, improper storage, long half-life, lipophilicity, bioaccumulation and applications. All these increase the chance of contamination, as only a small fraction of applied pesticides reaches the target organisms while the bulk is dispersed into the environment (Uçkun, 2017). The European Union in its decision watchlist 2015/495/EU classified pesticides as priority substances (Sousa et al., 2018). Pesticides were reported to be found in an environmental matrix including water, sediment, air and soil. Sediment is the ultimate sink of pesticides and a new source of pesticide contamination in the aquatic environment (Fang et al., 2017). Pesticides can be organochlorides, organophosphates, carbamates, phenylamides, Benzoic acids, phthalimides, dipyrids, pyrethroids, or others, based on their chemical structures, functional groups, toxicity, modes of action, targets and formulation (Kim et al., 2018). In this study, the exposure to Carbamates, organochlorine and organophosphate pesticides individually and in mixtures are investigated on biomarker response in fish.



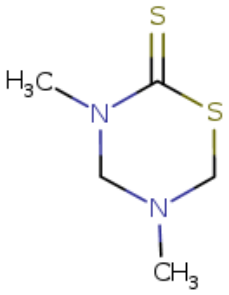
(a) Malathion



(b) DDT



(c) Dieldrin



(d) Dazomet

Figure 1.4a-d: Chemical structures of Pesticides applicable in this study

1.3.5 Phthalates esters

Commonly phthalic acid esters (PAE's) are produced as plastic softeners; they are found in almost all plastic materials, polyvinylchloride and other polyethylene materials such as building and packaging materials, toys, lubricants, paints and adhesive to improve flexibility and workability. Due to their global prevalence, they are found in the aquatic environment in a diffused state over time, because they are not covalently attached to the plastic (Zhang et al., 2018) and are likely to leach from the material. PAEs can occur in different environmental matrices, including in atmospheric air, the aquatic environment, in the soil, in sediments and in the body of humans and other animals resulting in far-reaching human exposure and aquatic environment contamination (Net et al., 2015a). PAEs have the ability to travel long distances in the aquatic environment and are likely to enter a food chain once discharged. Phthalates have different physical and structural properties, thus their environmental chemodynamics is affected. In the present research, the exposure effect of Bis (2-ethylhexyl) phthalates (DEPH) singly in the laboratory and their mixture in the field will be studied and documented in Nile tilapia species (*Oreochromis niloticus*).

1.3.6 Perflourochlorinated compound (PFC)

Since the 1950s, Perflourochlorinated compounds (PFCs), have been produced globally and used in numerous industrial and commercial applications. PFCs are organic compounds in which a fluorine atom substitutes all the hydrogens of the hydrocarbon backbones. PFCs have very high thermal and chemical stability due to very stable high energy fluorine-carbon bonds (Fauconier et al., 2020). This bond makes PFCs more resistant to hydrolysis, photolysis, microbial degradation and metabolism by vertebrates. PFCs are well known to be reactive with acids and bases, a property that makes them attractive globally. These properties make them exceptionally tolerant to chemical, thermal and biological degradation and biomagnify in the food chain, which resulted in a global concern on their persistence and toxicity (Fauconier et al., 2020). PFCs are categorised into Perflourinated Carboxylic acids (PFCAs), Perflourinated Sulfonic acids (PFSAs), high-molecular-weight fluoropolymer, and low-molecular-weight perfluoroalkanamides and fluorotelomer alcohol (Stahl et al., 2011; Fauconier et al., 2020). However, due to their persistence, stability and resistance to oil and water, the exposure to PFOS results in adverse effects in fish, including cellular apoptosis and changes in gene expression linked with apoptosis such as p53 and Bax; oxidative stress induction possibly due to the generation of ROS as a consequence (Liang et al., 2017). Therefore, in this study, the effects of exposure to individual PFOS in the laboratory as well as the mixture effect in the field will be documented.

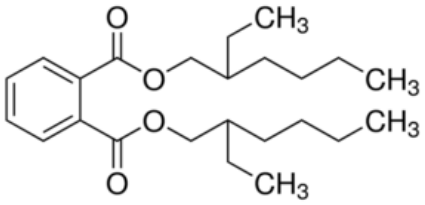


Figure 1.5: General structure of Bis (2-ethylhexyl) phthalate relevant in this study

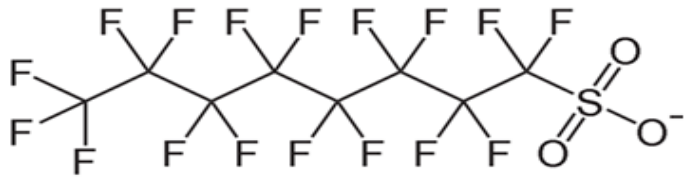


Figure 1.6: Structure of PFOS relevant in this study

1.3.7 Heavy metals

Due to the continuous loading of the aquatic environment with chemicals of both natural and man-made origins, the contamination of heavy metals is of paramount concern. This is due to their innate toxicity, accumulation, different sources, non-biodegradability, persistence and ubiquitous nature, as well as biomagnification through the food chain. Heavy metals are chemical elements with high toxic potential at reduced concentrations. Heavy metals are natural integral parts of the earth's crust and regarded as natural trace ingredients of the aquatic environment. Their allowed concentrations are restricted but have been elevated due to anthropogenic activity sources (Al-Taee et al., 2020; El-Khayat et al., 2020). The sources of heavy metals for the aquatic environment include the natural weathering of rocks containing metals and volcanic eruptions (Ali et al., 2019), as well as anthropogenic, domestic and industrial sources such as mining, smelting, municipal waste, aquaculture, and petrochemical industries. Cadmium exposure will be investigated individually in the laboratory and in combination in the field in the present study.

1.4 Mixture effects and chemical interactions

The challenges posed by mixture toxicity in the aquatic ecosystem are enormous. Domestic sewage, agricultural runoffs, industrial effluents, paper and pulp, and mining discharge various forms of xenobiotic metabolism chemicals including PAHs, PCBs, estrogen, metals, and pesticides. Data from individual chemical exposures could elucidate a mechanism of actions, but due to the chemical interactions in a mixture of chemicals, the individual chemicals may exert an additive (i.e., combination) effect on the organisms in the aquatic environment. A mixture of chemicals could have additive, antagonistic or synergistic toxicity on the organisms, depending on the interactions of various chemicals. Therefore, when different chemicals have opposite or similar pharmacological mechanisms of action, pharmacodynamic interactions can occur in the mixture exposure. As such, a chemical can exert influence on the other chemical mechanisms of action in processes such as excretion, metabolism, distribution or even absorption (Celander, 2011). Co-exposure to PAH and estrogenic chemicals has been observed to antagonise each other's effect. Therefore, as reported in many studies, the simple additive effects of an individual xenobiotic compound cannot predict either the laboratory or field xenobiotic mixture effects. Furthermore, most of the regulatory chemical risk assessments are still based on individual chemical exposure toxicity (Kienzler et al., 2016; Thrupp et al., 2018). Therefore, this present study will look into the understanding of both single and mixture exposure of chemicals.

1.5.0 Environmental Water quality monitoring

Monitoring the quality of water aims to contribute information to protect the environment from harmful biological adverse effects arising from various contaminants of anthropogenic, point and non-point sources. Monitoring the quality of water is set out to control the chemical input and safeguard the ecological status of the water body for specific use (Altenburger et al., 2019). The Sustainable Development Goals of the United Nations (UN, 2015), the EU strategy for a non-toxic environment (EC, 2016), and the Organisation for Economic Co-operation and Development's (OECD, 2016) Recommendations on Water, the Marine Strategy Framework Directives (MSDF, 2008/56/EC) as well as the European Water Framework Directive (WFD; EC, 2000/60/EC) stressed the need for the protection of water resources from any harmful chemical substance contamination (Hylland et al., 2017). Accordingly, the obligatory monitoring bodies have a key mission in the assessment of water quality and management. Different methods are employed depending on the monitoring's specified objectives. But any specific approach has to assess the limited costs, technological tools, and data obtained to be converted into a meaningful decision-making document to achieve the desired monitoring programme goals and management implementations (Brack et al., 2018). According to Hylland et al. (2017), it is important to have information on the concentration of chemicals in a biota and its environmental matrices (1) to relate a particular chemical with an observable effect for regulatory objective, (2) to make sure that no chemical concentration is beyond a set limit for human consumption and (3) to record the occurrence of the chemicals that potentially may or may not cause harm.

Reliable sampling and analysis techniques for different chemicals in the aquatic ecosystem for the monitoring of the quality of water may be challenging, since conventional chemical monitoring limitations have been explicitly recognised (Emelogu et al., 2013). It is therefore imperative to analyse chemical pollution in a comprehensive way within the limited available resources. This is in order to wholly diagnose the danger to biota, the ecosystem and human health and curtail the unforeseen from happening. The current perspective of an individual chemical analysis is not sufficient to estimate the probability that the substance's contamination can cause injury to aquatic biota and human health. Neither can the approach be used to establish schedules for actions to decrease the impact of chemical contaminations in the aquatic environment. Therefore, additional integrative alternative methods are needed to enlarge the number of chemicals to be evaluated, considering the limited number of chemicals under investigation by the current approach in the European Water Framework Directive.

For example, the use of conventional chemical analysis techniques cannot provide data on the toxicologically relevant concentration of mixture chemicals on the antagonism or synergism especially of pesticides (Kienzler et al., 2014). Furthermore, chemical analysis only considers information of individual chemical substances that are detected. Chemicals that are not evaluated or identified due to low concentrations or because they are below the detection limit are not considered, even though these chemicals might be biologically relevant and active (Kienzler et al., 2016). An accidental pollution can occur unnoticed, as well as other discharges during the time of annual pesticide and fertilizer applications, domestic and industrial discharges and flooding. Also, concentrations of a chemical substance may change over a period of time and this will affect the sampling analysis. Despite the advancement and excellent high precision and sensitivity of modern passive sampler devices (PSDs) and the analytical infrastructure in chemical monitoring little information is received in the approach on the possible adverse effects of biological complex mixtures of targeted or non-targeted compounds on the organisms in field studies, unless they are combined with other additional tests such as bioassays (Dopp et al., 2019). Passive sampler devices could possibly show the presence of, and the exposure to chemicals, but they have to be incorporated with bioassays to help in obtaining a comprehensive picture of the potential risks of exposure to these multiplex combined chemicals in the aquatic environment that could result in eliciting harmful effects (Toušová et al., 2019). Passive samplers absorb and preconcentrate free dissolved chemical substances in a long time of sampling period. They come up with a time-weighted average concentration of the desired analyte and a guess of a freely dissolved bioavailable aqueous compound of a toxicological relevant but yet are considered semi-quantitative (Miller et al., 2019).

State-of-the-art analytical tools that are sensitive, such as gas chromatography mass spectrometry (GC-MS//MS) and liquid chromatography mass spectrometry (LC-MS/MS) prove to be effective in detecting low concentrations of certain targeted chemical compounds in a specific relevant selected catchment area (Brack et al., 2016). However, the biological adverse effects of these chemical mixtures are seldom predicted in these instrumentations. Also, the quantity of known and unknown chemicals in multiplex water samples are far more than the quantity to be measured. Additionally, reaching the desired limit of detection (LOD) with an analytical chemical analysis in order to achieve the ecotoxicological relevant concentration is quite challenging (Itzel et al., 2017). Therefore, uncharacterised chemical mixtures, biologically relevant and active, such as transformed products, need a comprehensive analysis using additional approaches to complement the chemical analysis

of enriched samples of water (Escher et al., 2014). Thus, the chemical monitoring of a small number of preselected single chemicals cannot adequately provide useful information to deduce the probability that chemical mixtures or transformed products (TPs) may cause injury, whereas the likelihood of missing the consequential danger is enormous and escalating (Sousa et al., 2018; Brack et al., 2019). Due to this limitation, the use of additional alternative methods which are logical in forecasting missing and possibly harmful chemicals and assess the probability of harm due to complex chemical mixtures in the aquatic environment need to be developed. This would enhance the reliability and cost effectiveness of water quality monitoring. The use of biomarker tools in biological monitoring has gained momentum in the recent past, as a complement or as an alternative to replace chemical analysis in water quality monitoring of the aquatic environment (Brack et al., 2019).

1.6.0 Biomarkers

Biomarkers are essential tools used to quantify a chemical's sublethal effects in a natural wild environmental setting. In an ecotoxicological context, biomarkers are any biotic features of an organisms, covering cellular, biochemical, physiological, molecular processes and behavioural features, sometimes up-to population level alterations, that can be measured in organism samples (tissues or fluids) proving sensitivity to an exposure and/or to toxic effects of individual or mixtures of xenobiotic contaminants (Kroon et al., 2017; Van der Oost et al., 2020). However, in the context of toxicology, biomarkers are employed to encompass virtually any quantifications demonstrating an interaction between a biological system and a possible risk, which may be either biological, physical or chemical (Van der Oost et al., 2020). Biomarkers can be measured either in controlled laboratory exposure condition (Kroon et al., 2017); or in contrast to bioassays but similar to ecological tools, in field-exposed residential organisms, primarily focusing on sampled organisms to be analysed; and in active monitoring in situ in a cage exposed to contaminants, compared to references (Vieira et al., 2016).

The most promising benefit of biomarker investigations is the ability to detect and provide an early warning for deviations of normal organisms' health or disturbances at the population and ecosystem levels from significant irreparable adverse effect damage, providing a short-term yardstick for long term biological adverse effects (Paniagua-Michel and Olmos-Soto, 2016). These early signals can be viewed in both a time and a concentration approach, since ecosystem damage can take a long time to restore. It is clear that, when an organism is challenged by an environmental contaminant insult, the organism responds via observable

functional or structural changes. First, these changes occur at molecular or genetic level, followed by cellular, tissue, organ or whole organism responses. Therefore, before any major severe harmful effect could occur, the harm of any chemical agent can be assessed by monitoring physiological (such as growth rate and reproductive performance), biochemical or molecular changes (through organelle functions and structures, hormonal levels, gene expression patterns and some specific enzyme activities (Vander Oost et al., 2003; Lee et al., 2015).

For the purpose of environmental risk assessment, without bias, certain criteria are adopted for practical biomarkers (Van der Oost et al., 2003; Paniagua-Michel and Olmos-Soto, 2016).

- The test to measure the biomarker has to pass quality assurance and should be dependable, inexpensive, and easy to use.
- The response of the biomarker should be high enough to detect toxic effects of contaminants at an early stage.
- The relationship between the response of the biomarker and the long-term impact on the biota should be demonstrated.
- Biomarkers should primarily respond to a specific pollutant or group of pollutants.
- The response of biomarkers in a concentration dependent manner should be in tandem with the alteration in the ambient levels of the pollutants.

1.7.0 Application of biomarkers in Biomonitoring techniques

Biomonitoring was coined by ecology and ecotoxicology as an applied interdisciplinary science to refer to the routine use of organisms or their responses to establish a state or alterations in the environment. The underlying assumption in biomonitoring is that the intensity of contaminants in the tissues or body fluids of living biota are a proportionate quantity of the external levels of contaminants that are transported to the organisms. Consequently, they indicate whether an organism has been exposed to biologically relevant contaminants, the type of the contaminant present and the likelihood that the contaminants bioaccumulate in the tissues of the organisms affecting it (Mohan et al., 2016). This approach exploits the understanding that the chemical leaves a marker upon entering the organism indicating an exposure effect. The marker may be a variation in the organism's physiology or morphology as a result of the chemical contaminant. The outcome provides the required data for the measurement of the quantity of synthetic and natural chemicals that entered the

organism's body and the equivalent effects it induced. Biomonitoring helps in the holistic evaluation of the actual biological adverse effects of contaminants, anticipating the possible effects and the exact consolidated toxic effects of combined contaminants in the aquatic environment, prior to population, community and ecosystem adverse biological effects.

For the successful implementation of biomonitoring, certain biological monitors are usually employed. They are referred to as sentinel organisms or bioindicators. Aquatic biological communities are accurate and sound indicators of the aquatic ecosystem integrity as they are prone to wide arrays of physical and chemical impacts including antagonistic, synergistic and additive effects. Different organisms were recently employed as sentinel organisms to biomonitor aquatic contaminations. Such organisms include plants, planktons, insects, amphibians and fishes, with each one having different characteristic qualities. An effective sentinel organism should be stationary, be tolerant to contaminant exposure without dying, be cultured, maintained and resist stress in the laboratory, have a long life, be easy to acquire, have seasonal distribution and abundance, be big enough to provide experimental tissue, have well known ecological characteristics and significance, show dose-effect relationships, have a high sensitivity to environmental pollutants and be important in the food chain (Mohan et al., 2016). As different organisms are sensitive to different contaminants, their suitability for various bioindicators also differs in the natural environment (Nikinmaa, 2014).

1.7.1.1 Molecular Biomarkers of contaminants exposure and effect

The implications of the contaminants' exposure and effects can be observed through biochemical responses including proteins, enzymes and nucleic acids of the organisms. This might enhance our knowledge on the significant biological effects of these contaminants up to the community and ecosystem stage. Responses at cellular and molecular levels are used as early warning signals in the quantifications of biological effects in water quality monitoring. Certain biochemical responses are among the most responsive biomarkers to be employed as early warning signals for the deviation for a normal organism's health. They have demonstrated to be distinct indicators of sub-lethal exposure of organisms to certain classes of environmental contaminants. In the laboratory as well as in the field, changes of these biochemical biomarkers can precisely evaluate toxic insults from a particular class of environmental xenobiotics, and this serves primarily as an initial response of the adverse biological effect to the organisms (Van der Oost et al., 2020). Therefore, their quantifications can serve as an indicator of changed cell performance. In the aquatic environment, even at low concentrations of a contaminant, biochemical responses are evoked and detect changes

(Capela et al., 2016). An assessment of biomarker criteria shows that phase I biotransformation enzymes and biliary biotransformation products in fish are valued molecular biomarkers in water quality monitoring. Phase II biotransformation and antioxidant enzymes on the other hand, even though not as diagnostic, sensitive and specific as phase I enzymes, are regarded as supplemental biomarkers of exposure of different micropollutants in the aquatic environment (Van der Oost et al., 2005, 2020). Oxidative stress biomarkers in addition, has gained attention due to various environmental pollutants insult. As such no specific biomarker is identified to examined oxidative stress. But among the parameters investigated to study oxidative stress are *GSTs, CYP 1A* and *MT* genes. Other biomarkers include reproduction and endocrine disruption biomarkers and apoptosis biomarkers.

The use of pathological and morphological indices in biomonitoring studies are frequently regarded as excellent indicator of the effects of exposure to contaminants at top-level biological structures. The establishment of adverse effects through the examination of lesions, injuries, changes or even the development of tumours in specific tissues of fish (e.g., gills, kidneys, liver, muscles, intestines and spleen) can provide information on either acute or chronic exposure to contaminants in the aquatic environment (Azevedo et al., 2020). A considerable benefit of histopathological biomarkers is the identification of pathological changes in various tissues separately, consequently allowing clear connections with physiological processes such as reproduction and respiration (Yancheva et al., 2015). The morphological parameters, the so-called gross indices, which indicate the conditions of fish, manifestation and glaring features are also employed as biomarkers in water quality monitoring. These indices include the Gonadosomatic index (GSI), Spleen somatic index (SSI), length-weight relationship (LWR), Visceral somatic index (VSI), Growth index (GI) (Kroon et al., 2017), liver somatic index (LSI) or hepatic somatic index (HIS) and the Condition factor (CF) (Adeogun et al., 2016). The employment of length-weight relationship and condition factor were used as a biomarker of effect in this study.

1.7.1.2 Phase I and II Biotransformation enzymes biomarkers

Phase I biotransformation enzymes are biomarkers of exposure and are both measured using either protein levels, functional catalytic activity or the mRNA expression of their corresponding genes in different organisms. Cytochrome P450 IA is one of the Phase I biotransformation isoenzymes family and is related to the biotransformation of different contaminants such as Dioxins, Polychlorinated biphenyl (PCBs), Polycyclic aromatic

hydrocarbons (PCBs), and Halogenated aromatic hydrocarbons (HAHs). Different patterns of CYP 450s isoenzymes depend on the exposure to different contaminant types (xenobiotics). Cytochrome P450 (CYPs) are haemoprotein superfamily enzymes containing heme protoporphyrin attached to cell membranes found in almost all classes of eukaryotes and prokaryotes organisms that catalyse biological oxidation and reduction reaction. First reported by Klingenberg and Garfinkel in 1958, due to the spectrophotometric peak at the wavelength of the absorption of light at a maximum of 450 nm, when complexed with carbon monoxide (CO) in a specific liver derived pigment, hence the pigment 450 (P450) (Sharifian et al., 2020). Most of the chemical contaminants are lipophilic, hence not easily eliminated by the organisms. This system shows the crucial pathways in which these lipophilic substances are biotransformed, and the increased chance of being eliminated to avoid their toxic concentrations to bioaccumulate in the tissues of organisms. The process entails the transformation of foreign contaminants (xenobiotic), and endogenous chemicals to less active and more water-soluble metabolites to be more readily eliminated through either urine, gills of fish or bile. During the biotransformation of these lipophilic compounds, there may be a generation of metabolites through bioactivation by P450 enzymes that are more potent than their original compounds. These may result in toxic compounds to affect fish through distribution, persistence and bioaccumulations (Schlenk et al., 2008). This introduction of a functional group makes the compound more active for it to be metabolised in reactions involving phase II biotransformation enzymes.

The overall redox reaction for a typical cytochrome mediated monooxygenation is depicted below as follows:

$R\text{-}H + O_2 + NAD(P)H + O_2 \rightarrow ROH + NAD(P) + H_2O$

The *CYP* superfamily gene encoded more than 50,000 enzymes spread over divergent organism. Evidence from the phylogenetic analysis showed the ancestral *CYP* gene from the prokaryotes (Nelson and Strobel, 1987). The increased induction of *CYP 1A* mRNA expression through the aryl hydrocarbon receptor pathway is used as a biomarker of organisms exposed to planar aromatic hydrocarbons and dioxin-like compounds to determine their health status in a contaminated aquatic environment.

1.7.1.2.1 CYP1A as a biomarker gene in fish

Among the most frequently studied CYP isoforms in fish is the cytochrome P4501A (*CYP1A*) gene family. Having split from CYP2 about 450 million years ago, *CYP1A* has a

crucial function in the oxidative biotransformation of various persistent environmental aromatic hydrocarbons, such as planar halogenated aromatic hydrocarbons and PAHs (Schlenk et al., 2008. An increased expression of *CYP 1A*, is employed in the analysis of fish exposed to different structurally aromatic pollutants. The expression level of *CYP 1A* is usually low in fish, which have not been exposed to persistent polycyclic aromatic hydrocarbons and polychlorinated biphenyls and dioxin related compounds. The response in the induction of *CYP 1A* is a highly sensitive endpoint and reasonably accurate for PAHs, PCBs and dioxin like compounds. Quantification of the mRNA level using qPCR is reported to be a sensitive method in biomonitoring programs (Quintanilla-Mena et al., 2020). The induced expression of *CYP 1A* is mediated through the aryl hydrocarbon receptor (AhR) signalling pathway, and the initial induction response needs binding to the aryl hydrocarbon receptor. In this study, the role of *CYP 1A* in biotransformation of exogenous chemicals due to exposure to different classes of halogenated aromatic hydrocarbons through CYP1A-AhR signalling pathways will be explored.

The dioxin or aryl hydrocarbon receptor (AhR) is a ligand transcriptional factor belonging to the basic-helix-loop-helix/Per/aryl hydrocarbon receptor nuclear translocator protein/single minded protein Per-Arnt-Sim (bHLH/PAS) DNA binding protein family, a typical xenobiotic receptor and a classical transcription regulator of different batteries of genes including *CYP 1A* (Lv and Huang, 2020). The basic-helix-loop-helix (bHLH) is positioned in the N-terminus containing site b, and site HLH. The binding of the transcription factor to the target DNA is the function of site b, while site HLH functions in the dimerization of protein-protein (Nebert, 2017). Following the bHLH site is the Per-Arnt-Sim (PAS) site, designated in honour of three proteins as follows: Period circadian protein, aryl hydrocarbon nuclear translocator protein and single-minded protein. The Per-Arnt-Sim (PAS) contains PAS A and B, which also binds to the aryl hydrocarbon nuclear translocator protein (ARNT). The Per-Arnt-Sim site B is the ligand binding location of the aryl hydrocarbon receptor (AhR) (Sakurai et al., 2017). Apart from its role in xenobiotic metabolism, the aryl hydrocarbon receptor (AhR) is also involved in crucial functions in different biological responses such as embryonic development, sustaining physiological homeostasis, proliferation and differentiation of cells, modulation of cell gene expression, immune response, and hormone metabolism.

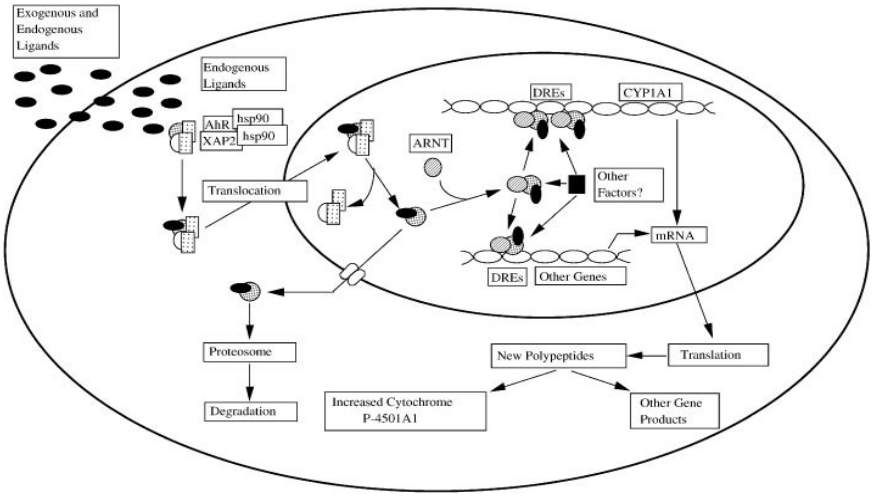


Figure 1.7: The molecular mechanism of AhR mediated CYP1A induction adopted from Denison and Nagy, 2003.

1.7.1.2.2 Phase II Biotransformation enzymes biomarkers

In the phase II biotransformation enzyme system, the major biomarkers use major signaling pathways involved in the conjugations of electrophilic contaminants and their metabolites through the most abundant non-protein thiol, occurring in reduced glutathione (GSH) and in oxidised glutathione form (GSSG). GSH plays a prominent role in the defense of organisms against oxidative stress and in the detoxification of foreign chemicals (xenobiotics compounds) through reactions with the compound interchanging groups of hydrogen, nitro and chlorine. The ratio between reduced glutathione (GSH) and oxidised glutathione (GSSG) as a thiol status is used as a biomarker of oxidative stress (Iqbal Dar et al., 2020). *Glutathione-S-transferases (GSTs)* is another biomarker of the phase II biotransformation enzyme system. *GSTs* play a prominent function in intracellular transport as well as in the defence mechanisms through catalysing conjugations of electrophilic compounds by reducing GSH via its cysteine rich thiol with both endogenous and exogenous compounds against oxidative damage and peroxidative DNA products and lipid. Conjugation or addition reactions entail the covalent addition of polar groups or large compounds such as amino acids or sugar to the exogenous compounds. The induction of GSTs is considered a biomarker of stress and detoxification (Paul et al., 2019). The use of *Uridine 5'-diphosphoglucuronic transferase* has been reported to act as a key signalling pathway in the biotransformation and elimination of potentially harmful endogenous and exogenous compounds in organisms. *UDP- GT* catalyses the conjugation of UDP-glucuronic acid to a lipophilic receptor substrate. The activity of *UDP-GT* is also considered a biomarker of detoxification (Leaver et al., 2007). Most of these phase II enzymes (*GSTs and UDP-GT*)

act as catalysts in facilitating these synthetic conjugation reactions, which consequently ease the eliminations of these compounds by supplementing the polar group (glucuronic acid and glutathione) to the molecules in the reaction. Like *CYP IA* genes, *GSTs and UDP-GTs* are also among the aryl hydrocarbon gene battery and their expressions are also regulated through aryl hydrocarbon receptors (Van der Oost et al., 2005).

1.7.1.2.3 GST as a biomarker gene in fish

Two types of xenobiotic compounds are categorised as monofunctional and bi-functional compounds based on their possibilities to begins either phase I or Phase II metabolism process (Fig. 1.8). Overall, the monofunctional compounds inducers are electrophilic substances that are able to interact with reduced glutathione (GSH) and choosily induce the transcription of phase II metabolism enzymes particularly via antioxidant response element or electrophile response element (ARE/EpRE) (Schlenk et al., 2008; Park et al., 2020). This highly grant the triggering of the detoxifying enzymes of phase II reactions and consequently elevated carcinogen detoxification (Buetler et al., 1995). Furthermore, elevated levels of the phase II enzymes were shown to be practically related to the elevated gene transcription, implying unequivocal response among antioxidant response element or electrophile response element (ARE/EpRE) and phase II metabolising enzymes responding to the monofunctional compounds inducers resulting to elevated excitement of detoxification of the xenobiotic (Bergelson et al., 1992). Conversely, bifunctional compounds inducers have the ability to induce both phase I and Phase II metabolising enzymes comprising of mainly the traditional CYP P450s chemical inducers (Buetler et al., 1995). In fact, in the promoter region of CYP 1A gene, bifunctional compounds inducers encompass distinct functional regulatory elements, dioxin responsive element (DRE) or xenobiotic response element (XRE).

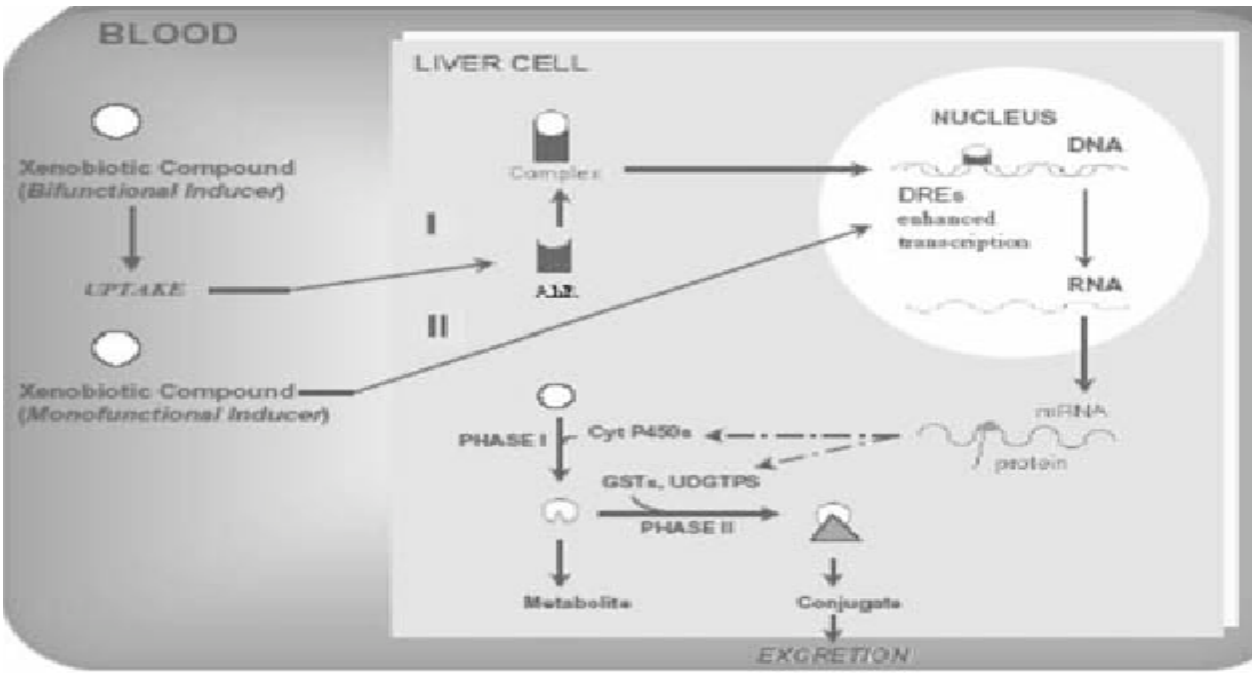


Figure 1.8: A simplified representation of the phase I and II metabolic pathways in a typical liver cell (detoxification center) adopted from Blanchette et al., 2007.

1.7.1.2.4 UDP-GT as a biomarker gene in fish

Uridine diphosphhate-gluconosyl-transferases (UDP-GT) are a super genes family of phase II conjugating enzymes crucial for the detoxification of exogenous compounds (Van der Oost et al., 2020). The main characteristic attribute of vertebrates is glucuronidation, while the utilisation of glycosylation is mostly preferred in invertebrates. The membrane bound conjugating enzymes UDP-GT, catalyse the transfer of glucuronic acid to the acid group of UDP-glucuronic acid to the functional groups of a particular substrate such as sulfur, carboxyl, hydroxyl and amino. Glucuronidation is the main route for catalysing the conjugation of various hydrophobic compounds (aglycones) and enhance the conversion or deactivation of xenobiotic and endogenous compounds to a polar, more hydrophilic form which facilitates their water solubility and elimination via urine or bile (Schlenk et al., 2008; Wang et al., 2014; Johnya et al., 2020 As commonly found in different phase I and II gene reactions, UDP-GT is induced through the reaction of aryl hydrocarbon receptors with a dioxin response element (DREs) or a xenobiotic response element (XREs) in the promoter region of an organism (planar phenol conjugation).

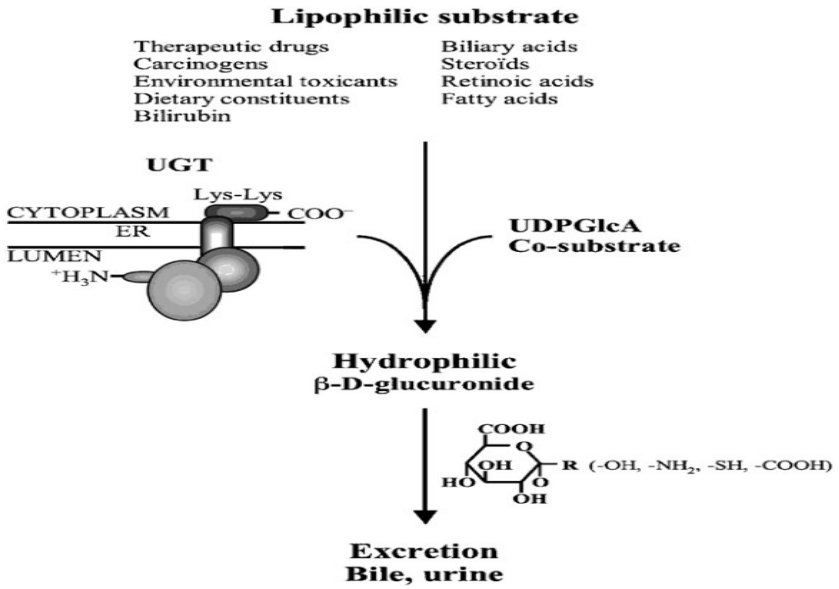


Figure 1.9: Glucuronidation of membrane-bound UDP-GT enzymes. Adopted from Guillemette, 2003.

1.7.1.3 Metal detoxification gene (Metallothioneins) as a biomarker gene in fish

Metallothioneins (MTs) are family of non-enzymatic, low molecular weight (6-8kDa), high cysteine rich protein with high affinity to divalent ions devoid of aromatic amino acid and heat stable with an isoelectric point of 8.3. Metallothioneins high cysteine rich protein (20-30 %) containing thiol group (-SH) that enables MT to bind specific heavy metals through thiolate bond. MT was discovered in 1957 by Margoshes and Vallee who reported first metallothioneins in horse renal cortex. Since then, metallothioneins and metallothioneins-like protein have been documented in various organisms. MTs are broadly distributed in the tissues of vertebrates such as fish and numerous invertebrates such as mollusc and crustacean (El-Khayat et al., 2020). It was reported that, the function of metallothioneins is influenced by the chemistry of thiol group, so that any metal having similar stoichiometric properties of zinc or copper can also bind metallothioneins.

Increased in metallothioneins concentration is a typical defence response in all organisms. Primarily, metallothioneins roles in metal detoxification is performed through high affinity binding of the metal to the MT and consequently sequestering the metal and rendering it biologically unavailable by blocking the interaction with the biomolecules of the cell such as lipid, DNA and protein (Ibor et al., 2020). Metallothioneins also performed a role of non-enzymatic antioxidant defense via the induction of metallothioneins, also through both essential and non-essential elements binding to sulfhydryl-rich proteins. Being

metallothioneins a thiol-rich protein, it binds metals with a status of prooxidant functions, example cadmium and make available a thiol group which forage hydroxyl radical (OH·) and singlet oxygen (Regoli and Giuliani, 2014; Adeogun et al., 2019). The crucial mechanism for the generation of ROS through trace metal rest on the capacity of losing an electron and initiate catalysing a Haber Weiss and Fenton reaction (Halliwell and Gutteridge, 2007, Regoli and Giuliani, 2014; Adeogun et al., 2019). The Haber Weiss reaction entails the reduction of oxidised metal by superoxide anions ($O_2^{-\cdot}$) and the reaction with hydrogen peroxide (H_2O_2) to generate hydroxyl radical (OH·).

$O_2^{-\cdot} + \text{metal}^{n+1} \rightarrow \text{metal}^{n+} + O_2$

$\text{metal}^{n+} + H_2O_2 \rightarrow \text{metal}^{n+} + OH\cdot + HO^-$

General reaction: $O_2^{-\cdot} + H_2O_2 \rightarrow + O_2 + OH\cdot + HO^-$ ($\text{metal}^{n+1}/\text{metal}^{n+}$)

The Haber Weiss is reported to be catalytically sluggish except when a transition metal ion interacts with hydrogen peroxide (H_2O_2) to produce hydroxyl radicals (OH·) and oxidised metal (Fenton reaction).

Fenton reaction: $\text{metal}^{n+} + H_2O_2 \rightarrow \text{metal}^{n+1} + OH\cdot + HO^-$

Transcription of metallothioneins gene is elicited through various inducers including exposure to metals, oxidative stress, cytokines, stress hormones such as thyroid and glucocorticoid (Regoli and Giuliani, 2014). Each of these inducers acts via a specific metallothioneins gene promoter regulatory element and begins a particular response such as heavy metals through metal response element (MRE), oxidative stress through antioxidant response element (ARE), cytokine signalling through cytokine response element (CRE), thyroid stress through thyroid response element (TRE) and glucocorticoid stress through glucocorticoid response element (GRE) in the expression of metallothioniens gene. Metallothioneins gene transcription begins through binding of metal transcription factor-1 (MTF-1) to a promoter of nuclear metal response element (MRE) via heavy metal initiation activation (Adeogun et al., 2019). Figure 1.10 shows schematic regulation of metallothionein gene showing (1) homeostasis of the essential trace metals Zinc and Copper; (2) detoxification of the non-essential metals Cadmium and mercury; (3) donation of essential metals to apo metalloproteins; (4) protection against oxidative damage; (5) free radical scavenger as thionein A.

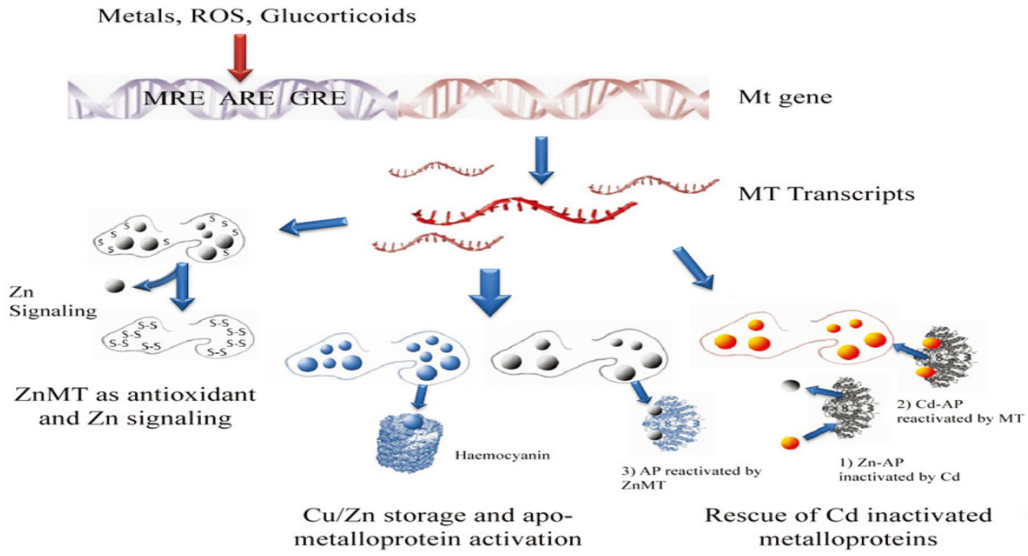


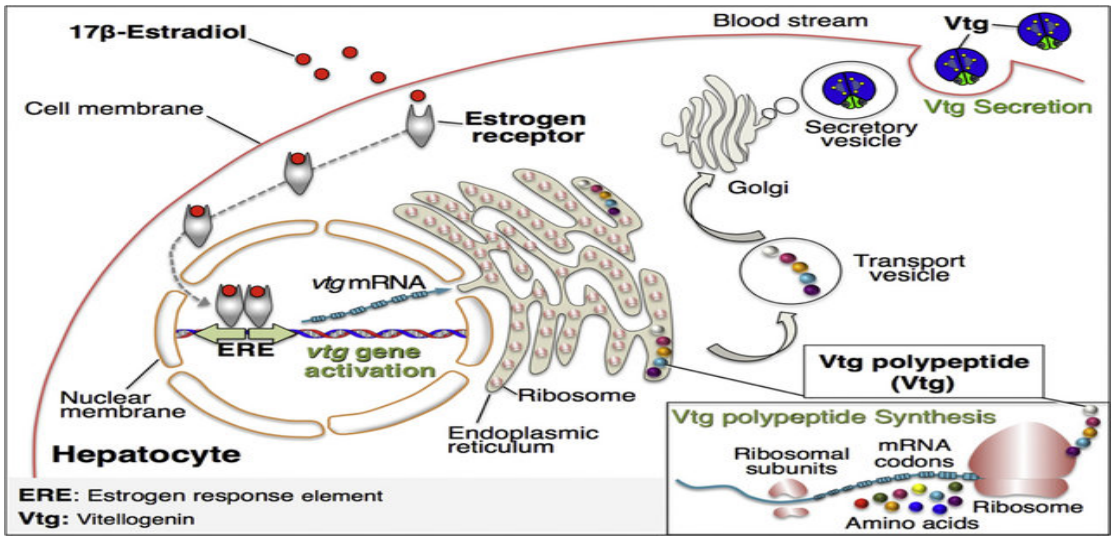
Figure 1.10: A simple schematic metallothionein gene regulation and transcription adopted from Isani and Carpene, 2014.

1.7.1.4 Reproduction and endocrine disruption biomarkers in fish

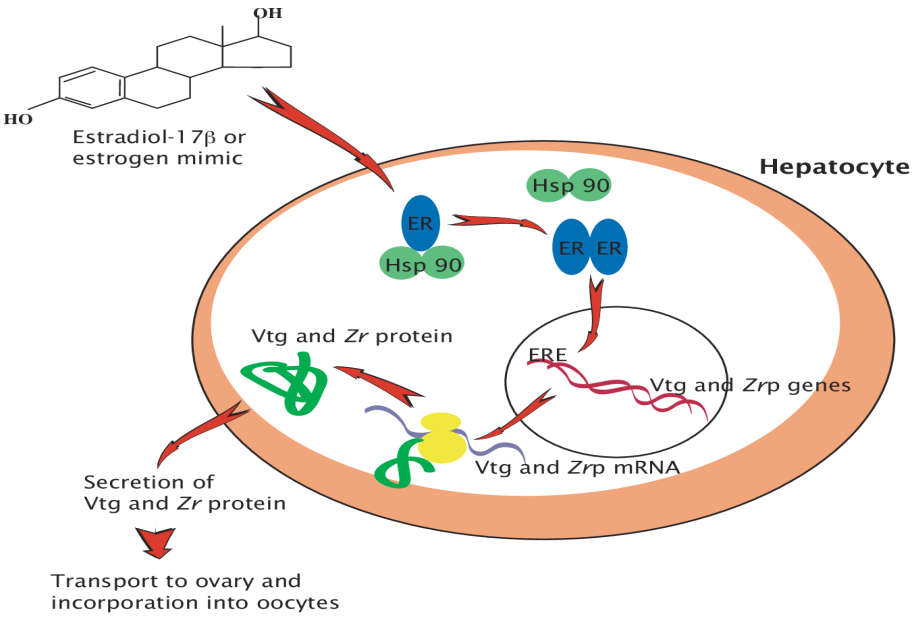
Contaminant specific endocrine modes of action (MOA) are frequently indicated by alterations in different biomarkers including Vitellogenin (VTG/ZRP), secondary sexual characters (SSC) and sex ratio. Among the sensitive biomarker for estrogenic activity in aquatic organisms is Vitellogenin (VTG) and Zona radiata protein (ZRP). Vitellogenin (VTG) is a lipophosphoglycoprotein, an egg yolk precusor expressed in female oviparous fish in response to estrogen signalling through binding to nuclear estrogen receptors (ER) existing in the liver and transported through the blood stream to the ovaries and absorbed by maturing oocytes (Ferreira et al., 2019; Olivera et al., 2020). Due to its function as an egg-yolk precursor, VTG is expected to occur in high concentration in female oviparous fish during the reproductive season. Normally, vitellogenin is low or undetected in male oviparous fish. But exposure to xenoestrogen (17-beta-estradiol) or estrogen-mimetics (17-alpha ethinylestradiol) can trigger VTG in adult and immature male oviparous fish and produce VTG like in their female counterparts (Ferreira et al., 2019).

In addition, the non-existence or decreased levels of Vitellogenin (VTG) in female oviparous fish during the reproductive season entails anti-estrogenicity and a possible consequence of decreased reproductive success (Yamamoto et al., 2017). Therefore, Vitellogenin in male oviparous fish is employed as a biomarker endpoint for xenoestrogenic contaminant detections in the aquatic environment (Olivera et al., 2020). Zona radiata protein (ZRP),

implicated in the production of eggshell in oviparous female fish is also employed in EDC. Zona radiata is secreted and transported through binding to the estrogen receptor (ER) into the blood stream to the ovaries and absorbed by the maturing oocytes to form an egg envelop (Adeogun et al., 2016). In the event of exposure to xenoestrogen (17-beta -estradiol or 17-alpha ethinylestradiol), fish accumulate estrogenic chemicals from their surrounding medium and trigger the expression of ZRP. Alteration in the synthesis of ZRP may result to decrease the mechanical strength and thickness of the eggshell, resulting in the depletion of the eggshell's ability to safeguard against mechanical disturbances in the premature time of oocyte development and determent of polyspermy during the course of fertilisation in oviparous fish (Adeogun et al., 2016). On the other hand, estrogens are pleiotropic steroid hormones widely integrated in the ovaries and testis of oviparous species and discharge their duties normally via cystosolic estrogen receptors located in the tissue of interest.



(a)



(b)

Figure 1.11a-b: Hepatic vitellogenesis regulated by estradiol. Fig. 1.11a: Estradiol (E2) passes from the bloodstream into the liver cell (hepatocyte) cytoplasm where it binds to estrogen receptors. The occupied receptors undergo conformational changes and are translocated to the nucleus where they dimerize and bind to estrogen response elements (EREs) in DNA sequences in the promoter region upstream of the target vitellogenin (Vtg) genes. Transcription of Vitellogenin genes is initiated and resulting in mRNAs transit to the rough endoplasmic reticulum where they provide a template for translation of Vtg polypeptides on the ribosomes. The nascent vitellogenin polypeptides are loaded with lipid and packed into transport vesicles for travel to the Golgi body where final post-translational modifications (glycosylation, phosphorylation) and dimerization are accomplished. Secretory vesicles carrying newly synthesized dimers vitellogenin bud off from the Golgi body and fuse with the peripheral cell membrane to disgorge their contents (Sullivan and Yilmaz, 2018). Figure 1.11b: Schematic representation of endogenous estradiol-17β (E2) or synthetic estrogen invigorated oogenic protein synthesis. Eggshell zona pellucida proteins and the egg yolk protein precursor, vitellogenin are synthesized and secreted by the hepatocyte. They are travelled in blood to the ovary and integrated into maturing oocytes in female teleosts. Adopted from Arukwe and Goksøyr, 2003.

1.7.1.5 Pro-apoptotic genes as biomarkers in fish

1.7.1.5.1 Diablo/Smac gene

Diablo (direct IAP binding protein with low pI) or Smac (second mitochondria-derived activator of caspase) is an important pro-apoptotic molecule that encourages programmed cell death (apoptosis) by decreasing the inhibitory influence of inhibitors of apoptosis proteins (IAPs) on caspases (Lv et al., 2019). Diablo is among the numerous proteins let loose into the cytoplasm from the mitochondria ensuing an apoptotic inducement (Zacchino et al., 2012). However, the process of apoptosis is executed through caspases, which are principally implicated in both intrinsic and mitochondrial pathways in an apoptotic mechanism. Moreover, these caspases are completely modulated by some molecules in the process of programmed cell death. For instance, inhibitors of apoptosis proteins (IAPs), are diverse protein family which actively inhibit the function of the primary effectors of apoptosis, caspases 3 and 7, and the initiator caspases 9 and damagingly modulate apoptosis (Zacchino et al., 2012; Lv et al., 2019). Conversely, these suppressive effects of the inhibitors of apoptosis proteins (IAPs) are eradicated by other molecules known as the inhibitors of apoptosis protein-binding proteins (IAPs-binding proteins) such as Diablo/Smac (Shiozaki and Shi, 2004). Diablo/Smac has hydrophobic tetrapeptide or homologous sequences which bestow them with the ability to bind and block the activity of the members of inhibitors of apoptosis proteins (IAPs) (Zacchino et al., 2012; Lv et al., 2019

It was documented that in the mitochondria-dependent apoptotic pathways, upon sensing lethal signals, there is an increase in the permeabilization of the mitochondrial outer membrane and transport of a number of pro-apoptotic proteins (including the apoptosis-inducing agent (IAF), B-cell-Lymphoma 2 inhibitor of transcription (BitL) (Lv et al., 2019); cytochrome C, mitochondrial serine protease (Anvi Far et al., 2018); Endonuclease G (EndoG) and Diablo into the nucleus via cystosol that contribute and speed up the activation of caspase to trigger apoptosis induction (Green and Fitzgerald, 2016). An increase in the permeabilization of the outer membrane of mitochondria was reported in vertebrates, indicating the release of Diablo as the downstream effect of the pollutant stimulation, causing a degree of mitochondria membrane permeability (Du et al., 2016). In the extrinsic apoptosis pathway, cell death occurs in the form of activation of extracellular signaling. This is achieved through the attachment of ligands to a specific-transmembrane receptor (death receptor) belonging to the TNF/NGF family. Immediately after the ligand is attached to the transmembrane receptor, various receptor molecules are transported and undergo

configurational alterations permitting a group of the multi-protein complex, the death initiation signaling complex (DISC), to steer the caspase induction (Fig. 1.12).

All death receptors function in the same fashion. Upon receiving a lethal stimulus, FAS, immediately after ligand attachment, engages Fas-associated protein with a DD (FADD), via a highly conserved 80 amino acid domain, referred to as the death domain (DD). The presence of the death effector domain, containing conserved protein interaction, allows FADD to bind to a homologous domain in caspase 8, leading to its activation. Once the chain activation of active caspase 8 has ensued, one caspase activates additional caspase 8, leading to the activation of downstream caspases, such as caspase 3 in the cell (Fig. 1.12) (Favaloro et al., 2012). In this thesis, diablo/smac was investigated in a Nile tilapia (*Oreochromis niloticus*), exposure in the laboratory and field as a promising biomarker gene of exposure to pollutants as was confirmed by other previous studies (Anvi Far et al., 2018; Lv et al., 2019).

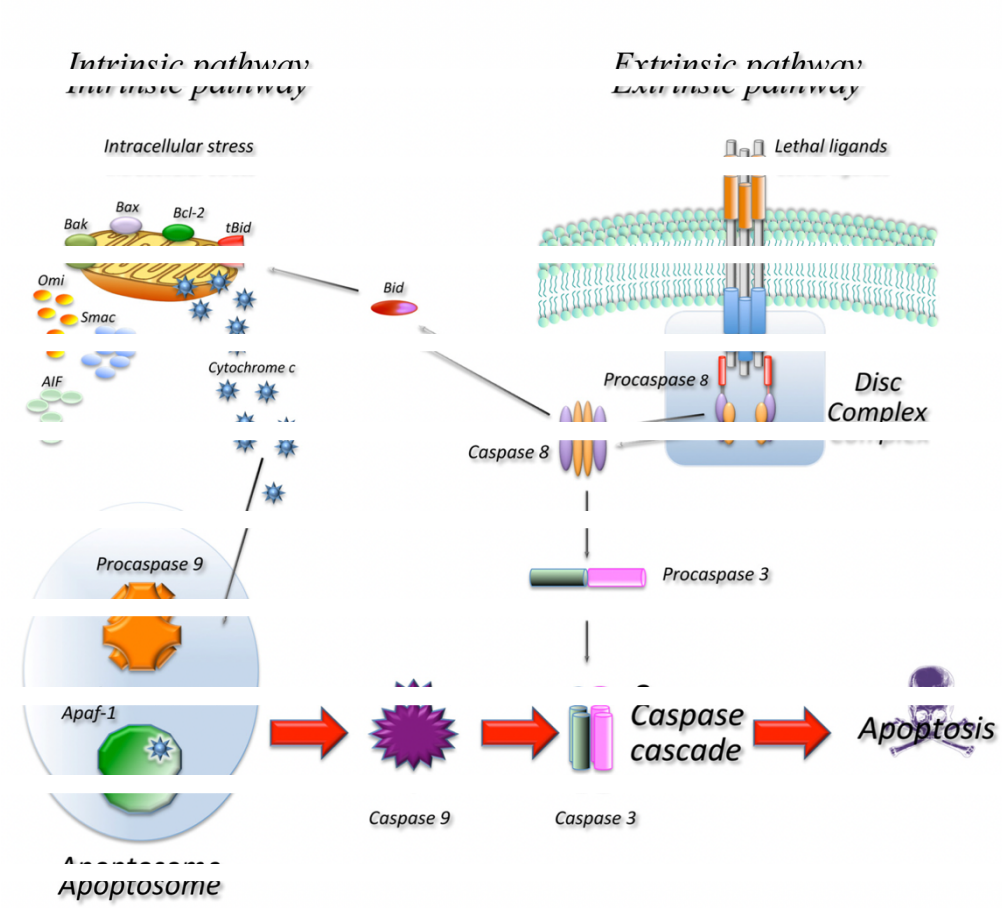


Figure 1.12: Schematic representation of the main molecular pathways leading to apoptosis adopted from Favaloro et al., 2012. In the extrinsic pathway upon ligand binding to specific

receptors a DISC complex is formed and caspase 8 activated. The intrinsic pathway release of cyt c from the mitochondria results in the formation of apoptosome and activation of caspase 9. Caspase 8 and 9 then activate downstream caspases such as caspase 3, resulting in cell death. The two pathways are connected through the cleavage of the BH3 only protein BID.

1.7.1.5.2 Siah 2 gene

Siah is a human homologue of protein of Drosophila seven In Absentia (SINA) proficient ubiquitin E3 ligase. Numerous studies speculated SINA and Siah performed functions as E3 ligase ubiquitin for proteasomal protein degradation (Qi et al., 2013). This is because, imposed Siah expression results in cellular growth arrest and can be pro-apoptotic (Nang et al., 2014). The extraordinary evolutionary conservation of Siah 1 and 2 implied that these proteins may have cellular functions conserved in all the vertebrates and invertebrates. In most normal and neoplastic human tissues, low level expression of these proteins was observed, even though more transcript level of Siah 1 was more than that of Siah 2. Hu et al. (1997) suggested that Siah gene may have a crucial role in apoptosis. It is noteworthy that, Bax a transcriptional target for tumor suppressor protein p53, has a crucial function in apoptosis and have a particular pattern of cell induction in response to p53. Therefore, in this study, Siah 2 was considered a target gene for study to define their roles in ecotoxicological response of Nile tilapia (*Oreochromis niloticus*) exposed to contaminants in the aquatic environment.

1.7.2.1 Biomarker of effect

1.7.2.1.1 Length-weight relationship and condition factor

Among the important factors of influencing organisms' survival and viability in an aquatic habitat is a biometric index. The biometric index can be analyzed through total a length / body weight relationship and a condition index (Sabarudin et al., 2017; Adeogun et al., 2018). The length-weight relationship (LWR) is a mathematical model that highlights the conversion of the weight of a fish into a given length and the weight into the length of a given species of fish, and an estimation of their biomass in length distribution frequency (Famoofo and Abdul., 2020). The condition factor relates to the physiological and wellbeing of the fish health in its aquatic environment (Adeogun et al., 2016). These data are required for the study of the fish population and their management and are fundamental as fishery resources. Data obtained are used to determine the growth rate and the age structure of the fish population dynamics in different species (Famoofo and Abdul., 2020). The Condition

factor is regarded as a genuine depiction of a fish' health status and presents a relationship between the environmental status and the physiological state of the fish, presuming that the exposure to environmental pollutants may bring a significant reduction in the condition factor (Vander Oost et al., 2003; Adeogun et al., 2016). The Condition factor (CF) may either be higher or lower than the normal range of (1) in response to contaminants in fish. Some arguments in past studies postulated that the condition factor was elevated in response to contamination and the availability of rich organic matter, which supplies different sources of food to fish. They use the opportunity to feed and cope with the impact of stress due to chemical toxicity (Ibor et al., 2019).

1.7.3 Gene expression profiling biomarkers integrating exposure and effect

Usually, a collection of different sets of biomarkers are employed to evaluate the biological effects of pollutants on fish. New technology using molecular approaches, such as microarrays, next generation RNA sequencing (RNAseq) and Quantitative polymerase chain reactions (qPCR), have the potential to provide in-depth assessments of the health of fish beyond a single biomarker to provide a global analysis of how a gene and protein, or the subsets of genes and proteins in the case of qPCR, respond to pollutants in the aquatic environment. These technologies are promising tools in ecotoxicology as they not only broaden the quantity of the analysed biomarkers, but also contribute to the understanding of exposure and pathways of harm (biological effect) and are moderately a bridge between the exposure and effects in an organism (Hook et al., 2014). Toxicogenomic technology is a common approach in the context of biomarker types, nonetheless, these gene expression profiling techniques require to be verified by observable effects which are measurable and obviously expressed in the organism, for example, the observable effects in the physiological status, tissues and organs of an organism. These approaches also potentially enable the detection of novel additional acceptable and separate biomarkers for regular water quality monitoring programs (Leaver et al., 2010; Su et al., 2020). Therefore, the establishment of a transcription of a target gene or its protein levels, implicated in the vital cellular pathways and functions, could be of significant regard in assessing the molecular influence of aquatic environmental contaminants (Gonzalez and Pierron, 2015).

1.7.3.1 DNA microarrays

DNA microarrays were first developed in the 1990s and since then have been widely employed in different studies, especially in humans. Microarray technology is important in ascertaining the influence of different classes of chemicals in the aquatic environment

regarding the sublethal toxic effects of chemical combination settings. The microarray global gene expression profiling is employed as a discovery tool to identify responses in fish exposed to contaminants in both field and laboratory studies. This profiling method allows the identification of unknown stressors as well as the detection of agents responsible for the deterioration of the organism's health through a comparison of laboratory with field studies results (Hook et al., 2014). Microarray technology is set up on the understanding of gene sequences.

The crucial advantage of DNA microarrays in this context is the possibility to analyse several thousand gene expression levels in the same experimental set up. Thus, DNA microarrays are employed to investigate tissue specific expression levels of genes or to determine the xenobiotic impacts on model aquatic biota (Kinaret et al., 2020). As a matter of fact, despite the multiplying number of genomes and transcriptomes accessible in databases, some ecotoxicological pertinent species are poorly represented at the genomic level. As such, microarrays have the disadvantage of identifying an unknown transcript, since the design of probes is based on nucleotides sequences that are known. Another limitation is the predicament to accurately link the expression patterns and to compare them to possible physiological perturbations in the organisms. Consequently, this technique could generate a false positive result, thus the status on the expression levels occasionally requires to be determined with quantitative polymerase chain reaction evaluations. In addition, there may be an occurrence of a cross hybridisation event while using highly tiresome genomes due to the repetition ensuing in an incorrect detected expression efficacy of the gene in contrast to the actual expression in the organism (Kinaret et al., 2020). Lastly, this approach is time consuming and costly due to the nature of the materials required, as such different ecotoxicologists thought that it could be substituted by high throughput approaches such as RNAsequencing (Gonzalez and Perrone, 2015).

1.7.3.2 Next-Generation RNA-sequencing (RNAseq)

The development of high-throughput technologies such as the DNA microarray hybridisation-based approach in determining the influence of chemicals on aquatic organisms has immensely improved the propagation of global gene expression profiling in organisms. The recent advent of the Next-generation RNA sequencing approach has further enhanced this ability (Kinaret et al., 2020). Its increased application in various fields of life science in recent years may be due to the advancement in sequencing technology and reduced expenses in sequencing analysis. RNAseq permits the identification of gene expressions

within an elevated control range resolving the difficulty of probe saturation for most expressed transcripts of an organism. The application of this approach to fish and other organisms is possible without prior knowledge of the complex genome sequences of a studied species or the hybridisation effect. In fact, the whole transcriptome-shotgun sequencing (RNAseq) supplied a great series of information. Gene sequences accessible by de novo assembly can be elucidated in contrast with familiar sequences obtainable in databases for some organisms or to identify new transcripts produced with alternative splicing (Chandhini and Kumar, 2019)).

The important advantage of this technique is that it is not restrictive and that it can analyse the impact at a transcriptome global level. Presumably it can establish every gene that is distinctly expressed in the exposure of biota to environmental contaminants or changes in the aquatic environment. Actually, the contrast between the transcriptome acquired with control fish, for example, could be employed to normalise transcriptome obtained from exposed fish. This would permit actual judgement of the molecular effects and the pathways implicated in the response to exogenous chemicals in the cells (Gonzalez and Perrone, 2015). The limitation of the technique of RNAseq usually produce broader and more complicated data that requires extended time and better advanced knowledge in bioinformatics (Gonzalez and Perrone, 2015) and analytical techniques in comparison to the DNA microarray approach (Kinaret et al., 2020). On the other hand, the bulk of the express sequence tag (EST) displayed could not be ascribed to an obvious role or name of a gene (Gonzalez and Perrone, 2015).

1.7.3.3 Quantitative polymerase chain reaction (qPCR)

Real-time or Quantitative polymerase chain reaction (qPCR) is regarded as state-of-the-art technology in the study of gene expressions of known sequences of fish and other aquatic organisms. Accurately executed, this technique may be employed for exact gene expression assays. It has turned out to be a standard technique of choice in diagnostics, life sciences, and medicine for the quantification of mRNA levels (Bustin, 2000, 2010). The PCR idea was conceived by the American biochemist, Kary B. Mullis in 1983, and developed into techniques in the late 1990s. The qPCR technique is an improvement of classical PCR by adding a fluorescent e.g., intercalating SYBR green dye, which permits the establishment of cycle of threshold (ct) and has demonstrated to be an excellent technique (Mishra et al., 2020). The techniques in Polymerase chain reaction employ the amplification and quantification of a specific section of target nucleic acids (DNA), defined by an array of two

primers (forward and reverse primers), at which the synthesis of the target cDNA from the mRNA transcripts begins with a heat resistant reverse transcriptase DNA polymerase by employing PCR to amplify and quantify the target gene of interest (Kinaret et al., 2020). The qPCR approach is rapid, sensitive and an easy measure of generating a reasonably substantial quantity of copies of target DNA molecules from a little number of the gene of interest. Generally, in the detection and analysis of the specific region of the target DNA molecules, at least a million-fold increase can be realized.

The qPCR monitors the amplification of DNA in real time mode via fluorescent monitoring at a separate time. This approach is the most practical due to a lower time consumption requirement to identify the amplified operation. Fluorescence emitted in the reaction as a sign of amplicon accumulation by each PCR cycle is monitored by qPCR and results in an amplification curve in initiation or lag, exponential, linear and plateau phases. The amplification curve forms the basis for quantitation. During the lag phase or initiation phase, amplification occurs but the fluorescence signal at the beginning of the amplification cycle is not strong enough to be detected above the background level signal. At the exponential phase, the reaction progress and the phase are used for quantitation, because it produces a fluorescence signal measurable above the background level, which contains an abundance of reaction components permitting a detectable doubling event at every PCR cycle. When the fluorescence qPCR signal is detectable over the background fluorescence, a threshold level is determined. The Threshold level is the epicentre of quantification, as the position in which all the samples that traverse this threshold are taken down as quantification cycle (Cq) or threshold cycle (Ct) values. In the exponential phase, the threshold value is set, and the record is not impacted by the paucity of the reaction component in the linear and plateau phases (Adams, 2020). It is therefore pertinent for the reference genes to correspond to the assay conducted. Relative quantification permits the calculation of the ratio between the reference genes and the genes of interest. The correctness of this method solely depends on the reference genes and thus, it is important for the reference genes to stay unaffected to avert incorrect outcomes (Adams, 2020).

qPCR is now a valuable asset for gene expression analysis and the preferred technique for confirming and validating results required from array analysis and other methods of assessing gene expression alterations. The reliability, specificity and sensitivity attained in a handy qPCR compel it the perfect tool to use in the ecotoxicological monitoring of aquatic environments (Mishra et al., 2020). One of the drawbacks of this technique is the deficiency of accessible gene sequences for some organisms, as only a small number of genes can be

assayed at a time, which makes the method time consuming and costly. For this reason, sequencing and cloning of genes of interest are required. This indicates that the assay could be restricted to only a few available genes and thus may be highly enlightening (Gonzalez and Pierron, 2015).

1.8.0 Overview of Nigeria's population, geography, climate and vegetation

Nigeria is the most populous black nation in Africa and the seventh largest in the world with a population of over 206,662,307 people as of Tuesday, August 11, 2020 based on the world meter elaboration of the latest United Nation data. Nigeria's population is 2.64 % of the total world populations (www.worldmeters.info). By 2025, Nigeria's population is expected to rise to 239 million and to 440 million by 2050, because of the momentous increase in population. It will become the 4th most populous country in the world (Etebong, 2018). Nigeria lies between the longitudes 2° 49'E and 14° 37'E and the latitudes 4° 16'N and 13° 52' North of the Equator (Imarhiagbe et al., 2020). Nigeria is located in west Africa. It has boundaries with Niger and Chad to the North, with the Atlantic Ocean to the South, with the Republic of Cameroon to the East and the Republic of Benin to the West (Fashae et al., 2017). The land mass of Nigeria is 923,768 square kilometres. Nigeria's topography spans from the Southern coastal swamps to tropical rainforest, open woodland, grassland in the Niger valley, to savanna and semi-desert in the far-reaching Northern part of the country with a diverse mixture of plants and wildlife. At its largest-scale, Nigeria has a distance of about 1200 kilometres from East to West, and 1050 kilometres from North to South (NHC, 2020).

The climate of Nigeria changes from the South to the North of the country and differs with altitudes. In the Southern part of the country, there is a warm, moist and south-westerly wind emanating from the sea, characterised by hot, humid and oppressive heat during most of the year. In the Northern part of the country, the climate is drier with a wide range of temperature. The temperature in the South has a daily average of 27 ^{o}C with small seasonal variations. In the North, the average monthly temperature ranges from about 21 ^{o}C to 32 ^{o}C. Nigeria is characterised by two basic seasons: wet and dry. The wet season lasts from about April to October, while the dry season last from about November to March each year. The rainfall is extreme during the wet season and can reach up to 1780 mm annually in the Southwestern part and 3810 mm annually in the South-eastern part. In the Northern part of the country, the rainfall is in the range of 635-1270 mm annually. The dry season ending in February begins with a harmattan, characterised by a dry chilly spell bringing lower

temperature and dusty and hazy conditions related to the North-easterly trade wind blowing from the Sahara and the Arabian Peninsula (NHC, 2020).

There are two types of vegetation: Forest and Savanna, moving virtually side by side the East to the West of the country. In the Southern part of the country, the vegetation includes tropical evergreen rainforests, saline water swamps, and freshwater swamps, while in the Northern part, the vegetation cover includes the Guinea savannah, Sudan savannah and Sahel savannah. In both the North and the South mountainous vegetation covers the land, separated by the high Plateau sites of the Northeast and the South of the country such as those in Jos, Plateau state, Mambila, Nasarawa state and Obudu, in Cross river state.

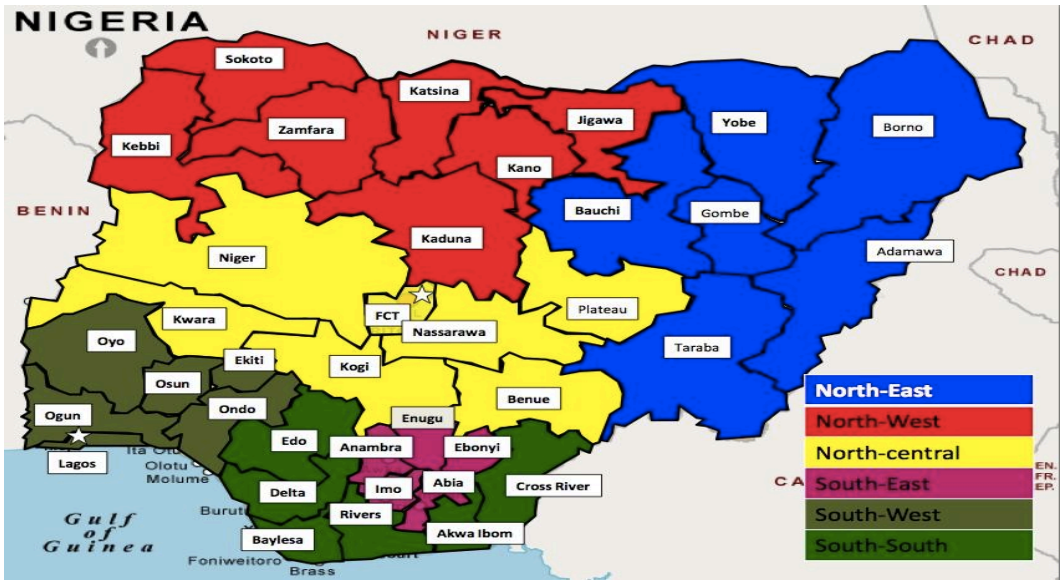


Figure 1.15: Map of Nigeria showing the different regions of the country as adopted from Nigeria United States Embassy in Nigeria, 2012.

1.8.1 Aquatic pollution in Nigeria

Pollution in developing countries originated mainly from the generation and handling of cheap waste. Matters of waste collection management need to have a synergistic approach involving local, regional and the federal governments to pre-empt environmental and health issues emanating from them. An exponential elevation of waste from different sources was seen over the last few decades in Nigeria. Depending on the type of waste under deliberation, three main sources in Nigeria are agricultural, municipal domestic and industrial waste. One of the major problems in a developing country such as Nigeria is aquatic pollution. Humans

rely on water for domestic, industrial and agricultural purposes. In Nigeria, very few chemicals have been tested ecologically for safety, despite their environmental consequences (Avoajah et al., 1997; Ivon et al., 2020). Due to the application of sewage sludge and manure, pesticides and fertiliser coupled with contamination during irrigation, farming is also a potential source of pollutants in Nigeria. During rainfall, all these substances are washed away to nearby rivers and lakes. Recent development in modern agriculture in Nigeria has come with the chemical control of a weed chemical fertilizer application for the enhancement of crop production. Among the basic indicator parameters of pollution are nitrates as a result of water runoff from land fertilized by nitrogen fertilizers. The widespread eutrophication of most water bodies resulted from diffused water pollution in agriculture and a resultant algal bloom. This is a normal scenario in Nigerian waterbodies. As a result, there may be interference in the aquatic ecosystem integrity and additional costs in drinking water treatment.

Another source of aquatic pollution in Nigeria comes from animal farms including poultry, dairy farms, pigs, aquaculture farms and livestock farms. Antibiotics, animal dung, litter from poultry, urine and milk pallor waste.

In Nigeria, animals are slaughtered on an open ground or close to streams, where blood and other excreta can be washed away. Leachates from open dumpsites and uncontrolled landfills are another source to aquatic pollution. As a result, there may be a flow of toxic chemicals into streams and rivers, which may also pollute the groundwater through seepage. The discharge of effluents from industries, either partially treated or untreated, are normally channelled to neighbouring rivers or streams. Domestic sewage from households, as a result of wastewater from laundry, kitchen and sewers, contains detergent, oil, pharmaceuticals, insecticides, and decayed organic matter. In developed countries residual waters from urban settlements and industries are usually treated to degrade the obnoxious contents of sewage in Sewage treatment plants (STPs) before being discharged into the receiving water bodies. However, in Nigeria, this is not the case, as there are very few environmentally functional centralized sewages treatment plants (except water treatment plants for pipe borne water) that treat wastewater before discharging it into the water bodies in the urban cities of Nigeria. This also affects residential water bodies that are close to the public. In most urban areas, sewage is discharged untreated, as there are no sewage treatment plants in the towns and villages of Nigeria. This is largely due to a lack of planning of town/village settlements to include facilities like adequate water supply, and an insensitivity towards environmental issues from the government. These residential water bodies are major tributaries to larger

water bodies during the rainy season; they empty their contents into rivers and the sea. On the other hand, urban runoff is another possible aquatic pollution in Nigeria. This is due to the nature of the marketplaces, where different harmful products are disposed of onto the market floor without bins in an appropriate place. These products are carried and washed away by rain and find their way into rivers and streams. Table 1 below summarises the industrial sources of aquatic pollution in Nigeria.

Table 1.1: Industrial source and effluents generated to aquatic environment

Industry	**Effluent content**
Tannery industry	Cobalt, lead, chromium, Nitrogen, Phosphorous, PCBs, chlorinated phenols, arsenic
Pharmaceuticals industry	Antibiotics, phenols, benzene, chloroform, heavy metals and Toluene
Pulp and paper industry	Mercury, transition metals, chelating agents, chlorides, benzene, methanol, sulfates, chlorates, and nitrates
Chemical industry	Hydrocarbon, acids, emulsifier, base, heavy metals, surfactants, other persistent organic pollutants
Iron and steel industry	Polycyclic aromatic hydrocarbons, naphthalene, cyanides, anthracene, benzene, hydraulic oil, phenols, chromium,
Mining industry	Heavy metals, metalloids, cyanides,
Fertilizer industry	Nitrate, urea, Phosphate, zinc, urea, iron, ammonia, salts, ammonium salt, cyanides, methanol, alkali, ash slurry
Textile industry	Heavy metals, Brominated flame retardants, Formaldehydes, sulphur dioxides, chlorine, bleaching reagents, volatile organic compounds, phenols and isocyanates
Food and beverages industry	Microorganisms e.g., bacteria, heavy metals, potash, nitrates, sulfates

1.8.2 Trends and the current status of pollution in developing countries: a case study of Nigeria.

For the last few decades, rapid population growth with resultant anthropogenic activities have been witnessed in developing countries such as Nigeria. Both of these changes led to different environmental burdens, specifically aquatic environmental contamination (Marais et al., 2014). The rapid increase in population growth, with people from villages flooding into towns and cities (urban pull) resulted in the evolvement of mega cities. Markets and flourishing businesses have giving rise to numerous distinct classes of industries. In addition, non-point source extensive agricultural activities and the lack of established methods for sustainable healthy environmental management from the authorities have also contributed to the aquatic environmental pollution (Adeogun et al., 2016). Moreover, despite the prevailing root causes, the pollution in Africa, especially in Nigeria, is a serious issue that requires urgent, timely and sustainable intervention (Babayemi et al., 2016). In a broad sense, pollution in Nigeria has attracted both local and global interest for anthropogenic activities, some of which are transboundary in nature and have come with serious alterations to the environment. Because of the geographical location of Nigeria, being located in the tropics and prone to heavy powerful rainfalls, many dumpsites and landfills are not covered, therefore toxic waste leaches out into ground water, and heavy rains wash waste into neighbouring surface water bodies. In spite of laws that ban the indiscriminate disposal of waste into aquatic water bodies, the regulations have not been effective in mitigating this menace (Ekiye and Zejiao, 2010).

The detrimental effect of pollution is clear, due to insufficient technology in dealing with and restricting the spread of the pollutants. Furthermore, the ignorance of the majority of people is linked to the cultural, traditional and other preindustrial attributes and practices that advance these hazards. The types of contaminants vary, they are produced in distinct quantities and are generally poorly handled. The processes of assemblage, processing, and disposal are inefficient, primitive and obsolete. This has high environmental importance, as a large majority of these contaminants ends up in the aquatic habitat, where they are seriously harmful to aquatic wildlife and humans. In furtherance of the detrimental effects on wildlife and human health, a significant portion of African countries, especially Nigeria, are worsening with uncovered landfills and dumpsites that litter most of the cities and towns. Thus, the impact of notorious climate change and global warming are of great concern on a global scale. The above implication tallies with the environmental consequences regarding food security and safety in agriculture.

In countries like Nigeria, poverty, social and economic underdevelopment and government negligence marr that process. Lack of sanitation, poor nutrition, and diseases are common due to the presence of open landfills within residential areas or waste disposal directly in water bodies. The lack of an existing regulatory framework and paucity of sufficient baseline data from disorganised studies by few individuals and scientific projects as well as the existence of limited reviews on inland waterbodies are a major constraint (Nweke and Sanders, 2009). Sewage, industrial and agricultural sources top the major contributors of contamination into the Nigerian aquatic environment. Industries discharge their effluents with little or no treatment. At an incidence at Koko village on the Southern coast of Nigeria in September 1987, an Italian by the name of Gian Franco Raffaella dumped about 3880 tons of toxic waste, suspected to be hazardous Polychlorinated biphenyl (PCBs), on behalf of an Italian company. Nigeria was not serious and was sleeping on environmental matters. This incidence awakened the country and hastened the creation of a Federal Environmental Protection Agency (FEPA) by military decree in 1988. FEPA decrees required the establishment of guidelines and standards for the moderation and control of different forms of environmental pollution. It was also mandated to commence policies in research and technology, and to plan and implement policies linked to environmental management. However, the lack of the necessary powers in the Nigerian constitution hampered the activities of FEPA in its oversight functions on environmental standards and regulations and it was therefore merged with the ministry of the environment. This necessitated constitutionally, that the country filled the vacuum by passing the NESREA act in 2007 (Ladan, 2012; Agbazue et al., 2017). NESREA was shouldered with the responsibilities for the protection and development of the environment, conservation of biodiversity, sustainability of the development of natural resources, coordination of environmental technology on matters regarding the enforcement of laws, standards, regulations, guidelines and policies by liaising with inside or outside relevant stakeholders (Ladan, 2012; Agbazue et al., 2017). The current status of pollution in Nigeria portrays environmental risks to human and aquatic wildlife and requires sustainable assessment and the investigation of different strategies. Therefore, it can be deduced that the protection of the environment is the rationale for environment laws, which can nurture the important harmony with other issues of life that might be impacted by environmental matters. Thus, formulation and implementation of such productive environmental laws remain uncertain for various governments, such as those in Nigeria (Chuks-Ezike, 2018).

1.8.3 Current status on water quality monitoring in Africa; a case study in Nigeria

Monitoring of water quality in Nigeria is crucial since it has an important impact on the wildlife, ecosystem integrity and human health. The rationale behind water quality monitoring is to acquire data, which will be useful in policy making and sustainable management of the water resources and will prove important in investigation of contamination, control plan and decision-making on the water bodies. This will allow regulatory management to detect the problem and proffer solution before it goes out of hand (Ramakrishna and Jagadeeswari, 2019). Knowledge on the occurrence, destiny, elimination and hazard of aquatic contaminants is highly rigorous and require so much human and infrastructural investment. On the contrary, in the developed nations, most of the research employed state of the art technologies in analytical approach and other high throughput technologies in water quality monitoring (K'oreje et al., 2020). Such technologies in analytical chemical analysis methods includes tandem mass spectrometry, high resolution-mass spectrometry (HRMS) have been known for long time (Perez-Fernandez et al., 2017). While high throughput technologies such as DNA microarray, next-generation RNA-sequencing (RNAseq), Quantitative polymerase chain reaction (qPCR) have taken root.

Majority of the studies carried out in in Africa particularly in Nigeria are faced with selective and less sensitive equipment especially on techniques based on High-pressure liquid chromatography with UV detectors (HPLC-UV), atomic absorption spectrometer (AAS), electron captor detector (GC-ECD), which may possibly restrict the scope of that research (Adeogun et al., 2015; K'oreje et al., 2020). Very few studies employed advanced analytical studies using Mass spectrometry such as gas chromatography mass spectrometry in Nigeria (GC-MS) (Ihunwo et al., 2019; Adekunle et al., 2020). This shows more logical connection and add more values in their studies in contaminants monitoring in Nigeria. In spite of the challenges in human and state of the art analytical instrumentation in Nigeria, the country started exploiting different opportunities positively. Nigeria has entered into collaborations with international collaborators to enhance research using advanced analytical instrumentations and high throughput technologies in molecular approach in order to overcome some of these challenges. Collaboration in research will enhance the research potentials of Nigeria as well as portray the country's research footprints globally and this will allow the local institutions to improve their monitoring capabilities.

Nigeria set up modern laboratory facilities in various regional universities with the little resource at hand in order to enhance research and training of staff and student in molecular

Biology. This will improve self-reliance and potentials to operate more advanced experimentations which can aid in forming more policy decision. For example, considering the local institutions circumstances, universities and other research centres in Nigeria can be involved in highly developed screening of emerging chemical contaminants ensuing in systematized chemical compounds for frequent monitoring in the country. Majority of the state-of-the-art analytical instrumentations are massively expensive for Nigeria to employ for routine monitoring program in different local institutions. Priority chemical compounds can be monitored with cheaper instruments that could bring quality outcome. Ecotoxicological tools have gained tractions as a complementary or substitute approach to analytical analysis of chemical compounds (K'oreje et al., 2020). This is because of their budget-friendly and efficiency in providing a comprehensive multiplex mixture effect of chemicals on wildlife and ecosystem integrity. Consequently, these ecotoxicological approach should be explored since they are very much limited in Nigeria to integrate risk-based and effect approach in monitoring chemical contaminants in a present time water quality management. In Nigeria policies and legislation guidelines are not lacking (Agbazue et al., 2017) but are ineffective mostly due to deficient data to rely on, or the attention of the studies is mainly on conventional physicochemical parameters, as most studies are undertaken by local researchers (Adeogun et al., 2016). If a more comprehensive data is produced on the existence of diverse chemical contaminants in Nigeria, it may provide an opportune moment for the policy makers to commence discourse on how to take care of these chemical compound. The discourse will pave way to a preparatory policy to lead to research and legislative plan of action.

Water quality monitoring in Nigeria mainly focuses on the study of physicochemical parameters of water, the concentration of heavy metals and other organic chemicals and pesticides in water and sediment, the bioaccumulation of heavy metals and other organic chemicals and pesticides in tissues of fish, use of biotic indicators such as macroinvertebrates, biochemical and molecular biomarkers, and other biomarkers of effect. Lack of proper and adequate wastewater treatment plants in major cities of Nigeria is hampering water quality monitoring in Nigeria. In major urban cities of Nigeria, there are only twenty-eight wastewater treatment facilities owned by government and other industries spread across six geopolitical zones with more prevalence in the southern part. Only twenty-three (23) wastewater treatment plants were reported in the Southern part, while only five (5) wastewater treatment plants were reported in the Northern part of Nigeria. Some functional while some non-functional (Adesogan, 2013).

The adverse effect of wastewater disposal practices in Nigeria water systems and lack of wastewater treatment plants in most part of Nigeria demands for a newer strategies and technologies into the pollution monitoring approach. The fact that some important chemical concentrations are often subtle on aquatic biota and may not be detected through conventional monitoring methods. Most of the monitoring methods in Nigeria do not represent the real exposure to the aquatic wildlife as they may not likely account for the bioavailability of the pollutants. It is the internal concentrations that are the initiating factors and are clearly the key to understand and predict the risk of exposure of biota to pollutants. Thus, it is important to have a practical working tool in Nigeria that can detect and quantify such exposure through internal concentrations in order to attribute cause and detrimental effect. Therefore, a more sound and advance approach is required in inferring the health status of Nigeria aquatic system. Nucleic acid carried and expressed by the aquatic organisms during a chemical insult can be quantified through Polymerase chain reaction (PCR). This can be regarded as useful part for a molecular-based monitoring method. Deoxyribonucleic acid (DNA) and Ribonucleic acid (RNA) can be used to detect an actively expressed genes stimulated by the chemicals and this may be considered as a functional approach in aquatic body monitoring in Nigeria water system. PCR allows a precise quantification of a low concentration of a specific nucleic acid in a complex mixture of chemicals compared to a reference. This approach has been used to quantify genes or transcript from environmental samples which allows deeper understanding on the development of a potential molecular biomarkers to be employed in a specific polluted environmental assessment (Zolkefli et al., 2020). Table 1.2 summarises some of the studies conducted using bioaccumulation, biochemical, molecular, morphological and histopathological biomarkers in Nigeria.

Table 1.2: Summary of Bioaccumulation, biochemical, molecular, morphological and histopathological biomarker studies in fish in Nigeria.

Biomonitoring technique	**Fish species**	**Pollutants**	**Location**	**Observed response**	**Reference**
Bioaccumulation	*Clarias gariepinus*	Copper, lead, Arsenic, Nickel, cadmium	Igbokoda river, Ondo State	Change in GST, SOD, CAT, GSH, and GPx activities	Arojojoye et al., 2018
Bioaccumulation	*Chrysichthys nigrodigitatus, Arius heudoloti, Trachinotus teraia, Sphyreana barracuda, Liza dumerilli, Pseudotolithus elongatus, Tilapia guieensis, Pomadasys jubelini, Caranx hippos, Polydactylus quadrifilis.*	PCBs, lead, zinc, copper, Nickel, arsenic, iron mercury, cadmium	Lagos lagoon, Lagos	high condition factor	Oluwakemi et al., 2020
Bioaccumulation	*Sarotherodon melanotheron, Sardinella maderensis, and Liza falicipinis*	Vanadium, zinc, cadmium, nickel, mercury, lead, copper,	Bonny River, River State	Metal toxicity	Obeka and Numbere, 2020
Biochemical response	*Clarias gariepinus*	Pharmaceuticals	Fisheries farm, Oyo State	Change in AST, ALP, ALT activity	Melefa et al., 2020

Table 1.2 continues….

Biomonitoring technique	Fish species	Pollutants	Location	Observed response	Reference
Biochemical response	*Clarias gariepinus*	Zinc, lead, copper, manganese, nickel	Oshogbo and Yakoyo fishpond, Ogun State	Change in the antioxidant enzyme activities (CAT, GST)	Aladesanmi et al., 2017
Gene expression	*Sarotherodon melanotheron*	Lindane, Dieldrin, 4-iso-Nonylphenol, 4-t-octylphenol	Lagos lagoon, Lagos	Endocrine disruption response	Adeogun et al., 2019
Gene expression	*Tilapia guinensis*	Heavy metal e.g., Lead, aliphatic hydrocarbon e.g., C10, Polycyclic aromatic hydrocarbon e.g., BaP	Eleyele Lake, Oyo State	Change in Phase I and II biotransformation enzymes genes response	Ibor et al., 2019
Gene expression/EROD/MROD activities	PLHC-1 cell lines	Aquatic sediment polar and non-polar extracts	Lagos lagoon, Lagos	Change in Cyp 1A mRNA expression, EROD and MROD activity	Menillo et al., 2020
Gene expression/Biochemical response	*Sarotherodon melanotheron*	Arsenic, cadmium, copper, mercury, zinc, chromium, lead	Awba dam, Oyo state	Change in MT, CAT, GPx, ZnCu-SOD mRNA and activities	Adeogun et al., 2020

Table 1.2 continues….

Biomonitoring technique	**Fish species**	**Pollutants**	**Location**	**Observed response**	**Reference**
Histopathological	*Auchenoglanis occidentalis*	Zinc, cadmium, iron, lead	Tiga dam, Kano	Lamellar edema, hepatic necrosis	Abalaka, 2015
Histopathological Biotic indices	*Clarias gariepinus* Insects	Nittol detergent Heavy metals	Challawa Dam	Fusion and damage of lamellar Loss of diversity	Ivon et al., 2020 Suleiman and Abdullahi, 2011

1.9.0 Tilapia as a test organism

Nile tilapia (*Oreochromis niloticus*) is a freshwater fish from the family of Cichlids. They are the most cultured fish grown apart from carp and are regarded among the most important commercial fish species in tropical and subtropical regions for aquaculture practice (FAO, 2012). Tilapia is a major source of animal protein and contributes to the generation of income globally. It is an ideal aquaculture species due to its characteristics of poor water quality, ease of reproduction in captivity tolerance since they are hardy and prolific, fast growth rate and omnivorous behaviour. Nile Tilapia lives in shallow water with an optimum temperature of 27-28 ^{o}C. It is an opportunistic feeder, feeding on a range of food from detritus, benthic fauna, planktons, small invertebrates and aquatic plants. This makes Nile Tilapia a good food resource to the poor rural populace. Presently, Nile Tilapia are the shining stars of aquaculture production in the world; they are even referred to as aquatic chicken (Prabu et al., 2019).

Tilapias are among the excellent fish species to study the mutagenic, carcinogenic and genotoxic potentials risk and adverse effect of pollutants present in the water bodies. Tilapia can metabolise, concentrate and store waterborne contaminants. Tilapia can function as a fish model for the assessment of toxicants in a tropical aquatic ecosystem. Tilapia can habitually react to contaminants in the same way to higher organism in the food chain. They can therefore be employed to monitor various chemicals that are possibly harmful to aquatic ecosystem and humans (Lionetto et al., 2019). One of the important functions of model species like Tilapia in biomonitoring is the determination of the distribution of chemicals to reflect exposure risk and effect in the aquatic animals. Since aquatic organisms including fish are employed in the bioassay in the monitoring of the quality to assess the exposure of different chemicals (El-Sappah et al., 2012; Hogan and Muldoon, 2016). Development in biological monitoring techniques employing fish have made it possible in the assessment of water contamination with a swift response on low concentrations of chemicals. Therefore, Tilapia species could possibly be used as an additional model fish for regulatory assessment and testing of chemical contaminants and ecological integrity monitoring (Hogan and Muldoon, 2016).

1.10 Justification of the research

Nigeria is the second largest aquaculture producer in Africa, behind Egypt. Nigeria has an annual production output of 300,000 tons of fish, dominated by African catfish (*Clarias gariepinus*) farming (Ozigbo et al., 2014; FAO, 2018). In Nigeria, aquaculture practice

commenced in the past five decades, but this practice was not able to bridge the gap between domestic consumption and production output (Ozigbo et al., 2014). The primary driving force for aquaculture development in Nigeria is socio-economic, with the ultimate objective of providing additional income, supplement beef protein consumption in the poor rural populace, and create jobs. Therefore, aquaculture production was improved to fill the national short fall of domestic fish provision in the country (Ozigbo et al., 2014). Fish is an important protein for humans and accounts for 40 % of animal protein consumption. Fish has a per capita consumption of 13.3 kg/year in Nigeria which is very low as opposed to world's average of 20.3 kg per capita /year (Adeleke et al., 2020). Nigeria roughly produces around 1 million tons of fish per annum, with above 750,000 from capture fish and 310,000 tons from aquaculture practice. Yet Nigeria has to import about 600,000 tons of marine related to bridge the gap of production and consumption (Bradley et al., 2020). To overcome these challenges, Nigeria introduced a caged system which has significantly increased domestic annual fish production in a natural aquatic setting. Nigeria is bestowed with natural coastal and inland water bodies, which are worthy of caged culture fish production. But the majority of these water bodies might be chronically contaminated, since there is no established routine monitoring strategy in the country (Adeogun et al., 2016).

Therefore, a comprehensive and methodical strategy that will help in the comprehension of the biological endpoints (biomarkers), influenced by the amount of these contaminants and the subsequent consequences on organisms, is of paramount importance. These strategies will provide a solid basis for risk assessments in human health, wildlife, and ecology. Furthermore, it will furnish regulators with the foundation to provide alternative means in the formulation of waste management policies and regulations as well as sensitizing the public to the dangers of indiscriminate waste disposal and its resultant long-term effects. An alternative biomarker approach to complement or serve as an alternative to analytical chemical analysis has been developed as an endpoint to measure mRNA as a regulatory guideline to assess xenobiotics in the aquatic environment (Adeogun et al., 2019). Therefore, this study hopes to provide baseline data on the development and validation of a biomarker approach for aquatic monitoring in Nigeria, especially in Northern Nigeria, where this approach is lacking. This research answered the following questions.

- Is biological monitoring an alternative to chemical monitoring for detecting pollutants in the environment?
- Can a liver be a suitable tissue for biomonitoring in Nile Tilapia (*Oreochromis niloticus*)?
- Can a PCR quantify selected genes of interest when exposing Nile Tilapia (*Oreochromis niloticus*) larvae to a range of chemicals under laboratory condition?
- What are the likely pollutants to be predicted in Nigerian water bodies based on samples of Nile Tilapia (*Oreochromis niloticus*) caught in those Nigerian water bodies without chemical analysis?

1.11 Objectives of the research

The ultimate objective is to provide a tool for assessing exposure pollutants using Nile Tilapia (*Oreochromis niloticus*) as an indicator species. Most of the research in Nigerian polluted water bodies has centred on the measurements of environmental heavy metals, some organic chemicals and physicochemical parameters due to limited human and infrastructure resources. So far, very little work (Adeogun et al., 2016 a, b; c; d; Adeogun et al., 2019; Ibor et al., 2016, 2019; Menillo et al., 2020) has been done on the use of gene expression biomarkers on fish to detect exposure to pollution in Nigeria. Water bodies in Nigeria were selected where common pollutants [e.g., toxic metals, polyaromatic hydrocarbons (PAH), polychlorinated biphenyl (PCB), polybrominated Diphenyl ethers (PBDEs), dioxin, phenols, adsorbable oxygen halide (AOX), organochlorine, some pesticides and other emerging pollutants] are suspected to be present, along with others where pollutants have not yet been detected and which are considered "clean". Of course, it is virtually impossible to undertake an exhaustive chemical analysis to ascertain all potentially toxic chemicals, and still harder to determine whether the level of any particular chemical is likely to have a biological effect. However, this is the point of the project - to develop a technique for analysing multiple gene expression variables, which can potentially provide "gene expression profiles" of the classes of chemicals present at biologically meaningful levels. It is therefore hypothesised that gene expression profiling is a good way to study the pollutant exposure in polluted Nigerian water bodies.

1.12 Aims of the Study

- Develop a practical monitoring tool using qPCR as an alternative to chemical analysis for the assessment of pollutant exposure, using Nile Tilapia *(Oreochromis niloticus*) as an indicator species.

- Generate expression profiling of certain set of genes of interest and their likely pathways due to exposure of Nile Tilapia (*Oreochromis niloticus*) larvae to individual chemicals in the laboratory exposure.

- Prediction of possible pollutants if present in a natural aquatic environment at a biologically meaningful level following the expression of certain set of genes of interest and the presence of some morphological indices.

2.0 CHAPTER TWO: DESIGNS AND OPTIMISATION OF OLIGONUCLEOTIDE PRIMERS AND TISSUE –GENE SPECIFIC mRNA PATTERN EXPRESSION

Abstract

Primers are conceivably the most important component of PCR assays. The optimisation matrix of the primers is used to enhance qPCR performance, specificity and sensitivity. In the setting up of qPCR assay, one of the most important steps is to determine the efficiencies of the newly designed or purchased primers for accurate data interpretation. Genome sequence interrogations were conducted in GenBank using Tilapia as a query species, obtained from the NCBI website. Therefore, varying cDNA concentrations on newly designed oligonucleotide primer pairs for xenobiotic target genes in Nile tilapia were evaluated. Five varying cDNA concentrations (x4, x40, x400, x4000, and x40,000) were measured using PCR array consisting of triplicate of liver tissue in a 96 wells qPCR plates for the 28 of the previously identified and selected genes. Moreover, the patterns of expression of xenobiotic metabolism genes in Nile tilapia was investigated, in order to determine the optimal tissue on which to focus field biomonitoring activities. Six tissues, liver, spleen, intestines, gills, heart and muscle were selected from adult Nile tilapia and validated using 28 genes already optimised.

Twenty eight qPCR assays were compared (*AHR 1, AHR 2, AHRR, CYP 1A, DIABLO 1* and 2, 16 isoforms of GSTs consisting of *GSTA,GST01LA-LC,GSTA2L, GSTK, GSTMA-MB, GSTR1-5, GSTT1-2, MGST, MT, SIAH 2, UDP-GT 1* and *2, VTG,* and *ZPC*) using a tenfold cDNA serial dilution to obtain a standard curve, in which ten of the twenty eight assays showed the best results and were within the target amplification efficiencies and coefficient of correlation ranges of 0.90-1.10% and 0.9-0.99 respectively. The ten assays are *AHR 2, CYP 1A, DIABLO 1* and *2, GST01LA, GSTA2L, GSTMA, GSTR2, MT* and *VTG.* The results of tissue validation showed differential expression patterns of 28 mRNAs measured in liver, spleen, Small intestines, gills, muscles and heart in an untreated fish. *Pan18S ribosomal RNA (18S rRNA)* and *Pan RPL 3* were used as a reference gene. Basal tissue mRNA expression of the genes was quantified in all the tissues with liver having expressed most of the genes.

2.0 INTRODUCTION

Several articles in peer-reviewed journal publications and databases contain references of oligonucleotides primer sequences employed in quantitative polymerase chain reaction (qPCR) assays or even supplied from the commercial industry. Obtaining an oligonucleotide primer are now inexpensive, most qPCR solutions and other reagents are becoming reliable, quicker, and cheaper to get. qPCR master mixes that are task-specific and affordable as well as user-friendly thermal cyclers are now available, as such a large number of data could be generated at ease. Considering the large number of qPCR related data that are published annually, it is vitally important to publish real and accurate results in order to avoid bias or inaccurate report (Bustin, 2017). It may be surprising, with all the accessible ready-made assays commercially, why would a researcher trouble himself designing a complex and tedious assay, rather than obtaining one that was validated from experienced commercial industry. This opinion can be wrong for some reasons. Oligonucleotides primers obtained from the commercial industry may not have undergone experimental optimization or validation and as such may not work to ones' satisfaction. Secondly, it cannot be assumed that primers from different assays to have similar efficiency for another assay. This is because assay's experimental conditions could differ from one laboratory to another, as well as templates extraction and purification methods, qPCR reagents, and the thermal cycler employed in the assay (Alemayehu et al., 2013).

The experiment in a laboratory to optimize and validate an assay could be a means of an assay's optimum performance and is sacrosanct to the researcher. It is better to catch the problem of poor synthesis, inconsistency in experimental data, and failed runs earlier before performing a lot of work on unrepeatable precious samples only to find out that the assay is performing below expectations. It was reported that many peer-reviewed journals published qPCR data that is deficient of critical information such as primer sequences, accession numbers or providing wrong data reports (Bustin and Nolan, 2017). The major problems associated with primer assay design affecting researchers in a laboratory are lack of knowledge on the basic parameters like appropriate design tools, that could generate optimum oligonucleotide primer that is suitable, robust, sensitive and specific to RNA (Bustin and Huggett, 2017). When designing optimal primers for accurate qPCR assays (nucleic acid quantification), it is crucial to have a full-scale workflow that needs mindful attentions of the primers, uniqueness of the amplicon and structures. Therefore, it is necessary to handle each step and the materials carefully to ensure accurate results (Kuang et al., 2018). The qPCR primer assay design workflow centers on some critical steps that

will impact its performance. Optimized and validated primers should result in qPCR assays that are sensitive and specie-specific in quantifying mRNA copy numbers. Appropriate design tools are always free online for use. Moreover, validation and optimisation of the primers is quite important. Whether a colleague designed them or bought from a commercial or even extracted from the databases. This would make the data from the qPCR assay results more reliable. In order to have specific and sensitive qPCR reactions, it is crucially important to complete the design and validation of primers. Primers are among the most important components of qPCR assays, as a poor design and a lack of validation and optimisation can lead to unbinding the cDNA, which may result in the non-detection of target gene amplifications (Applied Biosystem 2002a). The primers (forward and reverse) are designed short sequences of DNA that prime to specific sequences of target DNA template and allow synthesis of DNA in 5' and 3' end.

On the other hand, quantification of the external levels of the selected individual or mixture of pollutants due to the exposure to aquatic organisms of such pollutants is not sufficient to justify the environmental quality of aquatic ecosystem (Environmental monitoring). As such, a measurement of the internal dose of the pollutants in the organism is of paramount importance, as the fate of such contaminants in biotransformation, accumulation and bioavailability would be elucidated in the aquatic ecosystem. Nevertheless, monitoring of all pollutants of natural (e.g., Polycyclic aromatic hydrocarbons and heavy metals) and anthropogenic origins (e.g., halogenated hydrocarbons), which are potential threats to the aquatic environment, is impossible (Everaart et al., 1994). Therefore, numerous schemes have been adopted to monitor the exposure of xenobiotic metabolism with other persistent chemicals in fish by means of different metabolic biotransformation pathways by altering the chemical configuration of the contaminants that are alien to the normal processes of the organism's body, for example, changes in the activities of different mRNA transcripts or enzyme levels that function in cellular protection against the insult of a xenobiotic chemical. These mRNA/enzyme changes could be in two different ways, either by influencing the quantity of the mRNA/enzymes through induction or suppression of the synthesis of protein or by changes in the phenotypic activities of the exposed, compared to the non-exposed (activation or inhibition) organism (Karaca et al., 2014). It is therefore pertinent to note that specific proteins or mRNA transcript alterations are quantified in the fish tissues in relation to xenobiotic metabolism chemical discharges into the aquatic ecosystem, and the expression of different genes are influenced by the diversity of environmental stimuli (Auslander et al., 2008).

The development of gene expression pattern analysis in the recent past has contributed immensely to the understanding of how fish health is impacted by xenobiotic contaminants. Data from gene expression analysis can contribute knowledge on the exposure and possible consequences of the potential effects of contaminants on the organisms at the top of the food chain (Pauletto et al., 2019). Therefore, this valuable knowledge can be used to a certain extent to investigate how the presence of persistent and other emerging chemicals impact the health of an aquatic organism in its environment. This type of knowledge can also contribute to knowing the fate and effects of xenobiotic chemicals that are not yet known and classified. Previous studies in laboratories were conducted to demonstrate the effects of pollutants using either individual or mixed pollutants on gene expression studies (Vidal-Dorsch et al., 2012). Therefore, the early response of genes in fish such as *CYP1A, GSTs, MTs, VTG, AHR, DIABLOs,* etc to xenobiotics, including pesticides, metals, PAHs, PCBs and other emerging chemicals, could trigger the induction of gene transcription in various tissues such as the liver, gills, heart, spleen, intestines and white muscles.

Biomarkers represent induced toxicant alterations in biological systems and serve as a link between causes of environmental pollution and its exposure and effects, thereby allowing a distinctive knowledge on the health of the ecosystem as well as providing important data on any possible pathological processes in fish (Osman et al., 2019). Therefore, transcriptomic techniques using microarray and RNAseq gene expression analysis development could be used in biomonitoring xenobiotic chemicals, since this method measures simultaneously the expressions of thousands of genes. It is also a means of assessing a mechanism of action of the effects of such chemicals' exposure to environmental or non-model fish species (Leaver et al., 2010). A similar approach of microarray and RNAseq gene expressions, using RT-qPCR to measure a small number of genes was adopted in this present study, to test whether gene expression profiling could be used to detect the toxicity of contaminants due to exposure in the laboratory and the field. Due to its high sensitivity, specificity and reproducibility, quantitative real-time PCR (RT-qPCR) is presently among the most popular methods for mRNA gene expression analysis. It has become the technique of choice for examining changes in one or more genes of interest, due to its excellent approach for quantifying cellular RNA. Ultimately, the aim of this chapter is to validate and optimise the 28 genes and to compare gene expression patterns of different target tissues using a quantitative polymerase chain reaction (qPCR) to identify a tissue optimally expressing different target genes of interest in Nile tilapia for aquatic environmental monitoring to be used in wild tilapia sampling. It is therefore to note that only the 28 genes optimised, as

described, were used for screening and validation of different tissue in this study. Any results from the genes that did not meet the criteria for amplification efficiency are not reliable.

2.2.0 MATERIALS AND METHODS

2.2.1 Pollutant responsive gene selections

The selection of pollutant responsive target genes was based on previous studies. The sequences were extracted by interrogating the Tilapia genome sequences using Tilapia as a query species from the NCBI website at http //: www.ncbi.nim.nih.gov. Based on these transcripts, representative sequences were selected to uniquely represent the target genes. The transcript, primer name, accession number, primer sequences, melting temperature, G+C contents were obtained after interrogating the primer design tool NCBI website www.ncbi.nim.nih.gov which implement the programme primer 3. The transcript sequences were also uploaded to nucleotide BLAST on www.ncbi.nim.nih.gov for a similarity check, to ensure that the sequences are specific to the Tilapia species.

2.2.2 Oligonucleotide Primer Design

With the availability of a template sequence, the next step was to design primers (forward and reverse) using a primerBlast from the NCBI website. Primers are the focal point of any qPCR assay. They are short pieces of a single stranded DNA designed specifically to bind to a target gene of interest sequences and allow the synthesis of DNA in both 3' and 5' primer ends. The PCR amplicon size is set to a maximum of 250 base pairs (bp). All the primers have a melting temperature (T_m) value in the range of 57-61^{o}C. In addition, in order to ensure a uniform primer annealing, the primer G+C content was restricted to 45-60 % (Table 2.1). Additionally, the primers were chosen to span intron-exon boundaries in order not to not amplify genomic DNA but result in a specific mRNA amplification product. As such, there is a need to span an intron such that 5 or 6 bases of the 3' end of one primer hybridizes to one exon of the gene and hybridizes to the adjacent exon for the remaining portion (Raymaekers et al., 2009).

2.2.3 1 Aquarium tissue harvest

Five juvenile Nile tilapias were harvested from the University of Stirling tropical aquarium. The fish were euthanized (using Benzocaine, 3ml into 1 litre of water). Fish biometric data (total length, weight and sex) were determined before the fish were dissected to remove the tissue. Six tissue samples were collected from each fish. The tissues samples were taken from the liver, gill, heart, spleen, intestine and white muscle. The samples were collected in microtubes containing 1ml RNA for preservation, according to the manufacturer's protocols (Sigma) and taken to the laboratory freezer for storage (-20 ^{o}C) for further analysis. All the

protocols followed was in accordance with the University of Stirling AWERB and in compliance with UK regulations.

2.2.4 RNA extraction protocol

About 100 ± 20mg of liver tissue from Nile Tilapia harvested from University of Stirling tropical aquarium was added to 2 ml screw cap microtubes (Alpha labs) containing 1ml TriReagent (Sigma, UK) extraction buffer, according to the manufacturer's protocols, and homogenised using a mini bead beater 24 (Bio spec product) until it was disrupted. The homogenised samples were incubated at room temperature for 5 minutes and centrifuged at 12,000 x G for 10 minutes at 4°C (Sciquip). The supernatant was transferred to newly labelled 1.5ml nuclease free microtubes (Axygen). One hundred µl BCP (1- bromo-3-chloropropane, (Sigma, UK) were added to the sample and shaken vigorously for 15 seconds, using a vortex mixer, then incubated at room temperature for 15 minutes to effect phase separation. The samples were then centrifuged at 20,000 x G for 15 minutes at 4°C. The aqueous upper phase was slowly transferred (500 µl) to a newly labelled Eppendorf tube using a 200µl tip (Gilson) from the top, gradually lowering the tip as the supernatant decreased to avoid mixing the white upper phase and the pink interphase. An equal volume of RNA precipitate and isopropanol (250 µl) was added and gently inverted 4-6 times to mix. The samples were then incubated for 10 minutes at room temperature and centrifuged at 20,000 x G for 10 minutes at 4°C. After centrifugation, a pellet formed at the bottom side of each tube. The bulk of the supernatant was removed carefully by decanting to avoid the RNA pellet falling, and then pulse spun to remove the remaining supernatant residue by auto pipetting. 1000µl of 75% ethanol (Fisher scientific) was added to each tube, flicked and left on the bench for an hour to wash the pellets. The RNA pellet containing the ethanol was spun at 20,000 x G for 5 minutes. The ethanol was removed by decanting carefully to avoid the pellet, which was difficult to see, and then pulse spun to remove the residual supernatant by auto pipetting. The RNA pellet was then allowed to dry for 5 minutes on the bench with the lid open. The RNA pellet was resuspended in miliQ water. Keeping the RNA pellet in the fridge overnight allowed the dissolution of the RNA. 100µl MiliQ water was used for the bigger pellets, 50µl MiliQ water for medium pellets and 30µl MiliQ water for the smaller pellets.

Meanwhile, tissues (< 100 mg) from the liver, spleen, gill, muscle, heart and intestine (five samples per tissue) were cut to approximate 50mm^2 pieces and added to 2ml screw cap microtubes containing 1ml of TriReagent. The extraction of RNA was carried out as previously described above.

2.2.5 RNA quantification and quality assessment

The RNA purity and concentration were determined by measuring the absorbance of light at 260 (DNA and RNA) and 280 nm (for protein) using a NanoDrop ND-1000 spectrophotometer (Labtech International Ltd, UK). Usually a concentration of 70 ng/µl or above with an absorbance ratio of 260 – 280 nm (1.67- 2.00 purity) of RNA is required. The RNA was then standardised to a uniform concentration of 300 ng/µl.

2.2.6 Agarose gel electrophoresis

The RNA integrity and quality were evaluated using a 1% agarose gel electrophoresis (in Tris-acetate-EDTA-TAE-buffer) stained with ethidium bromide (EtBr) and visualized using Syngene UV illuminator bio imaging (for possible degradation) to check for the banding intensity of 28S: 18S rRNA by electrophoretic separation and visualisation. Prior to the loading, 2µl of RNA was taken and 2µl of 2x gel loading dye was added into a PCR tube and heated at 75 oC for 2 minutes in a thermocycler, for denaturation. 2 - 4µl (about 500ng) incubated RNA were then loaded into the wells of the 12 minigel and run at 70 volts for 45-60 minutes. The current was checked regularly to see if the samples were moving out of the wells in the correct direction. The progress of the gel movement was monitored using the loading dye until it run about ¾ of the gel way.

2.2.7 Gel picture using UV transilluminator

After running the gel for an hour, it was taken to a Syngene UV transilluminator and visualised to check the RNA quality. Intact or degraded RNA was analysed by assessing 18s and 28s bands. If the 28s RNA band is almost twice as intense as the 18s RNA band, it is a good indication that the RNA is intact. Partially degraded RNA has a smeared appearance, lacks the sharp RNA bands, or does not show a ratio of 2:1. Completely degraded RNA appears as a very low molecular weight.

2.2.8 cDNA synthesis

The RNA (1µg) was reverse transcribed to produce cDNA, using a Precision Nanoscript 1 reverse transcription kit (Primer design) according to the manufacturer's instructions. The synthesis of cDNA was performed in a Biometra thermocycler with a mastermix of 3µL RNA template, 1µl RT Primer, 6µL RNAse/DNAse water, 2µl 10x Buffer, 1µl 10mM dNTP mix, 2µl DTT, 4µl RNAse/DNAse water and 1µl Nanoscript enzyme in a total volume of

20µl under the following condition: 5 mins (initial denaturation) at 65ºC, 5 mins incubation on ice, 25 ºC for 5 mins, 55 ºC for 20 mins and 75 ºC for 15 mins. At the annealing step, a mastermix of 3µl RNA template, 1µl RT Primer, 6µl RNAse/DNAse water was prepared in thin-walled PCR tubes to make a final volume of 10µl and taken to the PCR machine, then heated at 65ºC for 5 minutes. After 5 minutes, the samples were immediately transferred to the ice to cool the tubes. Then the mastermix of the extension step was added, 2µl 10x Buffer, 1µl 10mM dNTP mix, 2µl DTT, 4µl RNAse/DNAse water and 1µl Nanoscript enzyme to give a volume of 10µl to make a final volume of 20µl on the ice. The samples were mixed by vortexing, followed by pulse spin, before taken to the PCR machine and incubated at 25ºC for 5 minutes, and then at 55ºC for 20 minutes. Later the reaction was heat deactivated by incubating at 75ºC for 15 minutes. The cDNA mixture was diluted with 80µl RNAse water and conserved at -20ºC until it was used in a real time PCR reaction. The cDNA for primers optimisation was diluted into five different concentrations, first the main concentration and then ten times per point in the form of 4, 1/40, 1/400, 1/4000/ 1/40,000 to generate a standard curve for the efficiency of the primers. For the determination of the amplification efficiencies of a set of primers, the slope of amplification of different cDNA dilutions was applied. The amplification efficiencies (E) of the 28 Oligonucleotide primers optimised from the slope of amplification of the standard curve serial dilution was calculated as a percentage of the optimal slope of a ten-fold amplification per cycle from the linear regression of a plot Ct (y-axis).

2.2.9 cDNA Dilution series

During the cDNA dilution series, 10µl of each 10-cDNA-synthesis reaction were pooled together to give a final volume of 100µl. This reaction was then diluted 4x in the cDNA dilution buffer (1ml Tris, 0.1mM EDTA, 1ng/µl plasmid DNA). They were mixed well by pipetting and then serially diluted to 40x, 400x, 4000x, and 40,000x in tenfold dilution series to generate a standard curve.

2.2.10 Oligonucleotide primer dilutions

Typically, the primers (Eurofins genomics, Germany) ordered need to be diluted to a stock concentration of 100pmoles/µl. They were diluted appropriately according to the manufacturer's instructions. A stock aliquot of each was made at 1:10 (5µl of primer stock to 45µl of MiliQ water), giving 10 µM of the aliquot stock and a final concentration of 0.2µM primer in the final mix.

2.2.11 Quantitative -PCR assay

Real-time PCR was performed using a TOptical PCR machine (Biometra, Germany). Each 10µl reaction contained a mastermix reaction of 0.2µl 10µM forward and reverse primers (Eurofins genomics, Germany), 5µl SYBR Green Luminaris supermix (Thermoscientific), 2.6µl MiliQ water and 2µl of the diluted cDNA samples. The following cycling conditions were used: initial heating at 50 ^{o}C for 2 mins, followed by 95 ^{o}C for 10 mins for initial incubation to activate the DNA polymerase in the mix. Then, at 95 ^{o}C for 10 sec for denaturation, at 60 ^{o}C for 10 sec, and at 72 ^{o}C for 15 sec for annealing and extension steps. The primerBlast search was targeted at primers with an annealing temperature of 60 ^{o}C. This cycle was repeated 40 times. In the PCR machine, due to final rise of temperature from 60 ^{o}C to 95 ^{o}C allows the detection of the melting temperature of the gene of interest by the machine, and this pave way for the generation of melt curve. This indicated that a single sequence has been amplified resulting to a single narrow peak. The assay plate consisted of four different genes run in triplicates in five rows (containing five different cDNA concentrations of x4, x40, x400, x4000, and x40, 000), one row consisting of controls, leaving two rows blank, all in a 96 well for each plate. In total, seven plates were used to run all the 28 genes. Controls lacking cDNA templates were included to determine the specificity of the target cDNA amplification as a no template control (NTC). Analysis of melt curve and agarose gel electrophoresis was performed to confirm the specificity of the PCR reactions. The melt curve provides accurate identification of amplified products and distinguishing them from primer dimers and other small amplification artefacts resulting into a single peak. The dissociation curve was automatically analysed from the machine due to successful amplification of a single specific cDNA. While Agarose gel electrophoresis specificity check employed the primers while binding with the cDNA, a single intense DNA band of expected size is observed.

2.2.12 DATA ANALYSIS

The quantification of the relative amount of mRNA was performed by generating a standard curve (plotting the Ct values against the logarithm of the initial copy numbers of the standards tenfold serial dilution cDNA). The qPCR machine software calculated the amplification efficiency and Coefficient of determination (R^2) automatically for each reaction plate, resulting in a standard curve after determining background fluorescence. The Ct value or threshold cycle is the number of cycles needed for the fluorescence signal to cross the threshold or background level and is also referred to as the quantification cycle (Cq), crossing point (Cp) or take-off point (TOP). The target range of the efficiency and

Coefficient of determination (R^2) were between 0.90-1.10 % and 0.9-0.99 respectively. For tissue expression pattern, all the analysis of the results was calculated using delta-delta Ct method (Pfaffl model equation, 2001) in Excel spreadsheets and IBM SPSS version 25 (IBM, Armonk, NY, USA). *18S ribosomal RNA* and *Ribosomal Protein L3* housekeeping genes were used to normalise the expressions of the target genes differences for each tissue. The geometric means of two housekeeping genes *(18s* and *Rpl 3*) were calculated and later used to normalise the resulting Ct values of each gene. All quantitative values were presented as the mean ± standard deviation of the normalised Ct values relative to reference genes expression. Following a failed test of normal distribution, the data was log transformed but was still not normally distributed; therefore, the non-parametric Kruskal Wallis tests was used appropriately on the Ct values of the normalised tissues/gene. A null hypothesis (H_o) was stated as: "there are no significant differences between the genes expressed in all the tissues." The alternate hypothesis (H_1) was stated as: "there are significant differences between the genes expressed in all the tissue." The level of significance was set at $P \leq 0.05$.

2.3 RESULTS

2.3.1 Serial dilution for standard curve and efficiencies

The genes optimised includes those of phase I and II biotransformation enzymes, apoptosis, endocrine disruptions, metal detoxifications and reference genes. The primers GC contents and melting temperatures (Tm) of all the genes were presented in the Table 2.1 below. The efficiencies of the target genes *AHR 2, CYP 1A, DIABLO 1and 2, GST01LA, GSTA2L, GSTMA, GSTR2, MT, VTG, I8S ribosomal RNA (18S),* Ribosomal *protein S5 (RPS5)* and *Ribosomal protein S7 (RPS7)* were within the target range of 0.9-1.1 for most genes. Meanwhile *Aryl hydrocarbon receptor repressor (AhRr*) has an efficiency of 1.78 %, *Glutathione S-transferase alpha (GSTA)* has an efficiency of 0.76 %, *Glutathione S-transferase01LB (GST01LB)* has an efficiency of 1.5 %, *Glutathione S-transferase01LC (GST01LC)* has an efficiency of 1.35 %, *Glutathione S- transferase Kappa (GSTK)* has an efficiency of 1.77 %, *Glutathione S- transferase Mu (b) (GSTMB)* has an efficiency of 0.57 %, *Glutathione S- transferase Rho 1 (GSTR1)* has an efficiency of 2.02 %, *Glutathione S-transferase Rho 3 (GSTR3)* has an efficiency of 1.76 %, *Glutathione S- transferase Rho 4 (GSTR4)* has an efficiency of 2.01%, *Glutathione S- transferase Rho 5 (GSTR5)* has an efficiency of 1.53 %. *Glutathione S- transferase Theta 1* (*GSTT1),* has an efficiency 0f 1.33 %, while *Glutathione S- transferase Theta 2 (GSTT2),* has an efficiency of 1.72 %. *Microsomal Glutathione S- transferase (MGST)* has an efficiency of 2.88 %, while *UDP-glucuronyl-transferases 1(UDPGT 1)* has an efficiency of 1.95 % and *UDP-glucuronyl-transferases 5 (UDPGT 5)* has an efficiency 1.90 %. *Zona Pellucida C (ZPC)* has an efficiency of 0.70 %.). The charts of the 28 primer standard curves showing the efficiencies and correlation of determination (R^2) are listed in Annex 2 (Fig. 2.1-32).

TABLE 2.1: Transcript, primer sequences, annealing temperature, GC content, melting temperature, amplification, efficiencies and corresponding target GenBank accession numbers used in the Quantitative -PCR.

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
1.	Aryl hydrocarbon receptor 1	XM_003446578.2	QtnAHR1f	CGTGTTTTCTGCCACCCAAG (20)	59.4	50	0.47
			QtnAHR1r	ACTGCGTTCCCATTGGACAT (20)	57.3	50	
2	Aryl hydrocarbon receptor 2	XM_025903995.1	QtnAHR2f	AGCCACCAAAACCCAGTCAA (20)	57.3	55	0.93
			QtnAHR2r	CTGCCAGCTGTTGGAAAAGC (20)	59.4	55	
3	Aryl hydrocarbon Receptor repressor	NM_131264	QtnAHRrf	TGCTCCCCTCTATCACGGAA (20)	59.4	55	1.78
			QtnAHRrr	ACAGGAGAGCCGGAGAGAAT (20)	59.4	55	
4	Cytochrome P450 1A	NM_001279586.1	Qtn CYP 1Af	GCGAGGACAGAAAGCTGGA (20)	59.4	55	1.02
			Qtn CYP 1Ar	AAGGGGCAAGTTGTTCCGAT (20)	57.3	50	
5	Diablo 1	XM_005455754	QtnDiablo 1f	CCACCTTGGCTTACGTCGAT (20)	59.4	55	0.90
			QtnDiablo 1r	ACACAGGCTGATGGCATTGA (20)	57.3	50	

Table 2.1. continued…….

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
6.	Diablo 2	XM_005474790.3	QtnDiablo 2f	GCCAGCGTGCAAAGGTTAAT (20)	57.3	50	1.03
			QtnDiablo 2r	GAGTTTTCGTGCCTCCTCCA (20)	59.4	55	
7.	Glutathione S-transferase alpha	NM_001279634.1	QtnGSTAf	ACTGCACACTCATGGGAACA (20)	57.3	50	0.76
			QtnGSTAr	TCCCGAGTTGTCAGAAGCAC (20)	59.4	55	
8.	Glutathione S-transferase 01LA	XM_003448885.2	QtnGSTO1LAf	TGTGGCCATGGTTTGAGAGG (20)	59.4	55	1.02
			QtnGSTO1LAr	AAAGGGACGGTTGAGGGTTTT (21)	57.9	47.6	
9.	Glutathione S-transferase 01LB	NM_001279634.1	QtnGSTO1LBf	CTCAGTCTTCACAGCCCGTC (20)	61.4	60	1.50
			QtnGSTO1LBr	AAAGGGCAGAACCTCATGCT (20)	57.3	60	
10.	Glutathione S-transferase 01Lc	NM_001279635.1	QtnGSTO1LCf	TTCGTTACTTCAAGCGCCAAC (21)	57.9	47.6	1.35
			QtnGSTO1LCr	ACTAGTCTGGTCCTTTGGGC (20)	59.4	55	

Table 2.1. continued…….

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
11.	Glutathione S-transferase Alpha 2L	XM_003460305.2	QtnGSTA2Lf	CCCTTGGACTTCAATAGGCGT (21)	59.8	52.4	1.05
			QtnGSTA2Lr	ACTCGACTTCTGCGACTGTT (20)	57.3	50	
12.	Glutathione S-transferase Kappa	XM_005455409.1	QtnGSTKf	CACACGCTGCGTTAGGTTTT (20)	57.3	50	1.77
			QtnGSTKr	CAAACCAGGAGGCTTGTTGC (20)	59.4	55	
13.	Glutathione S-transferase Mu (a)	XM_003444817.2	QtnGSTMAf	CTGTGGGGAAGCTCCAAACT (20)	59.4	55	0.92
			QtnGSTMAr	TGTAGCACAGCCTCACGAAC (20)	59.4	55	
14.	Glutathione S-transferase Mu (b)	XM_003444817.3	QtnGSTMBf	CCCAGGTTGCCTTCACGAAC (20)	61.4	60	0.57
			QtnGSTMBr	CTCTTGTCGTAGTCGGGAGC (20)	61.4	60	
15.	Glutathione S-transferase Rho 1	XM_003444815.4	QtnGSTR1f	AGAGAGACACGACTCTGCCA (20)	59.4	55	2.02

Table 2.1. continued…

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
16.	Glutathione S-transferase Rho 2	XM_003444816.4	QtnGSTR2f	ACTGTGCTGCTGCAGAAATCTT (22)	57.3	50	1.01
			QtnGSTR2r	ACTGTGCTGCTGCAGAAATCTT (22)	58.4	45.5	
17.	Glutathione S-transferase Rho 3	XM_003444817.4	QtnGSTR3f	TACGGTGCATGCTTCTTCCT (20)	57.3	50	1.76
			QtnGSTR3r	AACTCGCCCGTTAACGTCTC (20)	59.4	55	
18.	Glutathione S-transferase Rho 4	XM_019350597.1	QtnGSTR4f	CAGGGGACAGCTTCCAACAT (20)	59.4	55	2.01
			QtnGSTR4r	GACCCTCAAACATGCGTTGG (20)	59.4	55	
19.	Glutathione S-transferase Rho 5	XM_005451493.4	QtnGSTR5f	GTGCTGTTGTGTTTTGCGTG (20)	57.3	50	1.53
			QtnGSTR5r	GCGATCATCACCCTCCAACA (20)	59.4	55	
20.	Glutathione S-transferase Theta 1	XM_003456547.2	QtnGSTT1f	GGAGAGTGAAGCCCGTTTGA (20)	59.4	55	1.33

Table 2.1. continued…

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
			QtnGSTT1r	GTAGAGCTCCAGCGCCATAG (20)	61.4	60	
21.	Glutathione S-transferase Theta 2	XM_00346547.3	QtnGSTT2f	TGGGGAACTCAACATCGTTT (20)	59.4	55	1.72
			QtnGSTT2r	CGGCAGGAAACCAGTGATCT (20)	59.4	55	
22.	Microsomal Glutathione S-transferase	XM_013270914.3	QtnMGSTf	ACTGGGTGACAGGTGAGATTC (21	59.8	52.4	2.88
			QtnMGSTr	TGCTGAAAGCCCTCACTACC (20)	59.4	55	
23.	Metallothionein	XM_003447045.2	QtnMTf	CAACTGCAAAATGGACCCCTG (21)	59.8	52.4	0.91
			QtnMTr	ATGTCTTTCCTTTGCACACGC (21)	57.9	47.6	
24.	Siah 2	XM_003459533.2	QtnSIAH2f	GCCTGTTGAGGCTTTTGAGA (20)	59.4	55	0.72
			QtnSIAH2r	CCTTTTTCCTCCCCACACGA (20)	59.4	55	

Table 2.1. continued…

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
25.	UDP-Glucuronosyl transferase 1	XM_003445329.3	QtnUDP-GT1f	CCAGCGGACTTGGAAGAGTT (20)	59.4	54	1.95
			QtnUDP-GT1r	TGGCATGGGCTAGGAGATCA (20)	59.8	54	
26.	UDP-Glucuronosyl transferase 5	XM_005447168.3	QtnUDP-GT5f	TGCCGACTTCCTCAAAAGG (20)	59.4	55	1.90
			QtnUDP-GT5r	TGAACTCCATTGGTGCCTCC (20)	45.8	50	
27.	Vitellogenin	XM_005457331.1	QtnVTGf	TCTTGTCGGTCGAAACCCTG (20)	59.4	55	1.04
			QtnVTGr	ACAGCCACAGTCAACGAGAG (20)	59.4	55	
28.	Zona pellucida (C)	XM_003457432.2	QtnZPCf	GTTGCCCAAGCCATTGACAG (20)	59.4	55	0.70
			QtnZPCr	TGCCTGTAGTCCCTGTTCCT (20)	59.4	55	
29.	Pan 18s ribosomal RNA	XM_003200788	18sf	ACCACATCCAAGGAAGGCAG (20)	59.9	50	0.92
			18sr	CACCAGACTTGCCCTCCAAT (20)	59.9	55	

Table 2.1. continued…

No.	Transcript	Accession Number	Primer Name	Sequences (5'-3')	T_m (^oC)	GC Content %	Efficiency %
30.	Pan Ribosomal protein S5	NM_200750	Rps 5f	AACTCCATGATGATGCACGG (20)	58.3	50	0.97
			Rps 5r	GGTCTTGATGTTCCTGAAAGCA (22)	58.8	55	
31.	Pan Ribosomal protein S 7	NM_200752	Rps 7f	CAGAAGCGTCCCAGGAGC (18)	60.1	55	0.92
			Rps 7r	CCTGTGAGCTTCTTGTAGACACC (22)	60.8	55	
32.	Pan Ribosomal protein L 3	NM_001001590	Rpl3f	GGCAAGAAGCAGCTGGAGAA (20)	60.6	55	0.94
			Rpl3r	TTACGCAGACCACGATGGGT (20)	61.54	50	

2.3.2 Fish biometric

A total of five (both juvenile and adult) Nile tilapia were used for this experiment. The total length (cm), weight (g) and sex of each was determined. The mean total length and weight were 19.3 ± 0.5 cm and 82.2 ± 0.5 g respectively (Table 2.2).

Table 2.2**:** Biometric data of the fish sampled in the tropical aquarium

No.	**Length (cm)**	**Weight (g)**	**Sex**
1.	20.5	109	F
2.	19.5	100	M
3.	17.5	93	M
4.	21	113	M
5	18	96	F

2.3.3 Tissue distribution patterns of basal mRNAs level

The basal mRNA expression of each gene was measured in six different tissues from five individual male and female tilapia. The expression level of all genes between individuals was quite variable for both genes and varied greatly across all tissues. However, it was clear that the high expression level of most genes was in the liver, followed by the spleen, then heart, spleen, gills and small intestine. As was shown in the graph, (Figure 2.1) the tissue with the high number of gene expressions levels was the liver, with a highest gene expression level in 24 genes: *AHR 1, AHR 2, AHRR, CYP 1A, DIABLO 1, GSTA, GSTA01LA, GSTA01LB, GSTA2L, GSTK, GSTMA, GSTR 1, GSTR 2, GSTR 3, GSTR 4, GSTR 5, GSTT1, GSTT2, MGST, MT, UDP-GT 1, UDP-GT 5, VTG* and *ZPC*, followed by Spleen with higher gene expression in 12 genes: *DIABLO 2, GST01LC, GSTMB, GSTR 1, GSTR 2, GSTR 3, GSTR 5, GSTT 1, MT, SIAH 2, UDP-GT 1* and *VTG,* then gill with a high gene expression level of 6 genes: *AHR 1, CYP 1A, GSTR 5, GSTT 1, SIAH 2, and VTG*, and Small intestine with a high gene expression level of 6 genes: *GST01LB, GST01LC, GSTMA, GSTR 1, GSTR 4, GSTR 5, and MGST*, then heart with an expression level of 6 genes: *CYP 1A, GST01LB, GSTMA, GSTR 1, GSTR 2, and GSTR 5.* None of the genes was significantly expressed in Muscle.

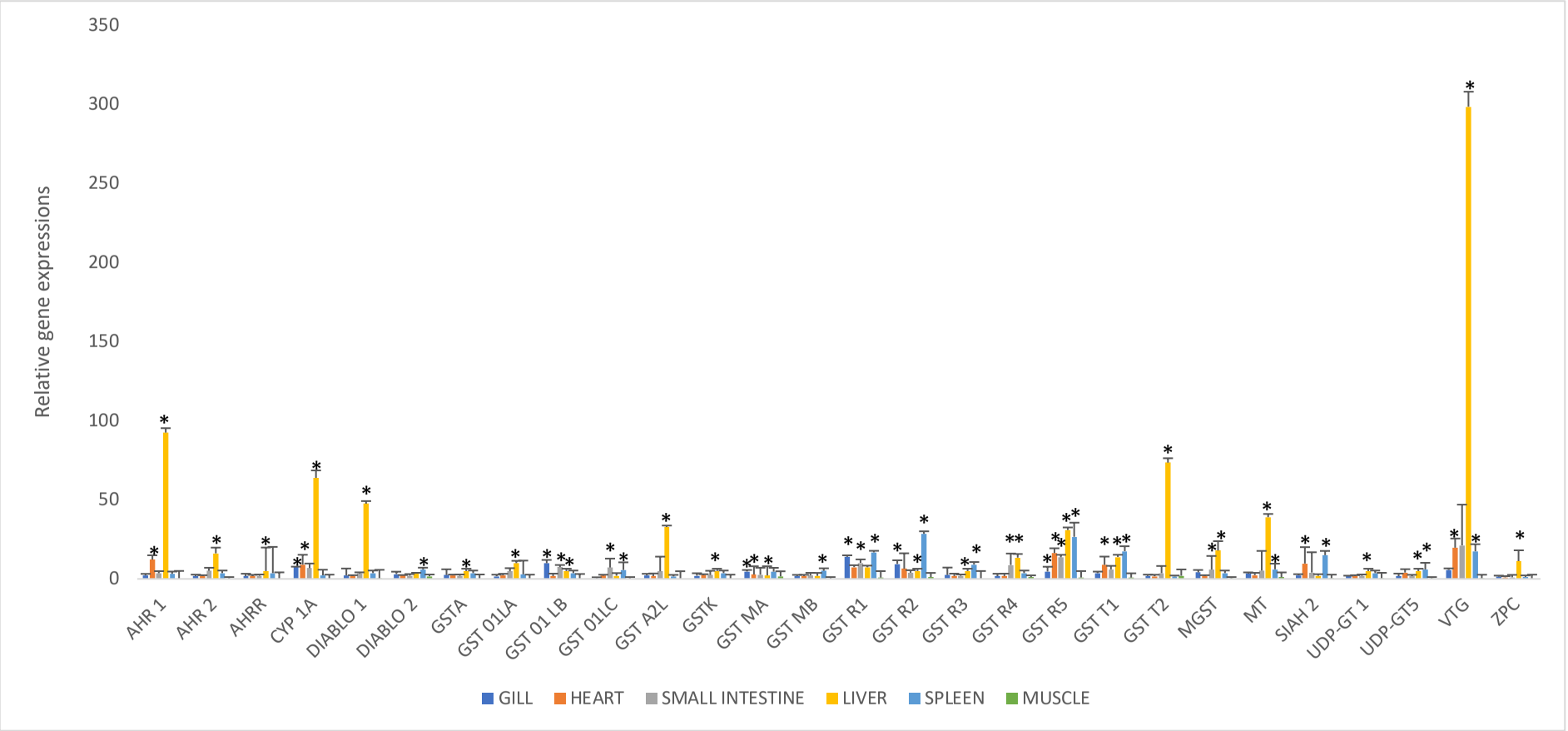


Figure 2.1: The mRNA expression of the laboratory Nile tilapia genes normalised relative to reference genes. The result was represented as (Mean ± Stdev). Error bars represent the standard deviation of the mean (n=5 replicate) across all the tissue for each gene. Asterisk denotes significant difference ($p \leq 0.05$).

2.4 DISCUSSION

2.4.1 Primers assay optimisation

2.4.2 Fish biometric and tissue distribution patterns of basal mRNAs level

zona pellucida

AHR

1, AHR 2, AHRR, CYP 1A.

2.5 CONCLUSIONS

3.0 CHAPTER THREE: GENE EXPRESSION IN TILAPIA LARVAE EXPOSED TO MODEL POLLUTANTS.

Abstract

Oreochromis niloticus

AHR 2, CYP 1A, DIABLO 1and 2, GST01LA, GSTA2L, GSTMA, GSTR2, MT AND VTG

Ribosomal Protein S5 S7 RPS5 and RPS7

3.1 INTRODUCTION

Peroxisome proliferator-activated receptor PPAR), Aryl hydrocarbon receptor (AHR), and Estrogen receptors (ER). PPAR

Oncorhynchus clarkii

Oncorhynchus shawytscha *collagen 2a1*

CYP IA

Oryzias latipes) *Danio rerio*

VTG *tilapia guinensis*

Gambusia affinis)

3.2 MATERIALS AND METHODS

3.2.1 Experimental animal

Oreochromis niloticus

3.2.2 Stock solution and preparation of chemicals

Oreochromis niloticus

Chemicals	Exposure (mg/L)	Concentrations

3.2.3 Experimental set up for the treatment

3.2.4 Primer design

AHR 2, CYP 1A, DIABLO 1and 2, GST01LA, GSTA2L, GSTMA, GSTR2, MT AND VTG

3.2.5 RNA extraction protocol

3.2.6 RNA Purification

3.2.7 cDNA synthesis

3.2.8 Quantitative PCR analysis

Rps 5 and 7

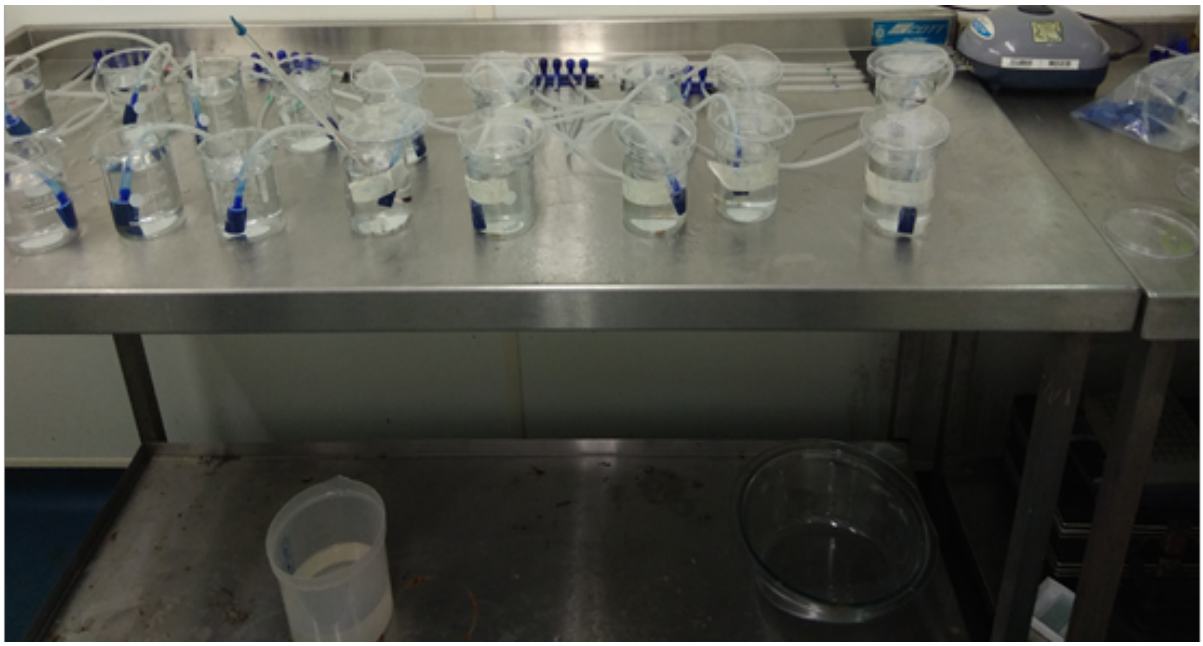
3.3 DATA ANALYSIS

(Rps 5 Rps7

≤

3.4.0 RESULTS

3.4.1 Fish survival in chemical exposure



3.4.2 Differentially expressed genes

CYP 1A *GSTMA* *GSTR2* *VTG* *MT*

CYP 1A *DIABLO 2* *GST01LA* *GSTMA* *MT*

CYP 1A *VTG* *DIABLO 2*

AHR 2 *GSTMA* *MT* *CYP 1A* *DIABLO 1* *GSTR2*

CYP 1A *DIABLO 1* *GST01LA* *GSTA2L* *MT*

CYP 1A *GSTMA*

VTG *GST01LA*

GSTR2 *MT*

GSTMA

GSTR2 *DIABLO 1*

CYP 1A

VTG

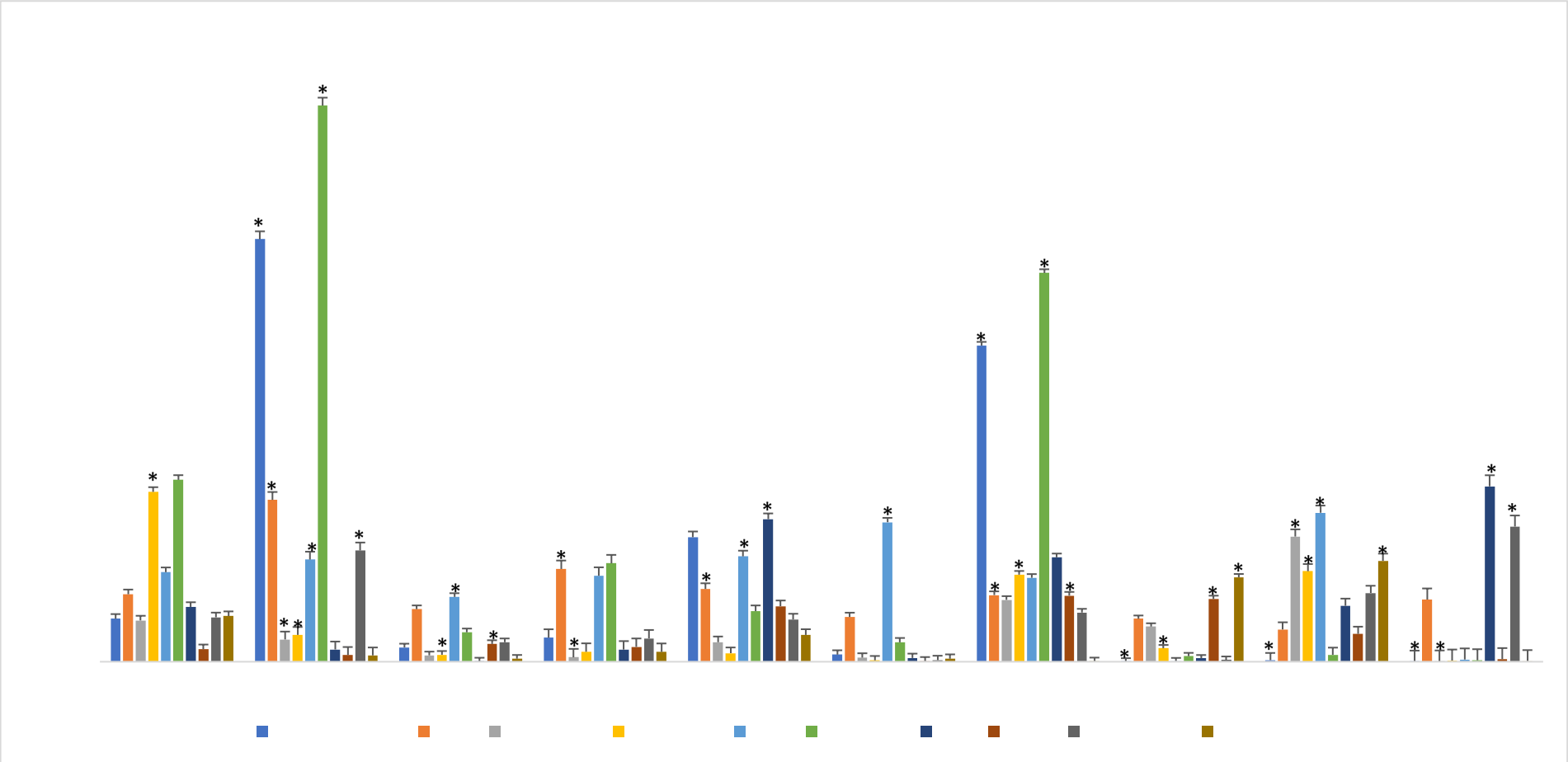


Fig. 3.2: The mRNA expression of 6-7 days prefeeding Nile tilapia exposed to10 different chemicals for 24 hr. Data is presented as mean ± std (n=10 containing 10 individuals /treatment) and control (n=10 replicates) using Kruskal Wallis test. Asterisk denotes significance difference. Significance different level was set at $P \leq 0.05$

3.5 DISCUSSION

3.5.1 Distinctive gene responses between chemical stressors

The present study assessed the responses of 10 prototypical environmentally relevant chemical contaminants on mRNA expression in 6-7 days post-fertilisation (dpf) Nile tilapia prefeeding larvae maintained under laboratory conditions for 24 hrs. The acute toxicity was determined using the arbitrary values for each chemical agent (sublethal exposure concentration). Although no death was recorded, the different chemicals generated specific profiles of gene expression changes compared to the control samples. Using RT-QPCR, the study was able to differentiate genes that were differentially expressed due to different treatment chemicals in the laboratory. This study employed data from the RT-QPCR to make numerous hypotheses on the molecular response to these chemicals exclusively on quantitative measures of gene expression changes. In this context, the observed changes in the quantitative measures due to exposure to these chemicals could be employed in identifying possible candidate genes in molecular biomarker determination and assessment of toxicity of the chemicals to Nile tilapia in the wild. Also, the changes in the genes could help in predicting the molecular pathways that could be affected due to the exposure to these chemicals. These identified biomarker genes could be used in predicting a toxicity of these chemicals in a large population of Nile tilapia living in the contaminated wild environment.

Majority of these changed genes were ubiquitously altered in all the treatment during the exposure period. These genes are potential candidates to be used in the field biomonitoring as a tool in determining specific chemical pollutant and their possible health effect in the wild Nile tilapia in the event of exposure. The commonly modulated genes in response to the ten (10) chemical treatments were *AHR 2, CYP 1A, GST01LA, GSTA2L, GSTMA, GSTR2, DIABLO 1 and 2, MT, and VTG* were important in Phase I and II biotransformation, apoptosis, metal toxicity and reproductive impairment. Therefore, alterations in these genes and particularly their competence in determining exposure could reflect the effect of chemical contaminants in Nile tilapia by negatively disturbing different systems.

3.5.2 Differential AHR 2 and CYP1A response in Different chemical treatments

Expression of *AHR* ligand *CYP IA* was observed in different treatments in the present study. There were differentially expressed *CYP IA* in response to Aroclor 1254 at a concentration of 16 mg/l, B[a]p at a concentration of 1.6 mg/l, DDT at a concentration of 0.4 mg/l, PFOS at a concentration of 6.4 mg/l, and DEHP at a concentration of 3.2 mg/l against control. It was established that the expression of *CYP 1A* elevates in response to PAHs and PCBs as its

agonist. These chemicals activate xenobiotic-metabolizing enzymes by binding to aryl hydrocarbon receptors (Pauletto et al., 2019). Significant expressions of this gene in both Aroclor 1254 (13-fold) and B[a]p 5-fold), is not surprising in larvae of Nile tilapia. Another AhR ligand expressed in the exposure of larvae to the treatment of Dazomet is *AHR 2* gene (5.3-fold) against control. However, A *CYP1A* mRNA's significant induction in the larvae could be due to the compounds at a concentration most probably consistent with the larvae burden of the contaminant. The development of early life stage toxicities due to PCBs and PAHs has been documented in fish due to *CYP1A* induction through mediations of aryl hydrocarbon receptor (AHRS) pathway activations (Chambers et al., 2012; Roy et al., 2018). This induction could begin as early as the gastrula stage of development in vertebrates (Duan et al., 2018). The observed result is an indication of activation of a pathway involving AHR and this speculates that larval early life stage toxicity of Aroclor 1254 could be mediated by the AHR pathway. Attention was given to early life stage laboratory exposure to chemicals in the present study since the majority of the fish in the natural wild environment particularly larval stages are exposed to toxicities of planar aromatic hydrocarbons with recruitment failure as a possible effect. The majority of the responses in fish due to the toxicities of PCBs and PAHs due to exposure and consequent effect include hatching failure, morphometric changes, conceded development of the eye as well as the short life span of the malnourished larvae. All these responses are likely to reduce the effective recruitment of the larval stage of Nile tilapia to the adult stage in the wild. And as a consequence, helps in the failure to restore the impacted population and may lead to the eventual death of the affected population in the wild environment (Chambers et al., 2012). Various reasons can be justified concerning the pertinence of the present result to the possible effect of Aroclor 1254 induces toxicity to Nile tilapia in the natural wild environment. At first, the treatment of the larvae was through a water-borne exposure route with the employment of DMSO as a solvent vehicle at 6–7-day postfertilization. There is a likelihood that the larvae population is exposed to these planar aromatic hydrocarbons in a chemically impacted wild environment through transfer from water. As a consequence, the unfertilized eggs could be carrying high levels of these toxicants in the wild environment before their fertilization. However, exposure in the natural wild environment could begin at an early embryonic developmental stage in the impacted locales just like the use of larvae in the present study. This similarity could ensue in the same upregulation of CYP 1A response in both the two life stages and possibly higher biological level toxic response in the embryonic stage in the wild.

Secondly in the present study, the larvae were exposed to an individual Aroclor 1254 and B[a]p congeners, unlike in the wild natural environment, where the larvae could be exposed

to the mixture of planar aromatic hydrocarbons and other xenobiotic compounds. Numerous studies have indicated the presence of noncoplanar congeners and other pollutants in the natural aquatic environment and impacted the binding of these agonists to AHR (Cardosoa et al., 2019). Based on the known functions of AhR activation in overt toxicity and on the present results, it is likely that tilapia is sensitive to induced toxicities of Aroclor 1254 and B[a]P at a concentration of 16 and 1.6 mg/l respectively.

Nonetheless, the inducibility of *CYP 1A* has been established to be a biomarker of exposure in environmentally exposed fish in the wild. This observation from short-term exposure would be useful in the differential expression of *CYP1A* in a field sample to infer long-term exposure responses accumulated over months or years in tilapia. The induction of a *CYP1A* gene in a Nile tilapia larvae sample in the laboratory exposure could reflect the local pollution load of AHR agonists, including PCBs in the wild. A broad marker of any AHR mediated pathway gene involves uptake, metabolism, and excretion of a chemical for a response to be detected and thus suggest an active metabolism of that chemical by the gene. As only gene expression was quantified in the present study, it could not be determined to what extent the uptake of the chemical compounds was into the larvae during exposure. But what was apparent was that, from the observed result of Aroclor 1254-fold difference against control (Fig. 3.1), it could be deduced that the uptake of Aroclor 1254 could be constant throughout the exposure period. This indicates that the nature of the chlorination and the co-planar arrangement of Aroclor 1254 weaken effective degradation by the induced biotransformation enzymes gene. Therefore, metabolization of Aroclor 1254 is more gradual. And due to the strong lipophilic nature of Aroclor 1254, it accumulates in the larvae all the time restarting the aryl hydrocarbon receptor (AHR) and re-inducing CYP 1A. While B[a]P could be easily metabolized in the liver and commences its metabolism through eliciting of *CYP1A* gene 1A (Meyer-Alert et al., 2018). Biotransformation of B[a]P may lead to the production of a different compound in the larvae which are regarded as carcinogens as these compounds react with protein and bind to the DNA. Such compounds include electrophilic diol epoxides (BPDE) (Santos et al., 2018).

In the present study, BPDE or other metabolite production may have occurred in the larvae and necessitate the increased expression of CYP 1A. The larvae may have metabolized B[a]P to produce B[a]P 7,8 dihydrodiol which is the precursor of BPDE, which could produce a covalent DNA adduct. Although after B[a]P exposure, no metabolite was determined which is beyond the present study. Therefore, the larvae may have metabolized B[a]P and form an adduct biotransformation product (Bussolaro et al., 2019). Expression of CYP1A was also recorded in the treatment of the larvae with DDT and Dieldrin pesticide at the concentration

of 0.4 mg/L and 0.2 mg/L respectively for 24 hrs. It was reported that CYP 1A metabolized planar organochlorine pesticides including DDT (Sarasquete and Segner, 2000). In different life stages of fish, various studies indicated that organochloride pesticides have an impact on oxidative stress, the immune system, apoptosis, and mitochondrial dysfunction (Wu et al., 2019). Therefore, phase I biotransformation enzyme gene significant upregulation by both DDT and Dieldrin could be as a result of their continuous accumulation in the lipid of the larvae due to their lipophilic nature. Also possible in the present study, was the element of oxidative stress due to the toxicity of these pesticides. DDT and Dieldrin could be metabolized and incorporated into the fats of the larvae which maybe not be harmful, but after subsequent metabolization of the fats, this could result in oxidative stress in the larvae. Exposure to a waterborne concentration of 3.2 mg/L DEHP for 24 hr induced a significantly increased transcription of *CYP 1A* mRNA (3.497-fold). The result of the study speculates that DEHP can activate the AHR pathway in the larvae of Nile tilapia and may act as an AHR agonist. DEHP is among many Phthalates esters that are ubiquitous in the environment and its metabolite could accumulate in humans. In this study the potential role in DEHP metabolism by CYP 1A is unclear, but recent studies suggested that exposure to DEHP caused oxidative stress and consequent apoptotic wound through mitochondrial and CYP pathways (Wang et al., 2020). Several studies have indicated the role of DEHP in reproductive impairment leading to the increase of VTG level indicating estrogen-like potency of DEHP (Maradonna et al., 2013). It was observed in the present study that the induced expression of VTG in the larvae of Nile tilapia demonstrated the estrogenic action of DEHP. It was established that the transcription of the VTG gene occurred through the stimulation of estrogen. This happened when estradiol binds the specific estrogen receptor, and subsequent interaction to the estrogen-responsive element and their transcription is enhanced. The obtained result could also suggest a high concentration of DEHP might be responsible for the VTG expression due to direct differential interaction with diverse estrogen receptors in the larvae. Therefore, the observed increase of VTG due to exposure to DEHP supports the estrogenic potentials of the phthalates in Nile tilapia larvae. Taken together, significant expressions of *AHR 2* and *CYP 1A* may reflect metabolite's biotransformation. This might lead to ROS production in the larvae of Nile tilapia due to chemicals toxicity and might increase the expressions of their mRNAs.

3.5.3 differential GSTs responses in different treatments

In this study, the RT-QPCR analysis revealed that *the GSTMA* gene was significantly upregulated in the exposure of the larvae to Aroclor 1254 (10-fold), B[a]P (2-fold), Dazomet (2.7-fold), Dieldrin (12-fold), and PFOS (2.077-fold) against the control.

While *GST01LA* was significantly increased by B[a]p (2-fold), EE2 (4-fold), and DDT (3.30-fold). Significant upregulations of *GSTR2* were observed in Malathion (6.523-fold) and DEHP (1.972-fold) against control. Significant expression of *GSTA2L* was also observed in DDT (4.35-fold) against control.

In the present study, *GSTs* are widely studied conjugating enzymes and could have performed the function of either detoxification or contributing to the antioxidant defense against the induced production of ROS, which causes oxidative stress in the larvae (Yao et al., 2017). In the present study, the observed increase in GSTs indicated the augmented ability of the larvae to detoxify these chemicals through a conjugating pathway. In this context, different isoenzymes of GSTs were employed in the present study and they might react differently in their expressions. Detailed assessment of the function of each isoenzyme needs further study. Since the larvae had taken these chemicals, accumulate in its body tissues and could generate ROS leading to possible oxidative stress condition. Observed significant mRNA expression of GSTs in the present study may suggest removal of secondary oxidation products and Hydrogen peroxides (H_2O_2) by these chemicals. And it is also possible that increases in ROS production were a result of biotransformation of the chemical treatments to active metabolites in the larvae. Cellular defense mechanisms can effectively cope with physiological ROS levels. In the larvae, over-production of ROS could result in overwhelming or saturations of physiological defenses leading to oxidative damages of several cellular structures including DNA damage. Since no concentration's dependent response expression at time interval was measured in the genes of interest in the present study. It could be speculated that the tested chemicals that resulted in the significant change in *CYP1A* had also contributed to the oxidative stress in Nile tilapia larvae. Therefore, Phase II biotransformation in the larvae might have resulted in the conjugation of electrophilic diol epoxides (BPDE) with glutathione reducing their toxicity through different GSTs isoform during oxidative stress. The conjugation of reduced glutathione (GSH) with BPDE by *GSTs* reduces their effect or even changes them to diol or phenol, which facilitates their hydrophilicity as water-soluble conjugates for easy excretion (Santos et al., 2018). Previous studies related induced expression of *GSTs* mRNA following different chemicals exposure in zebrafish larvae to countering response against lipid peroxidation and ROS production (Dale et al., 2019). For example, an increase in *GSTMA and GSTR2* from the result indicated that PFOS long-chain molecular weight may have a lasting effect in the early development stages of fish such as the larval stage used here. This also indicated some PFAS might have shared molecular targets. Particularly PFOS can trigger this effect in the larvae due to its long-chain molecular weight. In different animal systems, available data showed PFOS

induced oxidative stress through lipid peroxidation, dysfunction in mitochondria, and decreased antioxidant capacity (IARC, 2017). Additionally, GSTs have interrelated signaling pathways, therefore, change in the expression of these genes may be related to one another. Many metals and other organic pollutants including EDC, pesticides, PAHs, PCBs, and heavy metals, both organophosphate and organochlorides pesticides, estrogenic compounds are prooxidants in fish (Ibor et al., 2020). The aforementioned chemicals could have disrupted specific biological processes in the larvae. These genes could be regulated and be involved to compensate for the chemical impact in the larvae. And this would play an important role in the survival of these larvae in the exposure regime.

3.5.4 Differential MT, DIABLO 1, 2 and VTG responses in different treatments

In the present study, cadmium at a concentration of 2.8 mg/L resulted in the significant upregulation of MT (4-fold), while EE2 at a concentration of 0.002 mg/l resulted in the significant increase of VTG (6-fold) in the larvae against control. This result showed that cadmium-contaminated water leads to an increase of MT gene expression at the level of mRNA due to metal pollution, as explained by amplification. One of the potent inducers of MT gene expression is Cadmium. And Cadmium sequestration involved MT protein. Sequestrations of Cadmium could have occurred by MT in the cells reducing their availability in the larvae. As such, MT-bound cadmium ions can be detoxified metabolically or kept in granules rich in metal. The observed result demonstrated a positive impact on the gene expression of MT mRNA and the Cadmium uptake. This indicates that there was evidence of a possible connection between Cadmium accumulation and increase production of MTs and its detoxification role with subsequent gene expression of MT in the larvae. Thus, heavy metal accumulation in fish could encourage a chemical reaction in tissues with consequent ROS production leading to oxidative stress. (Sun et al., 2019). The significant upregulation of the MT mRNA copies may suggest that the MT had chelated more atoms of free Cadmium in the larvae of Nile tilapia. This implies that the larvae of Nile tilapia had greater protection against Cadmium burden in the body (Sheikh et al., 2019). And this could have resulted in available Cadmium for induction and binding to MT mRNA. Meanwhile, it could be speculated that Nile tilapia species sensitivity to Cadmium is mediated by the sensitive sections of cellular organelles in the fish (Sheikh et al., 2018). Therefore, changes in the expression of metallothioneins gene mRNA levels in the larvae laboratory exposure could be an indication of heavy metal contamination and the degradation of environmental conditions of different biological matrices in the wild environment (Wang et al., 2019). Thus,

the extent of these free radical productions could be dependent on the concentrations of the chemicals and the duration of time of the exposure regime in the Nile tilapia.

On the other hand, the induction of VTG mRNA of the larvae due to EE2 exposure implies that this chemical is capable of interfering with its normal endocrine and reproductive processes. Previous studies have shown that weakened spermatogenesis is strictly related to heightened estrogenic effects in the different life stages of fish in response to xenoestrogen exposure. Therefore, enhanced estrogenic effect after exposure to EE2 in the present study had dramatically upregulated VTG mRNA of the larvae, and this could be as a result of altered endogenous sex hormone production (Wang et al., 2019). Taken together, the observed present result could demonstrate disorder in VTG abundance in reproduction which may trigger some physiological effect on growth, levels in steroid hormones, and fecundity in the larvae of Nile tilapia in the future. These pathways could be consistently affected by the exposure to EE2 when assessing the direct effect of EE2 on the fish (Voisin et al., 2019). The treatment of the larvae with B[a]p at a concentration of 1.6 mg/l resulted in the significant expression of *DIABLO 2* (3-fold), and DDT at a concentration of 0.4 mg/l resulted in the increased expression of *DIABLO 1(*2.04-fold), the genes that strongly promote apoptosis. In fish, immune regulatory mechanisms are preserved, showing their importance in immunological homeostasis maintenance. Exposure to polluted chemicals such as organic xenobiotics including PAHs, pesticides, and metals might induce *DIABLO* by binding to IAPs protein molecules to represses caspases-IAP's influence in inhibiting its proapoptotic activity in Nile tilapia larvae. Execution of cell death program is then achieved (apoptosis) when released caspases are activated by *DIABLO/SMAC* in the Nile tilapia. (Zacchino et al., 2012; Jeffrey et al., 2019). During the exposure to different chemicals treatment in the present study, the upregulation of *DIABLO 1 and 2* mRNA might indicate that the larvae were under the death stimuli favoring apoptosis.

3.5.5 Differentially repressed genes in the chemical treatments

In this study, VTG (0.002- fold), *GSTR2* (0.008-fold), *and MT* (0.049-fold) were significantly downregulated in response to Aroclor 1254 at the concentration of 16 mg/l. So also, was a significant downregulation of *CYP 1A* (0.698-fold), *VTG* (0.06-fold), and *DIABLO 2* (0.138-fold) in response to Cadmium at a concentration of 2.8 mg/l in the study. Dazomet at a concentration of 4 mg /L for 24 h in 6-7 dpf Nile tilapia prefeeding significantly downregulated *CYP1A* (0.84-fold), *DIABLO 1*(0.23-fold), and *GSTR2* (0.42-fold). On the other hand, PFOS at a concentration of 6.4 mg/l significantly downregulated *DIABLO 1* (0.558-fold). The upregulation and downregulation of *GSTR2* in

the present study may suggest these genes performed the different divergent physiological roles in the detoxification of different pollutants in the Nile tilapia. For example, in the treatment of larvae with Aroclor 1254 at a concentration of 16 mg/l and Dazomet at a concentration of 4 mg/l, the repression may be due to due to biphasic changes of the *GSTR2* isoform in the larvae i.e., after a short time *GSTR2* is induced and later it is downregulated within the exposure time. VTG repression in the Aroclor treatment may be due to crosstalk between AHR and ERs. A previous study suggested expression of *VTG* mRNA was disrupted while that of *CYP1A* was enhanced when exposed to PAHs and PCBs (Pauletto et al., 2019). Therefore, the reason behind the downregulation of VTG may be that the activation of the Ah receptor by Aroclor 1254 might result in the upregulation of *CYP 1A* and could negatively influence the estrogen-dependent expression of *VTG* in the Nile tilapia larvae.

VTG was also downregulated in Cadmium treatment. The downregulation of VTG in the treatment might suggest Cadmium could not have acted as metalloestrogen in Nile tilapia. Cadmium could have modulated estrogen receptors through binding to them with high affinity and blocking the binding of estradiol in the Nile tilapia larvae. In rainbow trout (*Oncorhynchus mykiss*), estrogen receptors binding to Cadmium appeared to reduce the transcriptional activity of estrogen receptors thereby several estrogen facilitated pathways including vitellogenesis are affected (Driessnack et al., 2017). It could be assumed that treatment of the larvae with Cadmium resulted in the inhibition of VTG synthesis, at the transcriptional level since little or no VTG mRNA was upregulated in the larvae. The present study suggests considering the binding affinity of estrogen receptors and estradiol in the future assessment of Cadmium and other EDCs in the Nile tilapia exposure.

Downregulation of *DIABLO 1* and *2* in the treatment of PFOS and Cadmium occurred in Nile tilapia respectively. Since it has been previously established that apoptosis occurred through *DIABLO* binding to IAPs protein molecules and help in reducing caspases-IAP's influence in inhibiting the proapoptotic activity of IAPs on caspases in Nile tilapia larvae. This suggests that the activity of the released caspases is not activated by *DIABLO/SMAC* due to the treatment of these chemicals in the Nile tilapia, hence no apoptosis (Jin et al., 2017). There was also significant downregulation of *CYP 1A* in response to Dazomet and Cadmium treatments in Nile tilapia. The downregulation of CYP1A could be attributed to the fact that the induction of *CYP1A* was established to be mainly through aryl hydrocarbon signaling pathways that metabolize xenobiotics in fish, and this may suggest the possibility of Dazomet and Cadmium not being an agonist of *CYP 1A* in the larvae of Nile tilapia. There could be *CYP1A* mRNA induction at earlier inception in the

larvae and after Dazomet and Cadmium were metabolized, there could be a clearance of *CYP 1A* and return to basal level in the larvae as was observed in salmon trout following treatment with ß-naphthoflavone in a laboratory exposure (Whyte et al., 2008). Generally, upregulations and downregulations of these genes in the laboratory exposure of Nile tilapia indicate that the concentrations of these treatments in the exposure might have exceeded those that may be found in the freshwater wild environment.

3.6 CONCLUSIONS

The present study demonstrated that chemical contaminants could induce changes in gene expression in the larvae of Nile tilapia, a non-model species, like those in other fish species exposed to an environmentally relevant chemical at an acute laboratory exposure. This data enhanced our understanding of the development of PCR arrays on multiple gene modulations. The expression of these genes inferred that environmental chemicals significantly affect the expression of genes that are involved in phase I biotransformation, metal homeostasis, apoptosis, estrogenicity, oxidative stress, and detoxifications in a controlled laboratory assay. PCR array screening in this study furnished substantial data to identify chemically responsive genes and the potentiality in quantifying expression changes of how different pathways involved different chemicals. These responses obtained at the gene transcriptional level will help in assessing some physiological changes and act as early warning signals in the ecological risk assessment at individual and community levels. The data permits us to differentiate between different classes of chemicals exposure and define their general stress response, which is of particular benefit in ascertaining the general effect upon exposed Nile tilapia to contaminant mixtures. Additional studies may help explain some important pathways regulating other gene-mediated transcription in concentrations and time-dependent manner in the exposure. This data also demonstrates that changes in gene transcription levels could be used as biomarkers of an organism's exposure to environmental contaminants. The question remains as to how this study can relate the results of gene expression of the larvae in an individual contaminant treatment in a laboratory to a response from a wild Nile tilapia in a complex chemical mixture of the aquatic environment.

4.0 CHAPTER FIVE: FIELD-TEST OF THE RT-qPCR ARRAY

Abstract

In this study, gene expression profiling was used to assess the impact on tilapia in multiple polluted aquatic sites: Dan Agundi sewage pond, in Kano municipal, Kano State and Daberam reservoir, and also at a clean site, Jibia dam in Katsina state, Northern Nigeria. Wild tilapia samples (n= 48 in total) were caught from both the polluted and clean sites, (16 fish /site). Length-weight relationship and the condition factor were conducted. RNA extraction and cDNA synthesis were used to measure tilapia hepatic expression using QPCR. In both the polluted sites the length-weight relationship results showed that the fish were undergoing negative allometric growth, while in Jibia the fish were undergoing isometric growth. The condition factor 'K' for both the polluted and reference sites was >1. Significant changes in the expression levels of genes predicted to be involved in different physiological and metabolic signalling pathways were observed in the polluted sites compared to reference site. The approach of biomarker studies can effectively yield knowledge of the effect of pollutant mixtures with actual risks resulting on fish inhabiting the aquatic ecosystem when exposed.

4.1 INTRODUCTION

Globally, industrial development is increasing with the consequent increases in environmental problems. Water resources are particularly threatened by industrial waste, sometimes with devastating effects. Despite the lower levels of industrialization in third world countries, in the increase of the significant amount of organic matter in the environment due to the consumption of various products (Arukwe et al., 2012) is comparable between those countries and developed industrial nations. The aquatic environment, except in the arid regions of the world, has until recently been regarded as free, and as an infinite sink into which industrial waste could be disposed of at low cost. Given the rapid industrial development over the last decades, it is clear that these views are no longer valid. In Nigeria, cities like Lagos, Kano, Kaduna, Port Harcourt, Calabar, and Ibadan are the industrial hubs of the country. The regions are endowed with numerous freshwater resources and depend much on them for water supplies, irrigation, transportation, recreation, power supply, and fisheries. But the urbanization and industrialization, agricultural activities, as well as lack of regulations from the government, led to the indiscriminate dumping of effluents into these water bodies (Ukenye and Taiwo, 2019). In developing countries, wildlife, the biota and human health effects of environmental pollution have become a serious concern both for the protection of fisheries and aquatic resources as well as for sustainable management (Adeogun et al., 2016). Especially the inland and coastal waters of Nigeria, including rivers, lakes, dams, and streams are the most vulnerable, because of an abundance of contaminants discharged through waste disposal due to increases per capita of waste obtained from the consumption of products. This increase in dumping resulted in a huge number of wastes in leachates and sediment in the rivers and lakes when compared to developed economies (Arukwe et al., 2017).

The aquatic environment is the ultimate sink for a wide variety of products of personal care, industrial chemicals and pharmaceutical, non-ionic-surfactants and organophosphate pesticides, Perflourooctane sulphonic acid and flame retardants, dioxins, furans, PAH, PCBs, Phthalates esters, sewages effluents and anti-fouling agents (Menillo et al., 2020). Most of these chemicals are dumped into the aquatic ecosystem knowingly by industries without treatment, while a few are discharged unintentionally through the use of pesticides and fertilizers through runoff (Tongo et al., 2019). Physico-chemical and climatic (atmospheric and hydrologic) processes distribute these released chemicals for onward deposition into the aquatic environment (K'oreje et al., 2020). Previous studies reported that, in teleost fish, exposure to such chemicals, persistent in the aquatic environment, give rise

to the invigoration of different toxicological signaling pathways, including the triggering of aryl hydrocarbon receptor conciliated reactions through phase I and II metabolizing enzymes (Jin et al., 2020). Phase I and II metabolizing signaling pathways consist of different varieties of gene batteries that are mediated through aryl hydrocarbon receptors. Such exposure to these exogenous compounds may modulate their (Phase I and II enzymes) mRNA expressions or their enzymatic activities and facilitates the removal of xenobiotics either through biotransformation responses or through oxidative stress (Ibor et al., 2017).

Exposure of aquatic organisms in the wild is normally to a mixture of xenobiotics chemicals. They are usually analyzed on the information of their bioaccumulation, bioavailability, toxicity, and persistence (Wu et al., 2019). Organisms are not necessarily exposed to high toxic concentrations in the aquatic environment unless they are within a chemical spill area or site. Dispersion and dilution may occur more than on the initial impact site, reducing the acute concentration to sublethal or lower levels (Zhang et al., 2018). It is therefore important to know the effect of exposure to both single and a mixture of chemicals (He et al., 2019). The majority of organisms are exposed to xenobiotic chemicals at sublethal levels other than acute and lethal toxic levels during chronic exposure in the wild (Adeogun et al., 2019). Sublethal effects of different toxic chemicals are commonly diagnosed through biomarkers. Biochemical reactions normally trigger the sublethal effect of chemicals and these chemicals employ their toxic effect at the molecular level by responding with some enzymes and another active component of the cell in the organism (Adeogun et al., 2019). Lower levels of chemicals may not bring death but may have a significant effect on the organism in its future survival (Asker et al., 2015). In the present study, Nile tilapia *(Oreochromis niloticus*) was investigated in two contaminated sites; Daberam and Dan Agundi as polluted sites and Jibia as a reference in the Northern part of Nigeria where these kinds of studies (gene expression profiling) are lacking.

Nile tilapia, *Oreochromis niloticus*, is a cosmopolitan freshwater fish that is hardy in a contaminated aquatic environment and a possible model species for the development of biological monitoring in aquatic environmental contamination. In this aspect, the analysis of responses in hepatic damage and the defense of a local fish species is of paramount importance in biomonitoring studies. The significant importance of the liver in various roles, including contaminant accumulation, biotransformation and excretion, and its susceptibility to elevated levels of chemical pollutants and their metabolites (Megid et al., 2020) will be examined. In this chapter, the study describes the use of hepatic gene expression to assess the biological responses of tilapia collected from anthropogenically influenced field sites

(wild environment). This was achieved by examining the expression “patterns” in tilapia liver from the sites by comparing them with the expression patterns under laboratory exposure condition and by considering known gene pollutant responses. Data obtained through this approach (gene expression) was related to some past studies elsewhere on the steroid’s hormones due to exposure to endocrine disruptors chemicals, other antioxidant genes alterations and tissues observable pathologies due to the effect of chemicals exposure in fish which may help in characterizing the ecological quality of the aquatic habitats particularly Northern Nigeria.

4.2 MATERIALS AND METHODS

4.2.1 Study sites

4.2.1.1 Daberam reservoir

Daberam Reservoir is located on two major seasonal rivers, Kigo and Riniyal, which drain their waters into the Dam. Discharges also come from the residential settlements of Dutsi and Daura and local industries around the towns of the Dam. The river Dan-nakola is a major tributary, together with other streams around Daura and Dutsi Katsina state lies at latitude 13°2′ N and longitude 8°2′ E. The dam lies in the Northern Sudan savannah region, the climate is characterized by different dry and wet seasons with an annual rainfall of 600 – 640mm. The reservoir has a total storage capacity of approximate 400 hectares of land. Unfortunately, the water of the reservoir is turbid due to siltation, which might be related to approximately 200 hectares for the reservoir capacity to be utilized. The depth of the reservoir is 42.6 meters with a crest length of 2377.44 meters (Lawal and Ahmad, 2014). Fish samples were collected at the following sampling points: point A (Hayin Daura), point B (Hayin Dutsi), and point C (Madawa). The reservoir was selected for sampling due to the fact that there are all year-round farming activities, several diffuse sources of pesticides, run off of municipal effluents from far distance towns and villages, as well as the presence of high commercial fishing activities.

4.2.1.2 Dan Agundi sewage pond

Dan Agundi sewage pond, as it is popularly known, is situated at the heart of Kano metropolitan city in proximity to residential areas as well as some local industries such as a tannery, blacksmiths, oil stations, local beverage manufacturers and flower garden vendors. Kano metropolitan city is located at (11º 59′ 53.7″N, 08º 31′ 24.4″E), 415 altitude in Kano state (Abubakar and Ademoh, 2017). It has an estimated length of 14km and a depth of 5.95m, with an annual temperature of about 26-32°C and an annual rainfall of about 884-1900 mm respectively (Ahmad and Daura, 2019). In Northern Nigeria, Kano lies in the central North and is one of the developed industrial cities in Nigeria. (Ahmad and Daura, 2019). The pond receives untreated sewage effluents from the residential areas as domestic waste as well as from the local industries around the area. Three sampling points (P1-3) were chosen within the pond for fish sampling. The pond was chosen because it functions as a drainage and for its fishing activities.

4.2.1.3 Jibia Dam

Jibia Dam in Jibia, Jibia local government, Katsina State, lies between latitude13^0 05^1 N and 7^0 13^1 E and longitude 13^0 09^1 N and 7^0 23^1 E in the Northern part of Nigeria. The reservoir is located on the coordinates Latitude13^0 04^1 18m N and Longitude 7^0 15^1 06m E. The dam is an earth filled structure with a geomembrane liner and a height of 23.5 metres and a total width of 3660 metres. It has a capacity of 14.2 million cubic metres of water. The dam was constructed in 1989 and was built to support water supply and irrigation. The tributary to the Dam is the Gada River, which flows only during the rainy season for about 400 km and supplies water to the dam. The dam supplies water to a network of irrigation canals of about 192 km for onward distribution to 3500 hectares of farmland (Abba et al., 2018). Three sampling points were visited; Point A, Gada, Point B, Mazanya and point C, Mallamawa. An assessment rated the Dam as having some physico-chemical parameters and its heavy metals within permissible condition (Shamsuddin et al, 2018). There were no agricultural activities nor domestic discharges within the Dam area as irrigation canals were provided to the farmers that conveyed water downstream from the dam to their various farms. Hence the reason for selecting the Dam as our reference site.

4.2.2 Sample collection

A total of 48 live spent adult and immature Nile tilapia samples were collected from the three sites visited (16 samples for each site) at three different points randomly selected in July 2018, to give an in-depth analysis of environmental interaction and biota. Identifiable male fish based on undeveloped testes or resorbed testes by experienced fishermen were sampled. Sampling was conducted at the post-spawning period based on the judgement of the expert fishermen. The fish sizes ranged from a total length of 12.05-22 (cm) and a weight of about 40-125 (g). Fish were caught with the help of artisanal fishermen using fleets of gillnets and cast nets of different mesh sizes. Fish were euthanized with an anaesthetic overdose of Benzocaine (3ml in 1 litre of water). Biometric measurements were taken, and fish liver tissues (< 100 mg) were harvested on the field sites and preserved in 1ml RNA later (Ambion USA) in a 2ml microtube screw cap. Collected samples were put into frozen ice packs in an icebox and transported to the Umaru Musa Yar'adua University laboratory and kept in a freezer at -4°C before being taken to the University of Stirling for subsequent preparation and analysis. The entire sampling procedures were performed under license and in accordance with UK guidelines for ethical handling and sacrifice of animals under field conditions, with oversight of an institutional ethics review committee (Zacchino et al., 2012, David, 2019).

4.2.3 Biometric measurement, length-weight relationship and condition factor

The total length (TL) in (cm) and body weight in (g) were taken using a metre ruler and an Ohaun digital weighing balance respectively. The length-weight relationship was calculated using the equation $W=aL^b$, where W is the total weight (g), L is the total length (cm), *a* and *b* are estimated log transformed regression coefficients using the linear equation log W= log a + b log L and R^2 as the coefficient of determination. The *a* value is the coefficient of length -weight relationship, while *b* value is the exponent describing the rate of variation in weight with respect to length. Log *a* value were later transformed back in order to obtain the linear values using Microsoft excel. The condition index was calculated based on the relationship between total length and body weight using the Fulton condition index as $K=100 \times W/L^3$ (cm). W = weight of the fish and L = total length of the fish (Fulton 1902) (Table 4.1).

4.2.4 Primers design

Gene specific Primers were used from the previously optimised primers for the assays to amplify genes of interest in Tilapia (Chapter 2)

4.2.5 RNA extraction protocol

Liver tissue (< 100 mg) was added to a 2 ml screw cap microtube (Alpha labs) containing 1ml TriReagent (Sigma, UK) extraction buffer according to the manufacturer's protocols, and homogenised using a mini bead beater 24 (Bio spec product) until they were disrupted. RNA was extracted according to the protocol discussed previously in chapter two. The concentration of the RNA was standardized to 300 ng/µl.

4.2.6 RNA Purification

RNA concentration and purity, integrity and quality were determined by the protocols discussed earlier in Chapter 2.

4.2.7 cDNA synthesis

RNA (1.5 µg) was reverse transcribed to produce cDNA using an AB High-capacity reverse transcription kit (Thermofisher) according to the manufacturer's instructions. The synthesis of cDNA was performed in a Biometra thermocycler with a 2 µl RNA template, and a mastermix of 1 µl RT Primer, 9.2 µl RNAse/DNAse water, 2 µl 10x Buffer, 0.8 µl 25 x dNTP mix, and 2 µl Random primers in a total volume of 20 µl under the following conditions: 10 minutes denaturation at 25 °C, 37 °C for 2 hours. The reaction was then heat

inactivated by incubation at 85 °C for 5 minutes. The cDNA mixture was then diluted with 80 µl RNAse water and conserved at -20 ºC until it was used in a Quantitative PCR reaction.

4.2.8 Quantitative PCR assay

Quantitative PCR assays were performed with the ten gene specific primers previously identified (Chapter 2), using a TOptical PCR machine (Biometra, Germany). *Rps 5 and 7* were used as reference genes to normalise the resulting ct values. qPCR analysis was conducted of duplicates of polluted samples and referenced in 96 wells qPCR plates for ten of the previously identified genes, assaying one gene per plate for all the polluted sites and control sites [(16 fish each from the polluted and reference sites (i.e., 48 fish/plate)]. Ten plates were used for all the polluted and reference sites.

4.3 DATA ANALYSIS

The statistical analysis of the results was done using the delta-delta ct method (Pfaffl model equation, 2001) in Excel spreadsheets and SPPSS version 25 (SPSS. Inc. Chicago, USA). Geometric means of the two housekeeping genes (*Rps 5 and Rps7*) were used to normalise the target gene differences for each sample. Later the $2^{-\Delta\Delta}$ Ct method was used to calculate the differences between target genes and references (Pfaffl, 2001). All quantitative values were presented as the mean ± standard deviation of the normalised expression values relative to the control expression. The resulting data passed normality tests after log transformation. Therefore, parametric one-way Anova and Tukey post hoc tests were used to compare the values of gene expressions between sites. The null hypothesis (Ho) was stated as: “there are no significant differences between the genes expressed at the Daberam and Dan Agundi sites compared to the genes expressed at the Jibia site.” The alternate hypothesis (H_1) was stated as: “there are significant differences between the genes expressed at the Daberam and Dan Agundi sites compared to the genes expressed at the Jibia site.” The level of significance was set at $P \leq 0.05$.

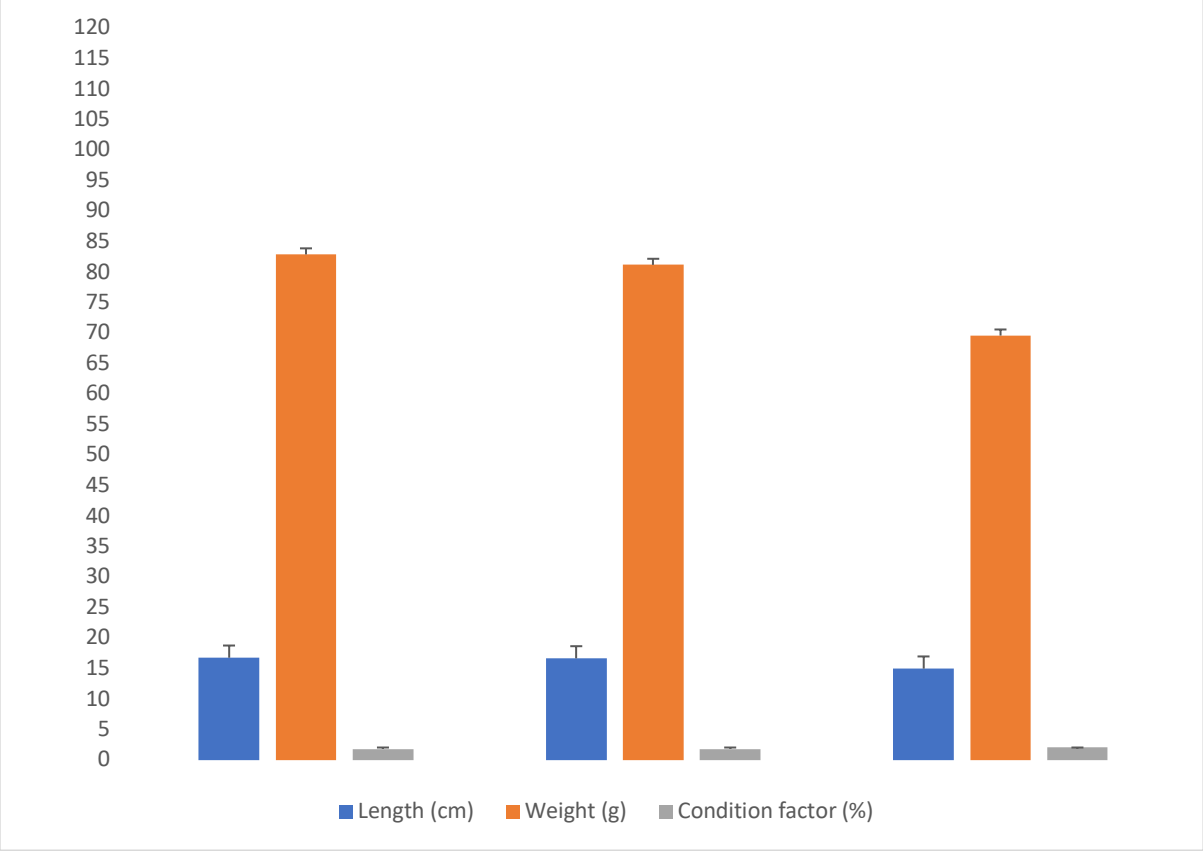
4.4 RESULTS

4.4.1 Biometric, Length-weight relationship and condition factor

A total of 48 live Nile tilapia samples were used for this study, (16 fish from each sampling site). The total length, body weight, and sex taken is shown in (Tables 1-4 in Annex 4). The average mean length/weight and standard deviation were also calculated. The mean total length (±Stdev) for the Daberam, Dan agundi and Jibia sites were between the range of 15.00 – 16.79 cm, while the mean weight between, Daberam, Dan agundi and Jibia sites were between the range of 69.60-82.90 g (Table 4.1). Linear regression coefficient *a* for both the polluted and reference sites were between 0.623-0.868, while the exponent *b* were between 1.79-3.01. The mean condition factor for both sites were in the range of 1.77-2.10. In length and weight, there was no significant difference between the sites. In the condition factor, no significant difference was observed between Daberam and Dan Agundi, but there was a significant difference between the Daberam and Jibia sites as well as the Dan Agundi and Jibia sites (Table 4.1 and Fig. 4.1).

Table 4.1: Mean length, body weight, condition factor and their significance differences with linear regression of length-weight relationship parameters (*a* and *b*) of the wild Nile tilapia caught at the Daberam, Dan Agundi and Jibia sites. Bold denote significant difference at P≤ 0.05.

Site	No. of Fish	Mean total length (cm)	Mean body weight (g)	Mean cond. Factor (K)	Sig. diff. between Length, Weight and Condition Factor	a (95%CI)	b (95% CI)	R^2
Daberam	16	16.79 ± 3.18	82.90 ± 27.34	1.77 ± 0.37	0.08	0.623 ± 0.13	1.94 ± 0.11	0.981
Dan agundi	16	16.68 ± 2.91	81.19 ± 23.65	1.79 ± 0.40	**0.02**	0.756 ± 0.14	1.79 ± 0.12	0.955
Jibia	16	15.00 ± 2.53	69.60 ± 21.18	2.10 ± 0.41	**0.04**	0.868 ± 0.16	3.01 ± 0.14	0.987


120
115
110
105
100
95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
Length (cm)
Weight (g)
Condition factor (%)

≤

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4.4.2 Tilapia Hepatic expression of target genes in wild environmental samples

AHR2, CYP 1A, DIABLO 1 and 2, GST01LA, GSTA2L, GSTMA, GSTR2, MT, and VTG

≤

AHR 2

AHR 2

CYP1A

DIABLO 1

DIABLO 2

DIABLO 2

GST01LA

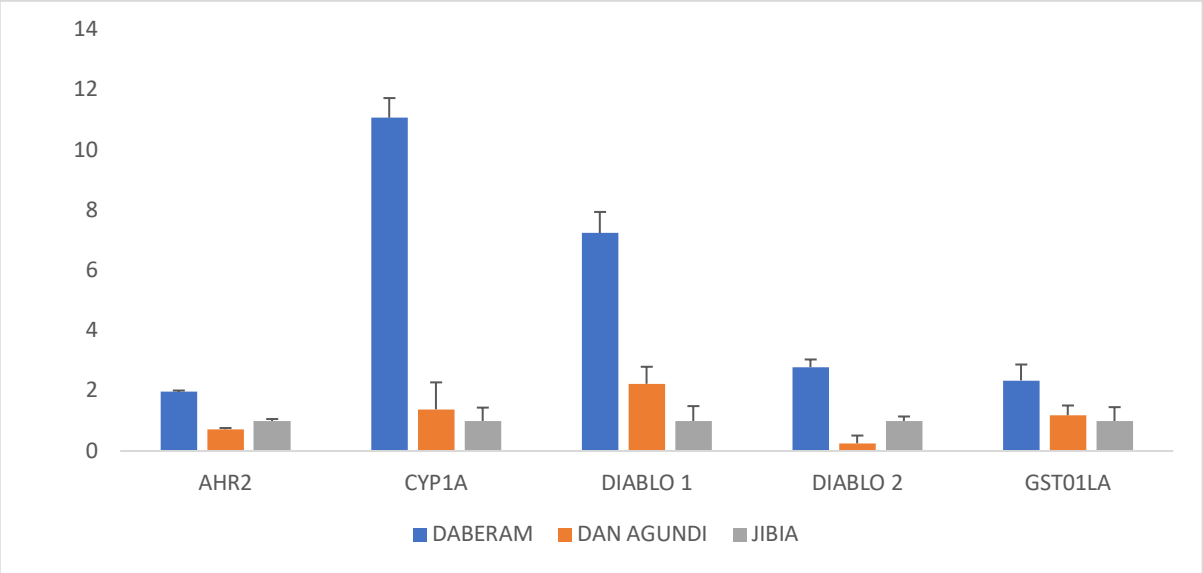
GSTA2L

GSTMA

GSTR2

MT

VTG



AHR 2, CYP 1A, DIABLO 1 DIABLO 2, and GST01LA

≤

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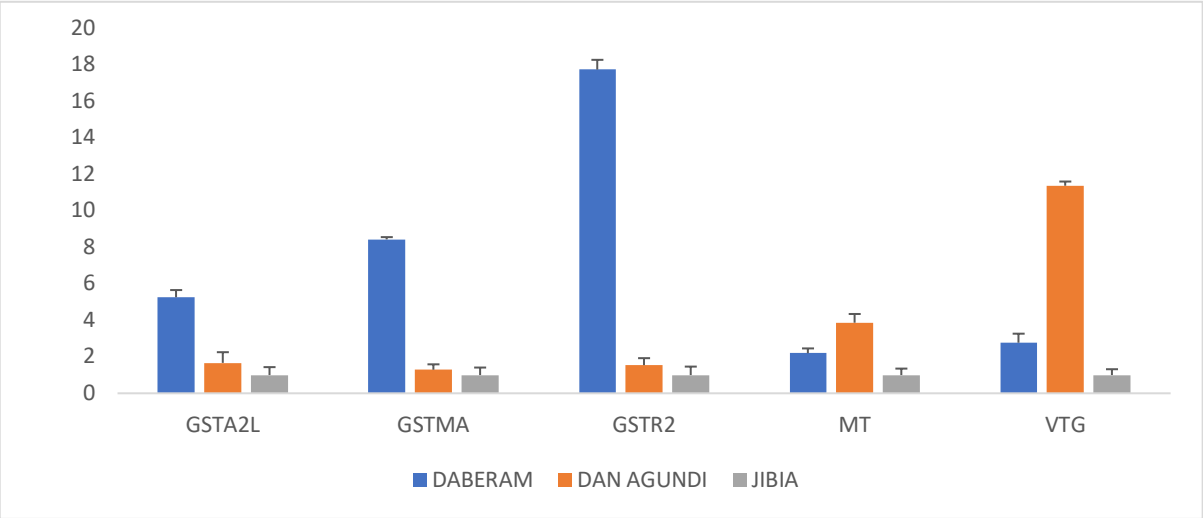

GSTA2L, GSTMA, GSTR2, MT, VTG

≤

! ""&

4.5 DISCUSSION

4.5.1 Length-weight relationship and condition factor

b

b *Oreochromis niloticus*

Oreochromis niloticus

Sarotherodon melanotheron

PPAR, CYP 1A

4.5.2 Phase I and II Biotransformation responses

CYP 1A

CYP 1A

CYP 1A

AHR 2 *CYP 1A*

CYP 1A *AHR 2*

CYP1A *AHR 2*

! "' '

AHR 2 CYP 1A

CYP 1A

AHR-CYP1A

AHR CYP1A

GSTR2 GSTA2L

GST01LA

GSTA2L), GSTMA GSTR2

GST01LA GSTMA

GSTs

GSTs

GSTs

GSTs

4.5.3 Response of apoptotic related genes in the field

Oreochromis niloticus Diablo 1 and 2

DIABLO

Platichthys flesus

Zoarces viviparus

Platycephalus bassensis

DIABLO

DIABLO 1 2 *DIABLO 1 and 2*

CYP 1A

CYP 1A

DIABLO

4.5.4 Response of metal-metabolism related gene in the field

MT

MT

MT

Oreochromis niloticus

CuZn-SOD *SOD* *GST*

CAT, GR, CuZn-SOD

GR, GPX, and GST *Sarotherodon melanotheron*

Oreochromis niloticus

4.5.5 Response of endocrine disruptors in the field

VTG

VTG *ZRP*

VTG

VTG

VTG

VTG

VTG

VTG

VTG

VTG

VTG

4.6 CONCLUSIONS

Oreochromis niloticus

! "' %

AHR 2, CYP 1A, DIABLO 1 and 2, VTG, MT, and GSTs (GSTO1LA, GSTA2L, GSTMA, and GSTR2),

5.0 CHAPTER FIVE: CONCLUSIONS AND FURTHER STUDIES

Oreochromis niloticus

Oreochromis niloticus

Oreochromis niloticus

5.1 FURTHER STUDIES

REFERENCES

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(Danio rerio

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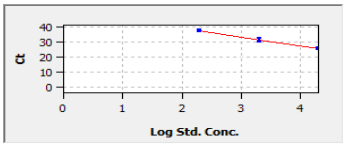
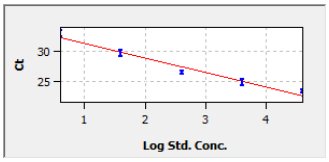
ANNEX 1: Supporting information for Chapter 2

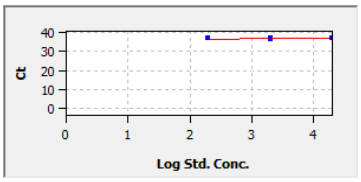
1.AHR
R^2 = 0.99952
PCR EFFECIENCY =0.47



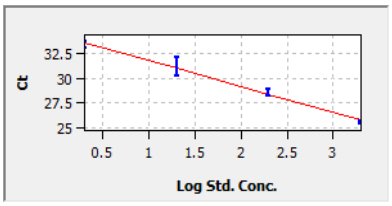
2. AHR2
R^2 =0.92119
PCR EFFECIENCY = 0.93



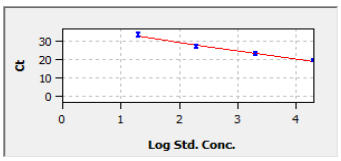
3. AHRr
R^2 =0.25724
PCR EFFECIENCY =1.78



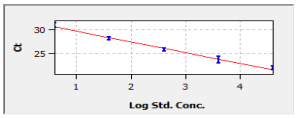
4. CYP1A
R^2=0.99485
PCR EFFECIENCY=1.02



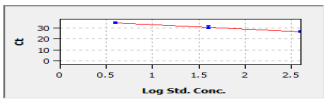
5. DIABLO 1
R^2=0.998114
PCR EFFECIENCY=0.90



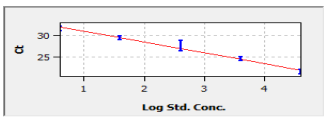
6. DIABLO 2
R^2 =0.99886
PCR EFFECIENCY =1.03



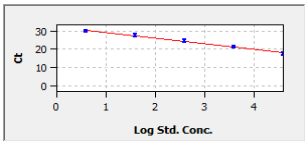
7. GSTA
R^2=0.99987
PCR EFFECIENCY=0.76



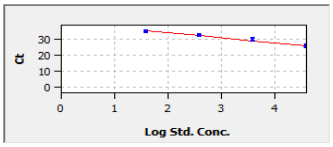
8. GSTO1LA
R^2=0.99276
PCR EFFECIENCY=1.02



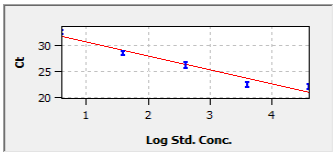
9. GST01LB
R^2=0.98909
PCR EFFECIENCY= 1.5



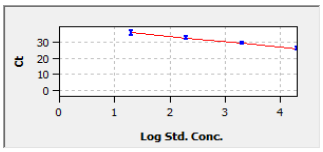
10. GST O1LC
R^2=0.95306
PCR EFFECIENCY=1.35



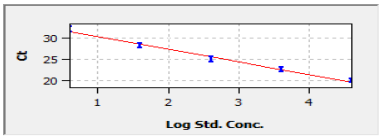
11. GSTAL2
R^2=0.97565
PCR EFFECIENCY=1.05



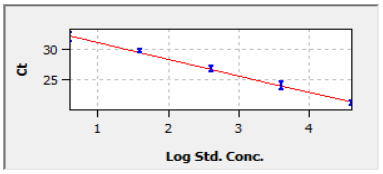
12. GSTK
R^2 =0.99158
PCR EFFECIENCY= 1.77



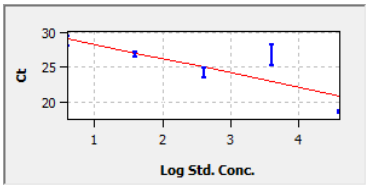
13. GSTMA
R^2=0.9894
PCR EFFECIENCY= 0.92



14. GST MB
R^2=0.98931
PCR EFFECIENCY= 0.57



15. GST R1
R^2=0.677447
PCR EFFECIENCY= 2.02



16. GST R2
R^2 =0.96651
PCR EFFECIENCY= 1.01

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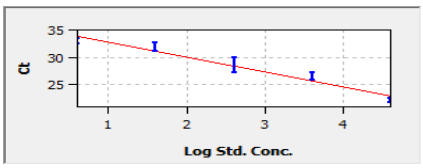
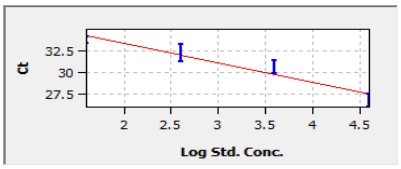
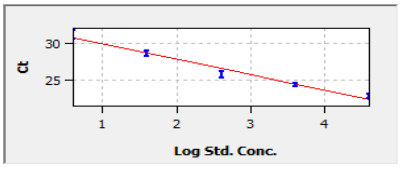


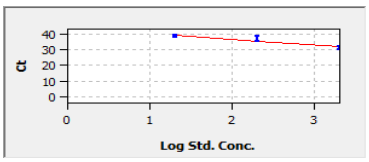
17. GSTR3
R^2 =0.93529
PCR EFFECIENCY= 1.76



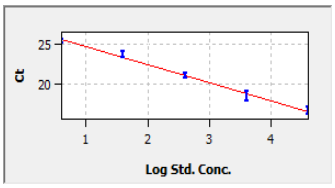
18. GST R4
R^2 =0.97265
PCR EFFECIENCY= 2.01



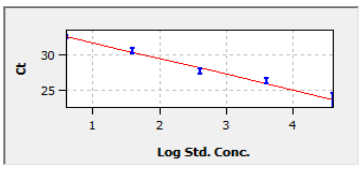
19. GST R5
R^2=0.9669
PCR EFFECIENCY=1.53



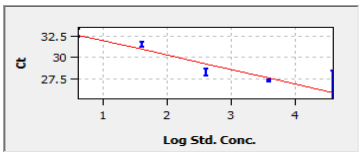
20. GST T1
R^2 =0.99492
PCR EFFECIENCY= 1.7



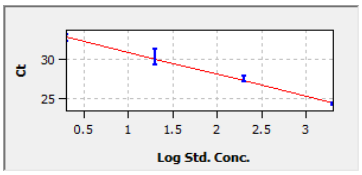
21. GST T2
R^2 =0.99125
PCR EFFECIENCY= 1.79



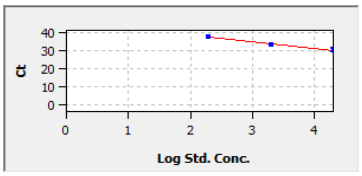
22. MGST
R^2=0.94288
PCR EFFECIENCY=2.88



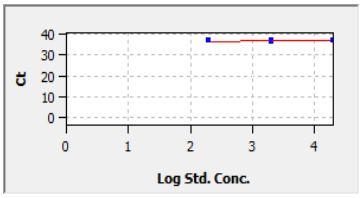
23. MT
R^2=0.998
PCR EFFECIENCY= 0.91



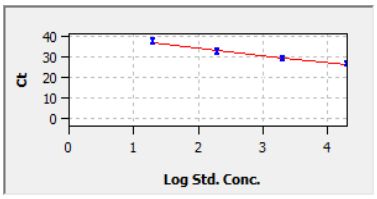
24. SIAH2
R^2=0.98098
PCR EFFECIENCY=0.72



25. UDP-GT1
R^2 = 0.98766
PCR EFFECIENCY= 1.95



26. UDP-GT 5
R^2 =0.97083
PCR EFFECIENCY = 1.90



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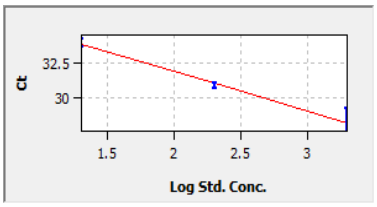
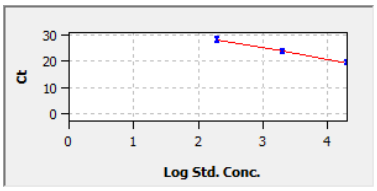
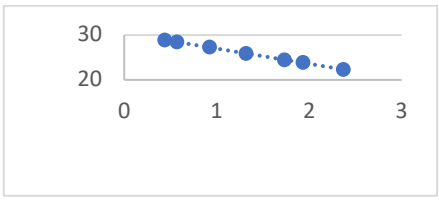
27. VTG
R^2=0.99766
PCR EFFECIENCY=1.04



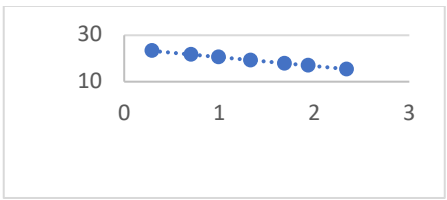
28. ZPC
R^2=0.99986
PCR EFFECIENCY=0.70



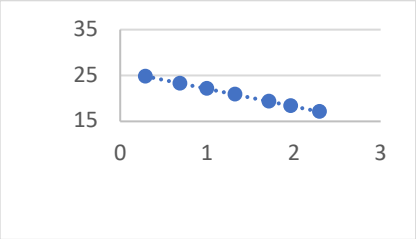
29.RPS 5
R^2= 0.98384
PCR EFFECIENCY = 0.97



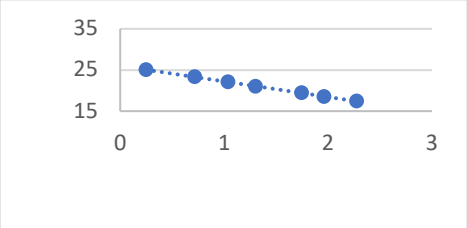
30. 18S
R^2= 0.99787
PCR EFFECIENCY = 0.92



31. RPS7
R^2= 0.99932
PCR EFFECIENCY = 0.92



32. RPL 3
R^2= 0.99723
PCR EFFECIENCY = 0.94



Annex 2: supporting information for Chapter 2

	≤
AHR 1	
	0.015
AHR 2	
AHRR	
CYP 1A	

0.001

	≤
DIABLO 1	
DIABLO 2	
GSTA	
GST01LA	**0.011**
GST01LB	
GST01LC	

	≤
GSTA2L	
	0.005
	0.026
GSTK	
GSTMA	
	0.007

	≤
GSTMB	
GSTR1	
GSTR2	
GSTR3	
GSTR4	
GSTR5	
MGST	
	0.008
MT	

		≤
GSTT1		
		0.023
		0.006
		0.035
GSTT2		
SIAH 2		
		0.014
		0.005

	≤
UDP-GT 1	
UDP-GT 5	
VTG	**0.035**
ZPC	**0.030**

	≤

Annex 3: supporting information for Chapter 3

		≤
Aroclor 1254		
	0.000	
	0.002	
	0.000	
	0.003	
	0.000	
Bap		
	0.000	
	0.000	
	0.002	
	0.003	
Cadmium		
	0.004	
	0.000	
	0.000	
	0.007	
Dazomet	**0.002**	
	0.000	
	0.002	
	0.000	
	0.004	
	0.003	

		≤
DDT		
	0.029	
	0.023	
	0.035	
	0.000	
	0.003	
Dieldrin		
	0.017	
	0.000	
EE2		
	0.005	
	0.000	
Malathion		
	0.000	
	0.023	

		≤
PFOS		
	0.019	
	0.035	
	0.000	
Phthalates		
	0.007	
	0.019	

ANNEX 2 FOR CHAPTER 2 cont.…

Table 2: Normalised tissue ct with RPL 3 and 18S Reference genes

NORMALISED TISSUE CT VALUES WITH GEOMEAN OF 18S AND RPL 3

TISSUES	AHR	AHR2	AHR R	CYP 1A	DIAB LO 1	DIAB LO 2	GST A	GST 01LA	GST 01 LB	GST 01LC	GST A2L	GST K	GST MA	GST MB	GST R1	GST R2	GST R3	GST R4	GST R5	GST T1	GST T2	MGST	MT	SIAH 2	UDP-GT 1	UDP-GT5	VIT	ZPC
L1	1.0775 E-04	6.111 7E-06	3.570 2E-07	1.080 7E-01	7.158 4E-05	9.915 2E-05	4.670 2E-07	1.379 2E-02	4.670 2E-07	2.243 6E-03	1.4340 E+00	4.670 2E-07	6.110 2E-11	9.433 0E-04	2.903 3E-04	4.670 2E-07	3.670 2E-07	9.136 0E-06	1.912 9E-03	5.018 8E-09	3.2490 E+00	2.397 6E-06	4.670 2E-07	9.094 9E-13	4.000 2E-07	2.371 5E-03	4.985 4E-04	2.855 7E-02
L2	5.3948 E-06	1.167 6E-07	3.467 6E-07	4.334 3E-03	1.167 6E-07	4.250 7E-04	1.167 6E-07	5.640 3E-03	1.167 6E-07	2.823 9E-04	7.2699 E-01	1.167 6E-07	5.630 0E-12	2.172 9E-05	1.167 6E-07	1.167 6E-07	3.167 6E-07	1.167 6E-07	3.579 2E-05	1.373 0E-09	1.4559 E-01	1.167 6E-07	1.167 6E-07	9.094 9E-13	2.067 6E-07	4.916 8E-04	9.752 6E-03	6.129 6E-03
L3	1.2023 E-03	1.473 9E-05	4.289 9E-07	2.534 9E-01	1.246 4E-04	8.763 9E-06	3.189 9E-07	1.541 0E-02	3.189 9E-07	1.145 3E-03	8.6454 E-01	3.189 9E-07	3.835 1E-10	3.030 7E-05	4.882 8E-04	3.189 9E-07	3.009 9E-07	4.795 1E-06	1.263 8E-04	2.024 2E-09	3.1167 E+00	2.377 8E-05	3.189 9E-07	9.094 9E-13	1.289 9E-07	4.310 1E-04	1.200 7E-02	1.142 3E-01
L4	7.4724 E-06	6.513 8E-07	4.513 8E-07	2.797 0E-02	5.211 0E-06	3.225 8E-05	6.513 8E-07	1.312 1E-01	6.513 8E-07	2.407 8E-04	2.1735 E+00	6.513 8E-07	9.094 9E-13	6.513 8E-07	1.222 3E-05	6.513 8E-07	4.513 8E-07	6.513 8E-07	1.220 7E-04	2.095 6E-09	3.2944 E+00	6.513 8E-07	6.513 8E-07	9.094 9E-13	7.413 8E-07	1.137 4E-03	5.137 9E-01	2.033 3E-02
L5	3.0812 E-07	1.161 0E-08	2.261 0E-08	6.354 2E-04	1.161 0E-08	9.778 7E-05	1.161 0E-08	2.444 7E-05	1.161 0E-08	4.862 1E-06	2.8164 E-02	1.161 0E-08	9.094 9E-13	1.161 0E-08	9.353 0E-08	1.161 0E-08	2.161 0E-08	1.161 0E-08	1.707 1E-06	2.344 5E-10	2.8164 E-02	1.161 0E-08	1.161 0E-08	9.094 9E-13	1.261 0E-08	2.309 7E-04	1.441 6E-04	1.902 3E-04
I1	1.5730 E-07	6.335 7E-07	4.651 5E-08	9.631 2E-04	3.851 5E-08	5.132 4E-05	3.851 5E-08	1.770 1E-02	4.676 5E-08	7.588 7E-02	1.2691 E-02	3.851 5E-08	1.473 9E-05	1.644 6E-04	1.482 2E-04	3.851 5E-08	6.851 5E-08	5.789 7E-07	6.632 9E-05	2.155 0E-04	3.3771 E-03	3.189 9E-07	2.541 0E-08	8.008 7E-06	2.751 5E-08	3.298 8E-01	3.851 5E-08	2.407 8E-04
I2	4.8015 E-07	7.227 5E-07	3.009 3E-08	2.231 0E-04	1.809 3E-08	7.942 8E-05	1.809 3E-08	3.644 7E-03	3.390 7E-06	6.215 1E-03	6.4877 E-04	1.809 3E-08	3.293 5E-05	8.801 3E-04	1.137 4E-03	6.650 7E-07	1.809 3E-08	4.094 0E-07	1.621 9E-04	4.021 4E-04	5.1612 E-04	5.005 4E-07	1.809 3E-08	2.268 2E-06	2.709 3E-08	8.740 5E-04	6.641 8E-06	2.863 4E-04
I3	5.1106 E+00	1.185 4E-08	5.424 5E-08	1.748 1E-03	6.162 4E-07	2.602 0E-05	1.185 4E-08	6.354 2E-04	3.081 2E-07	1.352 6E-03	4.1521 E-02	1.185 4E-08	5.240 3E-05	6.587 1E-05	4.315 8E-05	1.185 4E-08	1.185 4E-08	1.721 3E-07	8.763 9E-06	1.239 4E-05	7.3400 E-03	3.741 2E-07	1.185 4E-08	5.409 2E-08	2.185 4E-08	1.991 5E-02	7.029 9E-07	2.155 0E-04
I4	3.7462 E-08	3.256 9E-07	5.446 2E-08	1.246 4E-04	3.746 2E-08	1.612 9E-05	3.746 2E-08	9.355 3E-03	3.038 8E-07	2.683 0E-02	1.8073 E-02	3.746 2E-08	1.203 9E-04	4.849 1E-04	1.942 2E-04	3.746 2E-08	3.746 2E-08	1.377 0E-06	1.621 9E-04	1.588 6E-04	2.6313 E-03	4.574 1E-07	3.746 2E-08	2.221 6E-06	5.746 2E-08	5.197 1E-04	1.782 0E-07	4.855 6E-05
I5	4.4301 E-09	4.430 1E-09	3.830 1E-09	4.782 3E-04	4.430 1E-09	2.847 4E-05	4.430 1E-09	2.985 0E-04	1.083 3E-08	1.586 4E-03	7.4425 E-03	1.286 5E-07	9.644 1E-05	2.188 0E-05	8.936 1E-05	1.096 9E-07	4.430 1E-09	3.275 2E-06	4.387 8E-07	3.320 9E-06	6.2667 E-04	7.078 8E-07	4.430 1E-09	2.627 1E-07	2.330 1E-09	1.592 8E-06	9.224 2E-08	1.110 8E-06
G1	4.0151 E-08	4.015 1E-08	2.315 1E-08	7.662 0E-04	1.096 9E-07	5.570 1E-04	4.015 1E-08	3.381 6E-04	5.175 0E-06	3.235 2E-02	8.3154 E-03	4.015 1E-08	3.362 7E-05	3.574 4E-04	5.125 6E-04	4.015 1E-08	5.005 1E-08	4.015 1E-08	4.964 2E-06	1.319 2E-05	1.6198 E-03	4.015 1E-08	4.015 1E-08	1.386 6E-06	5.005 1E-08	1.412 0E-04	4.015 1E-08	1.506 9E-06
G2	1.7944 E-07	1.735 6E-08	1.673 6E-08	3.424 2E-03	1.735 6E-08	1.071 5E-05	2.635 6E-08	3.993 7E-04	1.560 0E-06	1.135 9E-02	9.1117 E-04	1.735 6E-08	1.306 6E-03	8.211 9E-04	2.182 2E-03	1.735 6E-08	1.832 1E-07	1.735 6E-08	1.827 2E-05	1.837 5E-04	2.5628 E-04	8.302 2E-07	1.735 6E-08	2.573 1E-07	2.005 6E-08	1.586 4E-03	3.710 4E-06	1.878 6E-05
G3	4.1856 E-08	7.554 6E-09	6.004 6E-09	7.109 0E-05	7.554 6E-09	5.417 8E-04	4.554 6E-09	1.055 3E-04	2.835 3E-07	1.016 7E-02	1.9023 E-04	7.554 6E-09	1.365 7E-05	1.149 9E-06	1.726 3E-04	5.334 8E-08	7.554 6E-09	7.554 6E-09	9.072 9E-06	1.394 4E-05	7.5143 E-05	5.047 0E-08	7.554 6E-09	1.104 6E-07	6.664 6E-09	2.602 0E-05	7.554 6E-09	1.985 7E-05
G4	2.4545 E-08	2.652 5E-09	3.442 5E-09	1.077 5E-04	1.419 5E-08	2.427 8E-05	1.628 4E-07	2.785 1E-04	2.682 3E-07	6.624 0E-04	5.0902 E-04	1.711 7E-08	2.377 8E-05	6.415 5E-06	1.431 7E-04	2.652 5E-09	2.652 5E-09	2.652 5E-09	3.664 2E-07	2.516 8E-06	1.6332 E-04	3.694 6E-08	2.652 5E-09	1.023 5E-07	4.452 5E-09	3.889 7E-05	8.898 1E-07	1.660 4E-06
G5	3.4043 E-09	3.404 3E-09	4.504 5E-09	3.700 5E-04	3.404 3E-09	1.161 0E-08	3.404 3E-09	2.545 1E-04	2.738 7E-07	8.986 2E-04	1.0336 E-04	4.363 3E-08	4.755 6E-05	1.829 7E-06	2.010 7E-04	3.404 3E-09	3.404 3E-09	3.404 3E-09	2.499 4E-06	1.525 9E-05	5.3504 E-05	4.015 1E-08	3.404 3E-09	8.019 4E-07	2.344 3E-09	7.410 9E-05	3.442 6E-07	2.381 0E-06
H1	7.6498 E-08	8.738 3E-09	9.838 3E-09	2.923 5E-04	8.738 3E-09	1.197 2E-05	8.738 3E-09	4.340 1E-04	8.738 3E-09	6.532 8E-04	3.2848 E-03	8.738 3E-09	4.855 6E-05	3.500 9E-04	2.299 9E-06	3.867 9E-06	8.738 3E-09	8.738 3E-09	3.525 2E-04	7.715 2E-04	6.3542 E-04	8.738 3E-09	8.535 6E-07	1.579 7E-05	5.638 3E-09	4.992 1E-05	8.198 9E-08	4.032 2E-06
H2	5.5925 E-07	6.668 5E-09	7.468 5E-09	8.042 9E-04	6.668 5E-09	1.557 9E-05	6.668 5E-09	1.068 6E-03	6.668 5E-09	9.111 7E-04	2.9439 E-04	6.668 5E-09	2.887 1E-05	3.500 9E-04	2.566 2E-05	1.175 7E-07	6.668 5E-09	6.668 5E-09	8.643 2E-06	7.410 9E-05	1.2464 E-04	6.668 5E-09	6.668 5E-09	2.101 7E-06	7.558 5E-09	4.561 9E-05	6.885 2E-07	2.296 8E-05
H3	6.6880 E-06	2.078 3E-08	4.058 3E-08	1.400 3E-03	2.078 3E-08	9.380 3E-05	2.078 3E-08	4.431 2E-04	2.078 3E-08	1.306 6E-03	6.2318 E-05	2.078 3E-08	1.335 8E-04	1.283 1E-05	1.326 6E-04	2.078 3E-08	2.078 3E-08	2.078 3E-08	7.735 9E-06	1.172 5E-05	9.1873 E-05	2.078 3E-08	2.078 3E-08	8.244 9E-07	3.178 3E-08	1.545 1E-04	5.439 6E-07	1.042 2E-05
H4	1.1725 E-05	2.387 4E-08	3.487 4E-08	3.747 1E-03	2.387 4E-08	2.024 7E-04	2.387 4E-08	1.262 1E-03	2.387 4E-08	4.280 3E-04	2.3097 E-04	2.387 4E-08	1.229 2E-04	8.348 8E-06	6.137 8E-04	4.638 0E-07	2.887 4E-08	3.087 4E-08	2.157 9E-05	2.215 6E-04	1.2464 E-04	2.387 4E-08	2.387 4E-08	1.579 7E-05	3.487 4E-08	2.746 7E-04	4.862 1E-06	3.167 8E-07
H5	1.3963 E-06	2.761 4E-08	1.861 4E-08	3.966 1E-04	2.761 4E-08	2.566 2E-05	2.761 4E-08	6.578 3E-04	2.761 4E-08	9.111 7E-04	7.2487 E-04	2.761 4E-08	2.788 8E-05	4.631 8E-06	3.889 7E-05	2.364 6E-06	2.761 4E-08	2.761 4E-08	1.915 5E-04	8.155 2E-04	3.2215 E-04	2.761 4E-08	2.761 4E-08	6.451 5E-05	3.614 5E-08	3.293 5E-05	2.761 4E-08	6.442 9E-04
S1	4.6442 E-08	4.644 2E-08	6.444 2E-08	6.415 5E-06	4.644 2E-08	2.172 9E-05	4.644 2E-08	7.391 1E-03	4.644 2E-08	6.121 4E-02	1.9129 E-03	4.644 2E-08	2.407 8E-04	7.289 3E-03	6.275 1E-05	5.944 6E-06	4.644 2E-08	4.644 2E-08	9.303 1E-04	5.013 4E-03	7.1489 E-04	4.644 2E-08	1.707 1E-06	1.229 2E-04	5.744 2E-08	4.493 1E-04	4.644 2E-08	4.985 4E-04
S2	6.7525 E-08	4.715 3E-09	7.415 3E-09	2.447 9E-06	4.715 3E-09	3.505 5E-05	4.715 3E-09	4.462 1E-04	4.715 3E-09	1.480 2E-03	1.0627 E-04	4.715 3E-09	5.380 4E-04	1.822 3E-03	1.310 1E-05	4.715 3E-09	4.715 3E-09	4.715 3E-09	1.134 1E-06	4.351 7E-06	6.6790 E-05	4.715 3E-09	4.715 3E-09	1.562 1E-07	5.515 3E-09	1.633 2E-04	2.287 1E-07	5.297 9E-08
S3	1.3807 E+00	1.849 8E-09	8.149 8E-09	4.449 0E-07	1.849 8E-09	1.101 6E-05	1.849 8E-09	4.923 4E-05	1.849 8E-09	1.203 9E-04	2.2496 E-05	1.849 8E-09	2.361 4E-05	5.742 1E-06	8.824 8E-06	1.849 8E-09	1.849 8E-09	1.849 8E-09	1.961 0E-06	1.496 5E-06	8.8862 E-06	1.849 8E-09	1.849 8E-09	3.767 2E-07	2.749 8E-09	3.731 2E-05	3.371 7E-07	3.873 1E-07
S4	6.6047 E+00	6.604 7E-07	4.304 7E-07	1.850 2E-04	6.604 7E-07	3.520 5E-03	6.604 7E-07	9.458 2E-06	6.604 7E-07	3.248 2E-05	6.7255 E-05	6.604 7E-07	5.090 2E-04	6.615 2E-03	4.645 3E-03	6.451 5E-05	6.197 0E-06	6.604 7E-07	6.763 2E-04	3.129 2E-03	3.6292 E-05	6.604 7E-07	6.604 7E-07	1.255 0E-04	5.404 7E-07	5.798 9E-03	9.458 2E-06	2.013 4E-05
S5	8.3133 E-08	1.288 3E-08	2.188 3E-08	8.643 2E-06	1.288 3E-08	2.070 0E-05	1.288 3E-08	3.111 7E-04	1.288 3E-08	9.833 6E-04	5.7267 E-04	1.288 3E-08	6.146 0E-05	1.740 7E-05	2.968 3E-05	1.288 3E-08	1.288 3E-08	1.288 3E-08	7.735 9E-06	1.356 3E-05	3.7521 E-04	1.288 3E-08	1.288 3E-08	2.773 2E-06	4.488 3E-08	1.077 5E-04	3.705 4E-05	7.629 4E-06

	4.8414	4.841	3.441	9.791	4.841	8.053	4.841	6.442	4.841	4.077	5.8877	4.841	2.041	3.500	4.964	4.841	4.841	4.841	4.841	8.824	1.6560	4.841	4.841	5.146	5.541	1.726	4.841	4.841
M1	E-08	4E-08	4E-08	8E-06	4E-08	6E-05	4E-08	9E-04	4E-08	6E-04	E-04	4E-08	5E-05	9E-04	2E-06	4E-08	4E-08	4E-08	4E-08	8E-06	E-04	4E-08	4E-08	2E-07	4E-08	3E-04	4E-08	4E-08
	1.8093	1.809	8.209	6.415	1.809	2.584	1.809	8.384	1.809	8.384	3.0057	1.809	3.181	4.683	2.101	1.809	1.809	1.809	9.804	8.898	1.2903	1.809	1.809	1.183	2.709	1.658	3.534	3.279
M2	E-08	3E-08	3E-08	5E-06	3E-08	1E-05	3E-08	4E-04	3E-08	4E-04	E-04	3E-08	4E-05	9E-04	7E-06	3E-08	3E-08	3E-08	9E-07	1E-07	E-04	3E-08	3E-08	9E-07	3E-08	2E-05	7E-06	5E-07
	3.1284	5.646	5.646	2.157	5.646	5.508	5.646	3.203	5.646	1.107	5.4555	5.646	2.638	1.907	5.985	5.646	5.646	5.646	5.646	3.564	4.8893	5.646	5.646	4.841	6.746	2.394	7.128	9.034
M3	E-08	5E-09	5E-09	9E-05	5E-09	2E-06	5E-09	5E-05	5E-09	8E-04	E-04	5E-09	4E-05	3E-06	9E-06	5E-09	5E-09	5E-09	5E-09	0E-07	E-05	5E-09	5E-09	4E-08	5E-09	4E-05	0E-07	4E-08
	5.1174	1.161	2.161	5.854	1.161	2.027	1.161	4.749	1.161	1.381	2.2592	1.161	8.948	5.313	4.599	1.161	1.161	1.161	1.043	1.752	1.4598	1.161	1.161	5.327	3.361	2.444	1.955	1.232
M4	E-08	0E-08	0E-09	9E-05	0E-08	4E-05	0E-08	3E-04	0E-08	1E-03	E-03	0E-08	0E-06	4E-05	8E-06	0E-08	0E-08	0E-08	6E-06	8E-05	E-03	0E-08	0E-08	6E-07	0E-08	7E-05	7E-04	5E-06
	8.5585	8.558	7.858	2.851	8.558	7.318	8.558	1.086	8.558	4.168	1.4199	8.558	1.042	2.319	1.087	8.558	8.558	8.558	3.564	1.829	5.9366	8.558	8.558	3.987	6.658	3.486	7.268	2.119
M5	E-09	5E-09	5E-09	2E-06	5E-09	6E-06	5E-09	5E-05	5E-09	8E-05	E-03	5E-09	2E-05	0E-07	9E-06	5E-09	5E-09	5E-09	0E-07	7E-06	E-05	5E-09	5E-09	3E-08	5E-09	0E-06	1E-06	2E-07

Annex 3: supporting information for Chapter 3

Table 1: Comparative expression of genes between Pseudoreplicates A and B beakers.

Genes	Kruskal-Wallis Test (P≤ 0.05)
AHR 2	
Beaker A-Beaker B	0.090
CYP 1A	
Beaker A-Beaker B	0.058
DIABLO 1	
Beaker A-Beaker B	0.095
DIABLO 2	
Beaker A-Beaker B	0.310
GST01LA	
Beaker A-Beaker B	0.162
GSTA2L	
Beaker A-Beaker B	0.222
GSTMA	
Beaker A-Beaker B	0.080
GSTR2	
Beaker A-Beaker B	0.091
MT	
Beaker A-Beaker B	0.080
VTG	
Beaker A-Beaker B	0.061

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Table 2: AROCLOR NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.00638986	1.80875876	0.03577263	0.13966089	0.08686989	0.00537321	0.61985385	6.5416E-05	0.00167979	3.2935E-05
2	0.07105106	0.96440451	0.25348987	0.37761815	1.02455682	8.948E-06	3.41053957	0.00051971	0.00666121	0.00025672
3	0.02768023	0.547925	0.04786119	0.09278272	1.02811383	0.01074642	0.01923663	1.6931E-05	0.00057069	6.6675E-05
4	0.06958885	0.50869922	0.00828662	0.04836141	0.59873935	0.00017204	0.07802066	2.2186E-05	0.00507457	9.3479E-05
5	0.03419668	0.84584523	0.01739721	0.03742121	0.13490353	0.01016673	0.23245124	4.468E-05	0.01165829	8.7673E-05
6	0.00056479	3.32879494	0.01838917	0.36602142	0.62633222	0.0043644	1.74110113	9.1044E-06	0.00078501	1.8786E-05
7	0.00754638	8	0.0438889	0.11149106	0.02503343	0.00347204	1.15268635	6.5051E-06	0.00379943	9.4129E-05
8	0.00583925	3.03143313	0.04703896	0.09278272	0.01350839	0.0009433	1.07549439	3.1594E-05	0.02119694	0.00019089
9	0.01950516	1.91189064	0.01910375	0.22531262	0.78730798	0.00041345	2.12137548	6.1673E-05	0.00854917	0.00015291
10	0.09246172	0.17745628	0.20877198	0.12985739	1.80250093	0.0016538	4.36203093	1.3469E-05	0.00594132	0.00140035

Table 3: BAP NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENE

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.08783867	0.17228031	0.15604132	0.3197464	0.06745176	0.0110869	0.25173889	0.00701666	0.0119239	0.3815648
2	0.07756564	0.64267933	0.19479114	0.77768536	0.07922824	0.13258597	0.17113655	0.05173889	0.89192852	0.08618499
3	0.04095424	0.07816833	0.85559503	0.86154616	0.38510947	0.00282997	0.63580412	0.00951883	0.01970902	0.05107546
4	0.00512909	0.69187468	1.23541864	0.72309232	0.79553648	0.00554345	0.54756499	0.00619363	0.00120229	0.33190018
5	0.06098372	0.51402675	0.01120278	0.04052624	0.0290564	0.00515433	0.05593907	0.00016107	0.00052697	0.55478474
6	0.02093126	0.24820691	0.00393342	0.67017584	0.69015868	0.00744248	0.74037108	0.00872881	0.00155378	0.12747857
7	0.38099848	0.50637684	0.00942037	0.80664176	0.29936968	0.00214471	0.06311599	0.00604518	0.00431926	0.36604026
8	0.2867208	0.02875586	0.01819896	0.92880901	0.13030822	0.00843147	0.00454974	0.03372588	0.00133402	0.10844234
9	0.20302504	0.0084219	0.00506578	0.22684324	0.52304247	0.05403358	0.48313122	0.00510225	0.36602142	0.21146131
10	0.09748105	0.48313122	0.08218392	0.91189064	0.58008262	0.00567958	0.16986304	0.00999207	0.04094979	0.85317612

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Table 4: CADMIUM NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENE

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.0485293	0.30460257	0.01584312	0.00017144	0.00650155	0.00647906	0.45218969	0.00053618	0.65292989	0.0055627
2	0.01493665	0.14014576	0.02186853	0.03820751	0.0402463	0.00121065	0.1995746	0.00010517	0.9930925	0.00048828
3	0.0168046	0.10153155	0.02335702	0.04671404	0.10438599	0.00111403	0.24827312	9.9152E-05	0.37241937	6.8194E-05
4	0.01341508	0.08304286	0.01116402	0.02826186	0.07694653	0.00062234	0.12718496	3.6671E-05	0.16070391	1.7589E-05
5	0.09087328	0.10956947	0.125	0.04152143	0.30992692	0.00333061	0.27456063	0.10019089	0.16941132	0.00010888
6	0.01059847	0.03716272	0.01819896	0.02319568	0.04109196	0.00059287	0.15283003	0.00013545	0.58845337	1.1644E-05
7	0.02787277	0.03491522	0.04038603	0.01606428	0.07031616	0.00125335	0.3077861	0.00011791	0.54756499	3.9714E-05
8	0.02701679	0.13397168	0.01209035	0.04655242	0.10806715	0.00129754	0.34989647	0.00011832	0.3375545	0.00054178
9	0.03941804	0.05613327	0.01264753	0.02655266	0.10657936	0.00346003	0.49654625	0.00057666	0.85960989	0.00141498
10	0.03060688	0.13397168	0.37113089	0.00458139	0.05751173	0.00223579	0.22531262	0.0001477	0.4665165	0.00010701

Table 5: DAZOMET NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.17434296	0.0686308	0.02845843	0.05347469	0.03384297	0.00149566	0.87964908	0.00133866	0.42337266	0.00531763
2	0.13584186	0.18049115	0.01398477	0.10769327	0.12032431	0.00010372	0.5377472	0.00716409	0.42044821	0.00161976
3	0.20447551	0.13821633	0.06698584	0.10547697	0.05851702	0.00365731	0.39229205	0.01074642	0.33564313	0.02664484
4	0.15072598	0.20804968	0.04166558	0.09375244	0.06515411	0.00099019	0.47139227	0.00433426	0.42928272	0.00978644
5	0.26701635	0.17313868	0.04450157	0.11110534	0.03326157	0.00013451	0.59873935	0.00076886	0.50522572	0.00335377
6	0.11703403	0.12032431	0.00982042	0.05292158	0.03092677	0.00055701	0.23406806	0.00164808	0.25971478	0.00485942
7	0.07179365	0.14408579	0.04358574	0.04313492	0.02295576	0.00023912	0.31316611	0.00149566	0.14916697	0.00364466
8	0.04066693	0.08626983	0.01360235	0.04195539	0.02248334	0.00012903	0.26794337	0.00047989	0.14968484	0.00067398
9	0.00668434	0.11744034	0.04607091	0.04066693	0.02209709	2.1579E-05	0.14660437	0.0152505	0.07588718	0.00019089
10	0.15550146	0.10547697	0.0320174	0.02141848	0.01104854	3.9167E-05	0.23651441	0.00011509	1.01291329	2.1729E-05

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Table 6: DDT NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.2822411	0.48220225	0.09944206	0.04972103	0.77110541	0.00374713	0.06910817	2.9478E-05	0.57889664	3.2821E-05
2	0.00081778	0.26096371	0.0640857	0.79626475	0.45614432	0.13490353	0.27967507	1.8209E-05	0.62078681	0.00126206
3	0.0995746	0.8345065	0.36223554	0.81790206	0.72397065	0.04972103	0.38705849	1.4536E-05	0.9862327	0.00013686
4	0.00777091	0.93865725	0.39639207	0.95594532	0.3375545	0.04920675	0.71355908	3.2595E-05	0.72309232	0.00189314
5	0.00174656	0.05554798	0.09827013	0.77646888	0.82323131	0.05111888	0.13983754	8.98E-07	0.9862327	0.00015886
6	0.00924441	0.49886575	0.18845337	0.32408891	0.42688829	0.08161624	0.81474589	5.1147E-05	0.85037777	0.01034444
7	0.20589775	0.33566005	0.9930925	0.19333574	0.37471644	0.11110534	0.49017798	0.00047658	0.04202425	0.01984611
8	0.00447158	0.56863307	0.01790206	0.48452357	0.55606329	0.15336062	0.09337189	0.00044931	0.4223801	0.06142629
9	0.01663078	0.62174565	0.6328783	0.82931955	0.19645458	0.02529507	0.55143356	0.00050375	0.36198532	0.00381262
10	0.00184138	0.50492795	0.09666787	0.40444488	0.58872814	0.00647906	0.28132198	0.00025896	0.61328352	0.00020889

Table 7: DIELDRIN NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.00418662	1.17690674	0.03703415	0.00184777	0.01104854	0.00269593	0.43830286	0.00017996	0.00634572	0.01457864
2	2.7552E-05	1.8276629	0.00214471	0.77916458	0.07588718	1.0422E-05	0.03983002	5.4891E-06	1.7772E-05	7.3951E-06
3	0.0008399	9.8833491	0.00704102	0.03269015	0.1391777	0.00134797	0.18111777	0.00064429	0.00133866	0.00340059
4	0.01804196	0.1767767	0.28420424	0.16379918	0.17862427	0.00061378	0.72951017	0.00019968	0.00184138	1.9181E-05
5	0.00089629	6.65758988	0.26061644	0.14458602	0.24064861	6.2534E-05	1.18920712	5.6751E-05	0.00036686	6.146E-05
6	0.00055269	0.00021927	0.00247218	0.01159784	0.21168633	0.00390625	0.06515411	0.00213729	0.00139309	0.00885066
7	0.52078146	3.95862663	0.7219646	2.37018554	1.54221083	0.00186062	10.4468783	0.0021299	0.06851198	0.00118574
8	0.30354872	0.0001983	0.00494436	1.32868581	0.04786119	0.00031881	2.54912125	0.00689612	0.11383373	0.0160087
9	0.01645877	0.01408204	0.12032431	1.81503831	0.0094859	3.6975E-06	0.24655818	0.00567958	0.01923663	0.02627801
10	0.55238651	4.11245531	1.9181E-05	0.00079322	0.03246435	0.09118877	2.32946717	1.6468E-05	0.07419676	0.00133866

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Table 8: EE2 NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.05478786	0.06100199	3.7702E-05	0.23488069	0.39093482	0.00096982	0.56252924	8.0536E-05	0.14968484	1.72309232
2	0.05691686	0.0842021	0.00058268	0.1372616	1.01747969	0.0013955	0.7631296	0.0004951	0.43226862	0.85263489
3	0.0842021	0.08747412	0.001011	0.09875516	1.21841026	0.00411469	0.44906619	0.00188659	0.57634317	1.01045145
4	0.06271698	0.07229301	0.00602426	0.07105106	0.95926412	0.00723897	0.66204446	0.00464534	0.10013373	1.0942937
5	0.08567393	0.09278272	5.2767E-05	0.04010706	0.6853914	0.00108356	0.32308821	0.00086501	0.16323247	1.58556827
6	0.01884075	0.04591152	0.00032103	0.01623217	0.40472111	0.00061591	0.30672124	0.00062234	0.05974658	0.44751254
7	0.01447794	0.05094202	6.0824E-05	0.0644802	0.57834409	0.00072487	0.49827013	0.00039251	0.1921094	0.3815648
8	0.00066011	0.02758447	0.00326206	0.04954901	0.48632747	0.00127968	0.43527528	0.00039251	0.15712667	0.60081802
9	0.00608722	0.03018551	1.3933E-07	0.02816408	0.57434918	0.00030902	0.40472111	0.00053618	0.26061644	0.10223776
10	0.04886685	0.07154526	8.0258E-05	0.0476956	0.46329403	0.00074524	0.49827013	0.00029746	0.06016215	0.78730798

Table 9: MALATHION NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.00229069	0.0066152	0.00837323	0.08717148	0.06185354	0.00065555	0.00154305	0.06768594	0.84089642	0.0003513
2	0.1609852	0.07856334	0.03071314	0.40053494	0.01551707	0.00708999	0.00789415	0.05251842	0.54912125	0.01745761
3	0.01727704	0.00958504	0.00316188	0.0336092	0.03246435	0.0010075	0.00050726	0.0028895	0.57434918	0.001011
4	0.00338882	0.00932294	0.00269593	0.00051791	0.06492869	3.4334E-05	0.00033934	0.0003708	0.02658806	0.00050201
5	0.06223554	0.07458348	0.04152143	0.00157002	0.54148752	0.00383914	0.00268661	0.00618464	0.48313122	0.00575886
6	0.04938489	0.10919661	0.04869779	0.05711447	0.40472111	0.00039251	0.00394708	0.00798828	0.36358566	0.0016538
7	0.01877556	0.00645664	0.00154841	0.00195313	0.00292976	0.00028535	0.00021402	0.01020203	0.96928982	8.3376E-05
8	0.02512034	0.00706546	0.00249802	0.0132304	0.05872017	1.5852E-05	0.00011469	0.08931213	0.3426957	1.4336E-05
9	0.00741674	0.00863852	0.00604518	0.06652314	0.0984135	0.00224355	0.0017481	0.00407792	0.04450157	0.00017504
10	0.00885066	0.00704102	0.00298098	0.00131566	0.03768149	0.00016733	6.3627E-05	0.03060688	7.998E-05	8.8981E-07

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Table 10: PFOS NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.00016967	0.05811281	0.0476956	0.06886907	0.09807301	9.3155E-05	1.0388591	4.1544E-05	0.22067575	0.00140521
2	0.00186062	0.02083279	0.44906619	0.23733553	0.79553648	3.8949E-06	0.02101213	1.8656E-05	0.02090512	0.08362047
3	0.00107982	0.05006687	0.11187813	0.35724853	0.29422668	0.00053804	6.8194E-05	0.15932008	0.03983002	0.01295812
4	0.0402463	0.04819409	0.02033347	0.04655242	0.27262693	3.2146E-05	0.62416527	1.6931E-05	0.2911834	4.4835E-05
5	0.02664484	0.06560729	0.03280365	0.05422117	0.28817159	0.00016163	0.01066983	0.00026995	0.24316374	0.00126206
6	0.00744248	0.0113986	0.00822938	0.0221738	0.17075503	2.7791E-05	0.23569613	9.4456E-05	0.06142629	0.00018248
7	6.197E-06	1.573E-07	0.00872881	0.00843147	0.05974658	0.00035497	0.16436785	0.00177724	0.01623217	0.00210057
8	0.00047989	0.01770131	0.01645877	0.00594132	0.20661258	0.00018826	0.3828895	0.00010054	0.03257706	0.00037914
9	7.7091E-06	0.03690602	0.01016673	0.01393638	0.29730178	0.00032551	0.50347778	0.00033006	0.02319568	0.00207885
10	0.00019557	0.64841978	0.20447551	0.51050606	2.96904714	0.00225916	0.20536742	0.0021822	0.8122524	0.00181603

Table 11: PHTHALATE NORMALISED MEAN CT VALUES USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
1	0.00032551	0.91768119	0.01838917	0.00054744	4.5522E-06	3.6975E-06	0.00028436	7.6559E-06	0.00186062	4.0840485
2	0.08620968	0.01727414	0.23733553	0.3597334	0.53961412	0.00027947	1.41912336	4.2417E-05	1.77153504	0.34412938
3	0.13713461	0.74697511	0.59666787	0.91066983	1.22264028	0.00044621	0.21913894	0.00464534	0.41161566	0.26359465
4	0.09807301	0.65520146	0.09909802	0.07510225	0.08717148	7.1584E-05	0.24064861	0.00147507	0.25266444	0.08087574
5	0.00012081	0.85705573	7.2429E-06	0.00021254	0.00022156	0.00014822	0.00044931	1.4436E-05	0.01509276	0.07113814
6	3.1376E-05	0.34477605	2.7125E-05	0.0007609	7.2835E-05	0.00093031	0.00037521	5.5008E-05	0.00153239	0.75434961
7	0.00808801	0.28563132	0.00315094	0.02768023	0.0160087	0.00028634	0.07641502	3.8412E-06	0.13678671	0.01383145
8	6.4515E-05	0.76676181	0.00073498	0.00101803	0.17800627	0.00396078	2.07E-05	6.1887E-05	3.1594E-05	0.09692763
9	0.01238725	0.19668692	9.0608E-05	0.15604132	0.02835997	0.00248938	0.31425334	5.4701E-06	0.24485507	0.90626815
10	0.00389274	0.77646888	5.4818E-05	0.04298568	0.00731463	1.8982E-05	0.0336092	8.0365E-06	0.02202064	0.06154616

ANNEX 3 FOR CHAPTER 3 CONT….

Table 12: MEAN CONTROLS CT VALUES NORMALISED USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
AROCLOR	0.00621513	0.00148791	0.03103414	0.08869521	0.34627737	0.00272411	0.24232245	0.00026902	1.10672207	0.00137868
	0.00821957	0.00705323	0.04512279	0.20236055	0.79005014	1.1564E-05	0.5691682	0.00250669	0.94493082	0.00058714
	0.00607091	0.00056284	0.03060688	0.10621062	0.43982454	0.00741674	0.00878952	0.00094986	0.00167455	0.00332484
	0.02845843	0.00355115	0.02112361	0.08161624	0.3842188	0.01703918	0.05403358	0.0024551	0.04822591	0.02664484
	0.03914576	0.0002949	0.03455408	0.0896222	0.00555561	0.01437793	0.00729216	0.00105759	0.07618857	0.0035788
	0.06515411	0.00048156	0.00531763	0.04404127	0.61985385	0.01147788	0.00797266	0.00091117	0.51365692	0.00507685
	0.00237147	0.00440238	0.01422922	0.05751173	0.01264753	0.01243026	0.00503831	0.00666121	0.1483754	0.0002746
	0.03081977	0.00147507	0.01634508	0.0204749	0.13537188	0.0016538	0.00278414	0.00361948	0.18735822	0.00158918
	0.04123462	0.00042803	0.07355667	0.07458348	0.56058304	0.00106126	0.15668818	0.00701666	0.06846117	0.00755947
	0.00511205	0.00022779	0.05953987	0.15020451	0.08008262	0.00019089	0.05236293	0.00014218	0.00626815	0.01350839
BAP	0.00717459	0.0094076	0.05974658	0.10366494	0.04607091	6.4515E-05	0.15712667	0.00061805	0.01393638	0.13167013
	0.00023059	0.0284356	0.02845843	0.758091	0.00422668	0.01917008	0.00017798	0.05129635	0.0005729	0.05525303
	0.00032747	0.00099101	0.16898885	0.00466038	0.02533546	0.10264962	0.00710678	0.00051612	0.05701337	2.00479443
	0.00029815	0.00140932	0.23114441	0.00031735	0.00048938	0.01651591	0.02987698	0.04195539	0.00658806	1.10322097
	0.00457765	0.00742929	0.00098584	0.13288389	0.00077308	0.10178483	0.00576752	0.00192624	0.00089642	1.00465607
	0.34989647	0.00028839	0.02555944	0.00254639	0.29320874	0.00046034	0.00530989	0.00233883	2.1341E-05	1.00313901
	0.64841978	0.01189064	0.00337247	0.00626475	0.43226862	0.10257716	0.01888443	0.01413093	0.00071081	1.00883609
	1.03495938	0.00083126	0.00919458	0.00625921	0.00236679	0.00526263	0.09050773	0.0084023	0.20580783	4.2863E-05
	0.45218969	0.00240763	0.18101547	0.0276629	0.00664176	0.00321715	0.03495938	0.00442533	0.00873935	0.11064083
	0.64718203	0.00948733	0.75262337	0.01328352	0.01395948	0.01634508	0.00063471	0.03874087	0.0019079	1.00825721

MEAN CONTROLS CT VALUES NORMALISED USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
DAZOMET	0.02555944	0.21538654	0.05440941	0.01745761	0.00010372	0.00026348	0.00311635	7.8879E-05	0.07634317	0.00213729
	0.03326157	0.30672124	0.0997873	0.05851702	0.0672184	0.00029033	0.00344562	3.8949E-06	0.00533492	0.00235509
	0.06652314	0.18946457	0.07484242	0.03157661	0.05059014	0.00020816	0.0028895	0.00021476	0.38024469	0.00106126
	0.07910979	0.27739237	0.07330218	0.11825721	0.03847326	0.00184777	0.00627319	0.00186708	0.03218509	0.01727704
	0.0672184	0.3077861	0.10957572	0.04937758	0.04736614	0.00015398	0.00100291	0.00033235	0.00502066	0.00071241
	0.00153155	0.25086794	0.12116123	0.09024557	0.02915728	0.00017687	0.00747955	0.00055701	0.24655818	0.00119398

	0.00215216	0.29627319	0.04419417	0.04527944	0.04639136	7.8334E-05	0.00167013	0.00016675	0.11226655	0.00017323
	0.00010706	0.24064861	0.07056028	0.00111888	0.04052624	0.00010372	0.00425451	0.00038045	0.07775073	5.4251E-05
	0.00900314	0.17194273	0.03955489	0.04343494	0.03071314	6.1247E-05	0.2030631	0.00033583	0.11703403	0.00082404
	0.03179624	0.23651441	0.05497807	0.03008108	0.0288557	2.9415E-06	0.23325825	0.00010813	0.00741416	0.00075827
CADMIUM	0.02090512	0.18685616	0.03729174	0.94278454	0.00073244	0.00157547	0.27932178	0.00011629	6.06E-07	0.00214471
	0.01628853	0.27357343	0.02711058	0.55286533	0.17313868	0.00149566	0.18428365	0.00188659	0.00587987	0.00628009
	0.02758447	0.34989647	0.0686308	1.65290064	0.16898885	0.00396078	0.2381595	0.00071241	0.00585953	0.00022922
	0.01551707	0.12413656	0.01745761	0.7219646	0.04836141	0.00255936	0.13121459	0.0001656	0.00560139	0.00323953
	0.01984611	0.23488069	0.01295812	1.04971668	0.03874087	0.00037392	0.18620968	0.00028436	0.01151773	0.0030861
	0.05422117	0.43527528	0.04937758	0.4966611	0.2030631	0.00379943	0.61344249	5.3504E-05	0.01595331	0.00027658
	0.01238725	0.11187813	0.00869861	0.38024469	0.0877778	0.00206449	0.00825721	0.00017026	0.00480916	0.00090801
	0.04166558	0.37892914	0.08391078	0.4794154	0.14063231	0.00929068	0.00074376	0.00070995	0.00013358	0.00467765
	0.02826186	0.24232245	0.02360113	0.90751916	0.26061644	0.0160087	0.00252924	0.00264962	0.00418662	0.018136
	0.05129635	0.2911834	0.02787277	0.02279719	0.24316374	0.00492726	0.00557221	0.00087102	0.00246363	0.00373416

MEAN CONTROLS CT VALUES NORMALISED USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
DDT	0.00627737	8.3376E-05	0.08105247	0.00749425	0.27547628	0.00047329	0.00221083	1.0279E-05	0.52057866	5.57E-11
	0.00465977	0.04358574	0.22375627	0.02826186	0.70956168	0.0006624	0.12747857	9.3929E-06	0.28535867	2.4788E-05
	0.00976137	0.01221671	0.45850202	0.01519773	0.1974787	0.00122755	0.00803164	2.0064E-05	0.09453579	8.813E-05
	8.494E-05	0.05366034	0.25792079	0.26609255	0.56058304	0.00359448	0.0039625	0.00037521	0.04263102	0.00037704
	0.00524675	0.53218509	1.8403753	0.33332467	0.00705849	0.01776277	0.11167584	0.00048828	0.3303828	0.00539186
	0.00218969	0.29235283	1.10573065	0.71449707	0.04915531	0.00721392	0.25897303	0.00012993	0.40151109	0.00196671
	0.00615084	0.2397077	1.74110113	0.32987698	0.05377658	0.00721392	0.44812372	0.00010336	0.41133848	0.00081552
	0.00968522	0.37526318	1.18099266	1.01747969	0.00433455	0.00469389	0.11221399	0.00047989	0.00228433	0.08161624
	0.0009175	0.07549439	1.01045145	0.51227841	0.09824251	0.00204313	0.6807506	9.9152E-05	0.00868529	0.00723897
	0.01282409	0.77113814	0.57461595	0.55671081	0.70762695	0.00071737	0.38024469	7.7256E-05	0.06676181	0.00428943
DIELDRIN	0.00353182	0.01977745	0.00412898	0.04094979	0.00015996	2.3401E-06	0.00308821	0.00112178	0.00925854	0.00433426
	0.0034841	0.00433426	0.00925854	0.02141848	0.05711447	0.00027851	0.00595542	0.00135734	0.00280069	0.00180974
	0.00045637	0.00043854	0.00849012	0.02127053	0.11383373	0.00148019	0.0110869	0.00056089	0.00047493	0.00051434
	0.00044776	0.00036495	0.00302259	0.06293472	0.00256825	3.5177E-05	0.00652412	0.00052697	0.00054934	0.0008269
	0.007725	0.00968522	0.00358205	0.21839322	0.06886907	0.00017626	0.00509277	0.0001412	0.00616151	0.00391981
	0.03931572	0.11703403	0.28917205	0.27898558	0.00546713	0.00051256	0.00677954	0.00016107	0.01320749	0.00149049
	0.00432037	0.11703403	0.00457765	0.00073996	0.0280535	0.01388817	0.00889258	0.74483873	0.04159959	0.2030631

0.00010992	3.7312E-05	0.11907975	0.05593907	0.00084427	9.9975E-06	0.07031616	0.00062234	0.00032382	0.00281042
2.4257E-05	3.21E-07	0.01070924	0.03337704	0.00094657	1.2351E-05	0.00101803	0.00067867	0.00011059	0.00229865
0.0045144	0.00239626	0.11462551	0.01557221	0.39229205	0.05574553	0.02409704	0.004534	0.00850484	0.03018551

MEAN CONTROLS CT VALUES NORMALISED USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
EE2	0.02171747	0.04559439	0.00010964	0.06492869	0.90125046	0.00023746	0.00345678	7.9153E-05	0.41421356	0.00268876
	0.03146736	0.04954901	0.00298098	0.05593907	0.25702846	0.00312918	0.85856544	0.00278135	0.00044821	0.00039389
	0.05273849	0.0476956	0.00061805	0.05516894	0.00204285	0.00080429	0.46009383	0.00079322	0.48801588	0.00068031
	0.06359248	0.06100199	0.00441002	0.06359248	0.01557221	0.00143473	0.76048938	0.00146488	0.0053914	0.00081326
	0.10547697	0.06910817	0.00012594	0.0953912	0.00664176	0.00123609	0.65067093	0.00086501	1.00037018	0.0004926
	0.04283696	0.04465607	0.00489322	0.04328467	0.07834409	0.0017062	0.62633222	0.00144471	0.00128625	0.00048903
	0.01030866	0.02720471	0.00022156	0.02171747	0.5	0.00033351	0.44596426	0.00067867	0.16323247	3.8961E-05
	0.00408627	0.04655242	0.00027467	0.04109196	0.34032853	0.00064206	0.46329403	0.0002644	0.82074161	0.00806715
	0.00184138	0.04195539	0.00025275	0.05516894	0.47467106	0.0013955	0.54336743	0.00013925	0.82074161	0.00031616
	0.00837323	0.09087328	0.00751173	0.14259546	0.08297505	0.00042507	0.37018554	0.00024755	0.0019646	2.7349E-05
MALATHION	0.00135849	0.06910817	0.00423037	0.04496667	0.08333117	0.00397453	5.3948E-06	5.0335E-06	0.00065706	1.0009885
	0.00655242	0.00496667	0.00988872	0.0591286	0.05831456	0.00215216	0.00898558	0.00016908	0.07922824	1.10037133
	0.00995401	0.05851702	0.01970902	0.03157661	0.05995401	0.00274306	0.00235283	9.3479E-05	0.48802331	0.00312918
	2.1666E-08	1.67E-09	0.02720471	0.01221671	0.0523742	0.00201501	0.00335377	1.3704E-05	1.11E-08	6.59E-11
	0.00024275	0.0523742	0.00176025	0.01668852	0.10806715	0.00172403	0.952638	2.3861E-05	0.005276	0.10019693
	0.06652314	0.08880907	0.02271832	0.1798667	0.31753775	0.00650155	0.0006699	0.00028933	0.08275556	1.00282017
	0.0523742	0.00473229	0.00754638	0.0187106	0.14259546	0.00322832	0.00186964	0.00010813	0.06675409	1.00181603
	0.04109196	0.00404537	0.01225913	0.08105247	0.21538654	0.00229865	0.00546741	0.00013038	0.01845301	9.3155E-05
	0.00985739	0.18492344	0.02149284	0.08508213	0.03507549	1.00095316	0.06058304	0.00047823	0.00062234	0.00029746
	0.01540989	0.01034444	0.00102157	0.01442785	0.03337704	1.3469E-05	0.00803214	2.6567E-05	0.00015186	0.00015451

MEAN CONTROLS CT VALUES NORMALISED USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
PFOS	1.1564E-05	0.63949279	0.43982454	0.765779	0.4127045	0.00070995	0.61160255	0.00015081	0.05111122	0.06359248
	0.00961412	0.60499704	0.49654625	0.35233019	0.00036848	0.00043401	0.40762046	0.0061722	0.82133967	0.00654677
	6.4964E-05	0.0402463	0.03235203	0.04010706	0.17016426	0.00011469	0.3842188	0.00062019	0.0238478	0.0009433
	0.01291329	0.15177436	0.04210105	0.14508799	0.34627737	0.00030371	0.20381023	0.00228277	0.00222244	0.00322832
	0.03794359	0.18364608	0.10474839	0.11622562	0.10621062	0.00034407	0.00841978	0.00111017	0.18492344	0.00894316
	0.00811281	0.36349313	0.41754396	0.17134785	0.4966611	0.00020038	0.06063471	0.00117347	0.00030798	0.00085903
	0.00224355	0.38031735	0.87964908	0.93303299	0.48967746	0.00343613	0.07438521	0.00680118	0.09824251	0.04989365

	0.00024499	0.02494682	0.03467405	0.00194637	0.10769327	0.00012948	0.20589775	5.8549E-05	0.01776277	0.00024669
	0.02105052	0.01395948	0.47963206	0.37241937	0.03901582	0.00095979	0.00508899	0.00108356	0.04343494	0.01567925
	0.00011629	0.00834409	0.32873569	0.25086794	0.02275482	0.00031552	0.23331664	0.00053432	0.15020451	0.00822938
PHTHALATES	0.05995401	0.36867952	0.00022156	0.03515808	0.00347204	9.0536E-07	0.13030822	7.8544E-07	0.2783554	0.08060393
	0.00265631	0.08904653	0.07536299	0.04458602	0.05219299	0.00034288	0.0238478	7.0205E-06	0.00465977	0.31589866
	0.04465607	0.21146131	0.01567925	0.06142629	0.0804926	7.1432E-06	0.13258597	0.00558201	0.00980494	0.00091482
	0.03050099	0.13613082	0.01143817	0.02720471	0.04298568	1.1326E-05	0.08021412	0.00579892	0.03077308	0.00877502
	0.01364958	0.06806541	0.01291329	0.00590028	0.01640182	0.00234695	0.05219299	0.00114535	0.07588718	0.00321647
	0.00033787	0.59271128	0.09310484	0.2783554	0.18492344	0.00325078	0.00792079	7.4366E-05	0.00580127	0.24991663
	0.02232803	0.00052824	0.02164234	0.05593907	0.02720471	0.00875911	0.08838835	0.0007742	0.07357343	0.37011797
	4.3761E-05	0.02791896	4.762E-06	0.00022622	0.00014218	1.1287E-05	0.00027947	1.6698E-05	0.0066152	0.25977104
	0.02503343	0.0094203	0.00028535	0.05478786	0.00789415	0.01059847	0.08333117	0.02295576	0.01950791	0.39240156
	0.00018375	0.04189034	0.00018502	0.02503343	0.0119239	0.00036243	0.04166558	5.6164E-05	0.10806715	0.74156561

MEAN CONTROLS CT VALUES NORMALISED USING RPS5 AND RPS7 REFERENCE GENES

TREATMENT	**AHR2**	**CYP1A**	**DIABLO 1**	**DIABLO 2**	**GST01LA**	**GSTA2L**	**GSTMA**	**GSTR2**	**MT**	**VTG**
PFOS	1.1564E-05	0.63949279	0.43982454	0.765779	0.4127045	0.00070995	0.61160255	0.00015081	0.05111122	0.06359248
	0.00961412	0.60499704	0.49654625	0.35233019	0.00036848	0.00043401	0.40762046	0.0061722	0.82133967	0.00654677
	6.4964E-05	0.0402463	0.03235203	0.04010706	0.17016426	0.00011469	0.3842188	0.00062019	0.0238478	0.0009433
	0.01291329	0.15177436	0.04210105	0.14508799	0.34627737	0.00030371	0.20381023	0.00228277	0.00222244	0.00322832
	0.03794359	0.18364608	0.10474839	0.11622562	0.10621062	0.00034407	0.00841978	0.00111017	0.18492344	0.00894316
	0.00811281	0.36349313	0.41754396	0.17134785	0.4966611	0.00020038	0.06063471	0.00117347	0.00030798	0.00085903
	0.00224355	0.38031735	0.87964908	0.93303299	0.48967746	0.00343613	0.07438521	0.00680118	0.09824251	0.04989365
	0.00024499	0.02494682	0.03467405	0.00194637	0.10769327	0.00012948	0.20589775	5.8549E-05	0.01776277	0.00024669
	0.02105052	0.01395948	0.47963206	0.37241937	0.03901582	0.00095979	0.00508899	0.00108356	0.04343494	0.01567925
	0.00011629	0.00834409	0.32873569	0.25086794	0.02275482	0.00031552	0.23331664	0.00053432	0.15020451	0.00822938
PHTHALATES	0.05995401	0.36867952	0.00022156	0.03515808	0.00347204	9.0536E-07	0.13030822	7.8544E-07	0.2783554	0.08060393
	0.00265631	0.08904653	0.07536299	0.04458602	0.05219299	0.00034288	0.0238478	7.0205E-06	0.00465977	0.31589866
	0.04465607	0.21146131	0.01567925	0.06142629	0.0804926	7.1432E-06	0.13258597	0.00558201	0.00980494	0.00091482
	0.03050099	0.13613082	0.01143817	0.02720471	0.04298568	1.1326E-05	0.08021412	0.00579892	0.03077308	0.00877502
	0.01364958	0.06806541	0.01291329	0.00590028	0.01640182	0.00234695	0.05219299	0.00114535	0.07588718	0.00321647

0.00033787	0.59271128	0.09310484	0.2783554	0.18492344	0.00325078	0.00792079	7.4366E-05	0.00580127	0.24991663
0.02232803	0.00052824	0.02164234	0.05593907	0.02720471	0.00875911	0.08838835	0.0007742	0.07357343	0.37011797
4.3761E-05	0.02791896	4.762E-06	0.00022622	0.00014218	1.1287E-05	0.00027947	1.6698E-05	0.0066152	0.25977104
0.02503343	0.0094203	0.00028535	0.05478786	0.00789415	0.01059847	0.08333117	0.02295576	0.01950791	0.39240156
0.00018375	0.04189034	0.00018502	0.02503343	0.0119239	0.00036243	0.04166558	5.6164E-05	0.10806715	0.74156561

Annex 4: supporting information for Chapter 4

Table 1: Hepatic gene expression between the Daberam and Dan Agundi sites compared to the Jibia site. Bold denotes a significant difference.

Genes	Sites	Fold difference	Tukey Test P ≤ 0.05
AHR2	Daberam	1.973	**0.016**
	Dan Agundi	0.723	**0.006**
CYP 1A	Daberam	11.089	**0.000**
	Dan Agundi	1.386	**0.000**
DIABLO 1	Daberam	7.249	**0.003**
	Dan Agundi	2.234	**0.002**
DIABLO 2	Daberam	2.789	**0.000**
	Dan Agundi	0.256	**0.000**
GST01LA	Daberam	2.348	**0.000**
	Dan Agundi	1.192	**0.000**
GSTA2L	Daberam	5.262	**0.000**
	Dan Agundi	1.668	0.199
GSTMA	Daberam	8.436	**0.025**
	Dan Agundi	1.292	**0.001**
GSTR2	Daberam	17.744	**0.000**
	Dan Agundi	1.542	0.426
MT	Daberam	2.198	**0.000**
	Dan Agundi	3.869	**0.000**
VTG	Daberam	2.773	**0.000**
	Dan Agundi	11.379	**0.000**

Annex 4: supporting information for Chapter 4

Table 2: Comparative expression of genes between the Daberam and Dan Agundi sites.

Genes	Tukey Test P ≤ 0.05
AHR 2	
Daberam-Dan Agundi	0.068
CYP 1A	
Daberam-Dan Agundi	0.057
DIABLO 1	
Daberam-Dan Agundi	0.087
DIABLO 2	
Daberam-Dan Agundi	0.072
GST01LA	
Daberam-Dan Agundi	0.054
GSTA2L	
Daberam-Dan Agundi	0.903
GSTMA	
Daberam-Dan Agundi	0.059
GSTR2	
Daberam-Dan Agundi	0.093
MT	
Daberam-Dan Agundi	0.519
VTG	
Daberam-Dan Agundi	0.074

Annex 4: supporting information for Chapter 4

Table 3: Daberam site Total length, weight, and sex of the wild Tilapia (Polluted).

No.	Length (cm)	Weight (g)	Sex
1	12.05	41	Unsex/Juvenile
2	17.3	90	M/Adult
3	17.6	95	M/Adult
4	17.9	98	M/Adult
5	12.6	42.3	unsex/Juvenile
6	12.5	46	unsex/Juvenile
7	17.4	89	M/Adult
8	12.5	41	unsex/Juvenile
9	18.8	103	M/Adult
10	19.0	106	M/Adult
11	17.9	92	M/Adult
12	20.6	110	M/Adult
13	19.8	104	M/Adult
14	22	125	M/Adult
15	15.9	75	M/Adult
16	14.6	69	M/Adult

Table 4: Dan agundi site Total length, weight, and sex of the wild Tilapia (Polluted).

No.	Length (cm)	Weight (g)	Sex
1	21.5	120	M/Adult
2	17.8	90	M/Adult
3	19.4	102	M/Adult
4	13.45	55	Unsex/Juvenile
5	14.43	63	M/Adult
6	15.33	76	M/Adult
7	18.67	100	M/Adult
8	16.95	85	M/Adult
9	20	105	M/Adult
10	17.41	95	M/Adult
11	16.04	79	M/Adult
12	13.96	53	Unsex/Juvenile
13	16.43	80	M/Adult
14	12.42	49	unsex/Juvenile
15	20.89	107	M/Adult
16	12.2	40	unsex/Juvenile

Table 5: Shows Jibia site Total length, weight, and sex of the wild Tilapia (Reference).

No.	Length (cm)	Weight (g)	Sex
1	14.5	69	M/Adult
2	15.6	75	M/Adult
3	13	52	Unsex/Juvenile
4	12.5	50	Unsex/Juvenile
5	14.3	55	M/Adult
6	14.8	60	M/Adult
7	15.1	73	M/Adult
8	15.6	78	M/Adult
9	14.9	70	M/Adult
10	14.81	69.5	M/Adult
11	19.54	100	M/Adult
12	14.51	77	M/Adult
13	22	130	M/Adult
14	12.45	52	unsex/Juvenile
15	12.39	48	unsex/Juvenile
16	13.95	55	unsex/Juvenile

Table 6: Condition factors of Daberam, Dan Agundi and Jibia sites.

No.	Daberam Condition Factor	Dan Agundi Condition Factor	Jibia Condition Factor
1	2.34	1.21	2.26
2	1.74	1.60	1.98
3	1.74	1.40	2.36
4	1.71	2.26	2.56
5	2.11	2.10	1.88
6	2.36	2.11	1.85
7	1.69	1.54	2.12
8	2.10	1.74	2.05
9	1.55	1.31	2.12
10	1.55	1.80	2.14
11	1.60	1.91	1.34
12	1.26	1.95	2.52
13	1.34	1.80	1.22
14	1.17	2.56	2.69
15	1.87	1.17	2.52
16	2.22	2.20	2.02

Table 7.: Daberam Normalised ct values of Daberam site using RPS 5 and RPS 7 reference genes

DABERAM	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
T1	2.58966158	0.00400985	27.0538794	0.57744825	0.1958422	0.00631397	2.6076741	0.12787123	0.00368981	0.41773511
T2	2.02003221	0.00159678	1.38584646	0.69465969	0.3065891	0.0018989	1.8395824	0.10341794	0.00232167	0.92209607
T3	2.11439672	0.00056685	1.6335084	0.67140077	1.64176324	0.00196705	2.07807265	0.04836966	0.00349679	0.93118517
T4	0.61789613	0.01670696	10.1540862	12.8758273	0.03298757	0.03733295	26.3840334	0.6014819	0.03973608	0.37247824
T5	1.19486943	0.00117092	2.82795499	0.56325221	0.88690592	0.00168487	1.02587359	0.00979902	0.00087214	0.46596735
T6	1.22630162	0.00050701	11.7477606	0.81751316	0.67563401	0.00422836	1.41844859	0.00296524	0.00374534	0.73425238
T7	1.29101976	0.00063918	7.82728018	0.55421247	1.09696013	0.00097556	1.00243666	0.02963634	0.00110519	0.59494988
T8	4.20964583	0.00411098	1.63801739	1.42276645	3.04976665	0.00627442	4.38841164	0.04416728	0.02169789	0.26841029
T9	1.44262248	0.00067337	15.6565432	0.45808709	0.64113222	0.00185894	1.75162483	0.00589494	0.00136555	1.68027085
T10	2.19754797	0.00358427	0.31802583	0.71246678	1.49577654	0.00174313	2.68681066	0.00288622	0.0155806	2.42989615
T11	4.65403119	0.00597635	0.46996181	0.62567306	2.11914291	0.00216485	1.8641021	0.00192136	0.01096068	3.3951631
T12	1.30361987	0.0012297	1.90292124	0.15469766	1.05155423	0.00057567	0.91860927	0.00161699	0.00360078	1.89542746
T13	1.93547768	0.00070636	2.38788777	0.30836046	1.00186607	0.00203987	1.58852706	0.00711554	0.00352708	0.87127453
T14	2.88350062	0.00136	1.19716452	0.55394757	1.71453972	0.00465443	3.04791217	0.02252735	0.00947158	0.74261728
T15	1.79741231	0.00049199	1.15933088	0.34172824	1.62554263	0.00213866	1.98745389	0.00920046	0.00283177	3.60730496
T16	1.56051648	0.00785196	1.01508428	0.63290168	1.88420524	0.01922227	12.9059948	0.05830531	0.50370426	0.86742211

Table 8: Dan agundi Normalised ct values of Dan agundi site using RPS 5 and RPS 7 reference genes

DAN AGUNDI	AHR2	CYP1A	DIABLO 1	DIABLO 2	GST01LA	GSTA2L	GSTMA	GSTR2	MT	VTG
T1	0.00096982	1.3628E-06	0.03257706	0.00088625	0.51405691	3.0493E-07	0.03303181	0.00154305	0.00026995	0.28519093
T2	0.00026532	2.6181E-07	0.01286861	0.00070749	0.2745232	1.3458E-07	0.06583506	0.0171577	0.00011791	0.16042824
T3	0.00075827	5.5732E-07	0.00414331	0.00151654	0.28817159	3.5149E-07	0.01864587	0.00210422	0.00081269	0.26517194
T4	0.08362047	7.998E-05	1.13681697	0.35848881	0.05111888	0.02758447	2.3375545	0.03564887	0.02529507	0.03246435
T5	0.41899357	0.00021625	3.27160823	0.00187356	0.27074376	0.00026348	0.64171295	0.00022427	1.00168855	0.12370708

T6	0.31643915	0.00030902	3.87715927	0.00057666	0.27643266	2.4597E-07	0.52485834	0.0001039	0.00205023	0.14968484
T7	0.1609852	0.00018891	1.33329868	0.00374713	0.21315872	8.8555E-06	0.21763764	0.00180974	0.0056993	0.15876887
T8	2.65737163	0.00126206	0.02194445	0.00383914	2.10672207	0.00069534	1.59107297	0.00341239	0.03349292	1.45902034
T9	0.24232245	0.00027851	2.19618563	0.00137153	0.25971478	1.4139E-05	0.29422668	0.00093354	0.0056993	0.11542279
T10	0.89192852	0.00256825	7.16020057	0.00268661	1.10956947	0.00019355	1.06437018	0.00055701	0.01884075	0.48296816
T11	0.3609823	0.00040776	3.06311599	0.00097318	0.38958229	0.00049168	0.42779751	0.00088013	0.0044871	0.18364608
T12	0.14358729	0.00011155	1.40932076	0.00149566	0.1995746	1.7896E-05	0.14458602	0.00014822	0.00117347	0.10083022
T13	0.1767767	0.0001255	1.23114441	0.0008269	0.13821633	0.0001255	0.24913507	0.00211519	0.0017481	0.11744034
T14	0.41754396	0.00019968	3.50642289	0.00191293	0.3426957	0.00019968	0.36729216	0.009068	0.00441002	0.26242917
T15	2.52275482	0.00051256	0.02460337	0.02156746	1.98618499	6.0195E-05	1.34723358	0.01188265	0.01776277	0.69255473
T16	2.64817782	0.00018826	0.02112361	0.00546713	1.26137741	5.5583E-05	1.20163605	0.0044871	0.02019301	0.55478474