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Molecular and biochemical effect-directed analysis of polluted sediment from the Lagos lagoon (Nigeria) using Atlantic salmon (*Salmo salar*) as a model

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Abstract

As a result of human interventions, such as agricultural, industrial, and mining activities and urbanization, there have been release of vast range of synthetic and natural environmental pollutants, such as persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs). The ultimate sink for these chemicals or their degradation products is the aquatic environment and eventually accumulated on the sediments, which may be taken up by benthic organisms, as a result of direct contact with polluted sediment or indirectly via food and water. Therefore, these have ultimately caused a constant threat for the aquatic organisms and significant loss of biodiversity in the aquatic ecosystem. It has been suggested that human activities in and around Lagos lagoon are the major sources of PAHs, which may lead to a huge reduction in fishery resources and threatening their sustainability. Previous studies revealed that water samples from the lagoon contains a wide range of organochlorine pesticides (OCPs) and phthalic esters and the sediment samples contain higher concentration of polychlorinated biphenyls (PCBs) and heavy metals. Moreover, different studies showed that fish species inhabiting the Lagos lagoon system suffer from severe intersex, developmental and reproductive effects and fish exposed to sediment extracts from the Lagos lagoon showed teratogenic, embryotoxic and genotoxic effects. Thus, more studies to establish the state of pollution in relation to detrimental effects to the health of the ecosystem and humans in the Lagos lagoon is needed and recommended. Therefore, the aim of the present study was to further investigate and document whether the overt physiological and health effects observed at the Lagos lagoon were due to accumulated contaminants in sediment samples, using the Atlantic salmon, *Salmo salar* as a model. The sediment samples were collected from Makoko and Ikorodu sites of the Lagos lagoon and both polar and non-polar pollutants were extracted from each sediment samples using an ultrasonic extraction procedure. In the present study, juvenile Atlantic salmon were exposed to polar and non-polar sediment extracts, 17α-ethynylestradiol (EE2: 2.5 µg/ml), and the carrier solvent dimethyl sulfoxide (DMSO: 0.016 %). As phase I enzyme, hepatic CYP1A1 catalytic responses were performed by ethoxyresorufin *O*-deethylase (EROD), methoxyresorufin *O*-demethylase (MROD), and benzyloxyresorufin *O*-debenzylase (BROD). The antioxidant enzymes, such as glutathione reductase (GR), glutathione S-Transferase (GST), glutathione peroxidase (GPx), and catalase (CAT) were also investigated. Gene expression levels of CYP1A1 and CYP3A genes in relation to their xenobiotic biotransformation activity and vitellogenin (Vtg) and *zona radiata* protein (*Zrp*) in relation to their estrogenic activities were also analyzed with

Real-time RT-PCR. Makoko polar and Ikorodu non-polar exposure produced higher EROD enzymatic activities whereas EE2 exposure produced a significant reduction of EROD. Regarding oxidative stress, generally, all the exposure groups exhibited reduced GR activities and increased GST catalytic activities. The gene expression patterns of hepatic CYP1A1 and CYP3A mRNA didn't show any significant difference from the control group. Salmon exposure to EE2 in this study produced significantly higher levels of Vtg, *Zrp*, and ERα and reduction in the expression of CYP1A1 mRNA whereas Makoko polar extract and Ikorodu non-polar extract created insignificant estrogenic responses. Thus, the higher EROD catalytic activities exhibited by both Makoko polar extract and Ikorodu non-polar extract may be a response to AhR ligand exposure and an indication of toxic planar compound uptake in fish. Generally, the higher GST catalytic activities in both the Makoko polar and the Ikorodu non-polar exposure groups may be a reflection of the amount of analyzed PAHs at the two sediment sampling sites. Regarding oxidative stress, the reduction of the antioxidant enzymatic activities in this study may be an indication of deficiency of the system as a result of high levels of pollutant exposure. This may indicate that GST might function more as a detoxification activity than as an antioxidant defense activity. In this study, all the exposure groups showed non-significant small increase and decrease of Vtg, *Zrp*, and ERα. Therefore, the unaffected estrogenic responses may indicate that the estrogenic compounds in the sampled sediments may not be found at the levels that produce estrogenic effects or other contaminants in the sediments may antagonize and reduce the estrogenic effects. Therefore, the exposure of salmon to sediment extracts from Makoko and Ikorodu sites of the Lagos lagoon system showed biotransformation of xenobiotics, failure in antioxidant defense system, and unaffected estrogenic responses, which may be an indicative of potential and adverse health effects that need further studies.

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Abbreviations

AhR	Aryl hydrocarbon receptor
AhRR	AhR repressor
ALDH	Aldehyde dehydrogenase
Arnt	AhR nuclear translocator
BaP	Benzo[α]pyrene
BHLH–PAS	Basic helix–loop–helix–Per–Arnt–Sim
BROD	Benzyloxyresorufin *O*-debenzylase
BSA	Bovine serum albumin
CAT	Catalase
cDNA	Complimentary DNA
CDNB	1-chloro-2,4-dinitro- benzene
Ct	Threshold cycle
CYP450	Cytochrome P450
CYP1A1	Cytochrome P4501A1
CYP3A	Cytochrome P4503A
DCM	Dichloromethane
DDT	Dichlorodiphenyltrichloroethane
DEPC	Diethyl pyrocarbonate
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DRE	Dioxin responsive element
EDC	Endocrine-disrupting chemicals
EDTA	Ethylenediaminetetraacetic acid
E2	Estradiol-17β
EE2	17α-Ethynylestradiol
ELISA	Enzyme-linked immunosorbent assay
EPA	Environmental Protection Agency
ER	Estrogen-receptor
ERα	Estrogen receptor alpha
ERE	Estrogen responsive element
EROD	7-ethoxyresorufin *O*-deethylase
FAD	Flavin adenine dinucleotide
FMN	Flavin mononucleotide
FSH	Follicle-stimulating hormone

g	Gram
GnRH	Gonadotropin-releasing hormone
G6PDH	Glucose-6-phosphate dehydrogenase
GPx	Glutathione peroxidase
GR	Glutathione reductase
GSH	Tripeptide reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione *S*-transferases
HMW	High molecular weight
HPG	Hypothalamic-pituitary-gonadal
HPLC	High-performance liquid chromatography
HSP90	90 kDa heat shock protein
KDa	Killo Dalton
LH	Luteinizing hormone
LMW	Low molecular weight
µl	Microliter
ml	Milliliter
MMLV	Moloney murine leukemia virus
mRNA	Messenger RNA
MROD	Methoxyresorufin *O*-demethylase
ND	Not detected
NR	Nuclear receptor
ng	Nanogram
nm	Nanometer
NTC	No template control
OCP	Organochlorine pesticides
PAH	Polycyclic aromatic hydrocarbon
PBDE	Polybrominated diphenyl ether
PCB	Polychlorinated biphenyl
PCR	Polymerase chain reaction
PMF	Post-mitochondrial fraction
POP	Persistent organic pollutant
PXR	Pregnane X receptor
qRT-PCR	Quantitative reverse transcription
RCEP	Royal Commission on Environmental Pollution

RER	Rough endoplasmic reticulum
RH	Substrate
RNases	Ribonucleases
ROS	Reactive oxygen species
RT-PCR	Reverse transcription- PCR
RXR	Retinoic acid receptor
SEM	Standard error of the mean
SOD	Superoxide dismutase
TBT	Tributyltin
UDPGA	Uridine diphosphate glucuronic acid
UDPGT	Uridine diphosphate glucuronosyltransferase
Vtg	Vitellogenin
XRE	Xenobiotic responsive element
Zrp	*Zona radiata* protein

1 INTRODUCTION

1.1 Pollutants in the aquatic environment

Chemicals are generally accredited for their positive contribution to human health, living standards and life expectancy for most people throughout the world. Over the years, however, the widespread use of these chemicals has been increasing the concern about their risk to human health and their damaging effects on our ecosystems (RCEP, 2003). Nowadays, environmental pollutants, such as oil spills, toxic metals, radioactive compounds, polychlorinated biphenyls (PCBs), and others take up a central role in social, economic and political discussions and will continue to do so in the near future (Plant, Voulvoulis, and Ragnarsdottir, 2012). This growing public concern about synthetic chemicals in the environment and their unintended effects have been ignited by Rachel Carson's landmark book "*Silent Spring*" in 1962 by pointing out the detrimental effects of pesticides which causes reproductive problems (eggshell thinning by dichlorodiphenyltrichloroethane (DDT)) and death in birds (Glausiusz, 2007). The damaging effects, which have been observed in both human and wildlife, have since been confirmed and extended to include a wide range of other health effects, from carcinogenicity, mutagenicity and reproduction toxicity to endocrine disruption, genotoxicity, neurotoxicity, and immunotoxicity (Plant *et al*., 2012).

The production and emissions of environmental pollutants to the environment are mainly due to human interventions, settlements and resource exploitation such as agricultural activities, industrial development, mining activities and urbanization (Islam and Tanaka, 2004). These anthropogenic activities lead to the release of vast range of synthetic and natural environmental pollutants, from plasticizers to pharmaceuticals and substances of agricultural and industrial usages such as persistent organic pollutants (POPs) and polycyclic aromatic hydrocarbons (PAHs) (Mansour, 2009; Plant *et al*., 2012). Furthermore, these chemicals include chlorinated and brominated aromatics (e.g., PCBs, polychlorinated dibenzo-p-dioxins and-furans (PCDD/Fs), and polybrominated diphenyl ethers (PBDEs)) and different organochlorine pesticides (OCPs) (Mansour, 2009). Although some of them are unintended combustion products or accidental byproducts of industrial chemical processes (e.g., PCDD/Fs), most of them are, however, produced deliberately for industrial uses (e.g., PCBs, PBDEs) or as agrochemicals (e.g., DDT, Lindane) (Chrysikou *et al*., 2008; Mansour, 2009). Many of these chemicals are highly toxic and persistent in the environment and biological systems (Islam and Tanaka, 2004).

The ultimate sink for most natural or man-made chemicals or their degradation products is the aquatic environment (Mills and Chichester, 2005). These chemicals enter the aquatic

systems through effluents, atmospheric deposition, runoff, and groundwater, which ultimately distributed in all parts of the water column and adsorbed on the sediment (Figure 1-1) (Erickson, Nichols, Cook, and Ankley, 2008). Eventually, large quantities of pollutants are accumulated on the sediments, which may be taken up by benthic organisms, as a result of direct contact with polluted sediment and water, and indirectly from food (Sumpter, 1998; Viganò, Arillo, Falugi, Melodia, and Polesello, 2001). Thus, as a result of the aquatic food chains and high lipophilic nature of these pollutants, the concentration of these chemicals has gradually increased through the trophic level and significantly biomagnified in top predators, including human. Therefore, these have ultimately caused a constant threat for the aquatic organisms and significant loss of biodiversity in the aquatic ecosystem (Islam and Tanaka, 2004; Sarkar, Ray, Shrivastava, and Sarker, 2006).

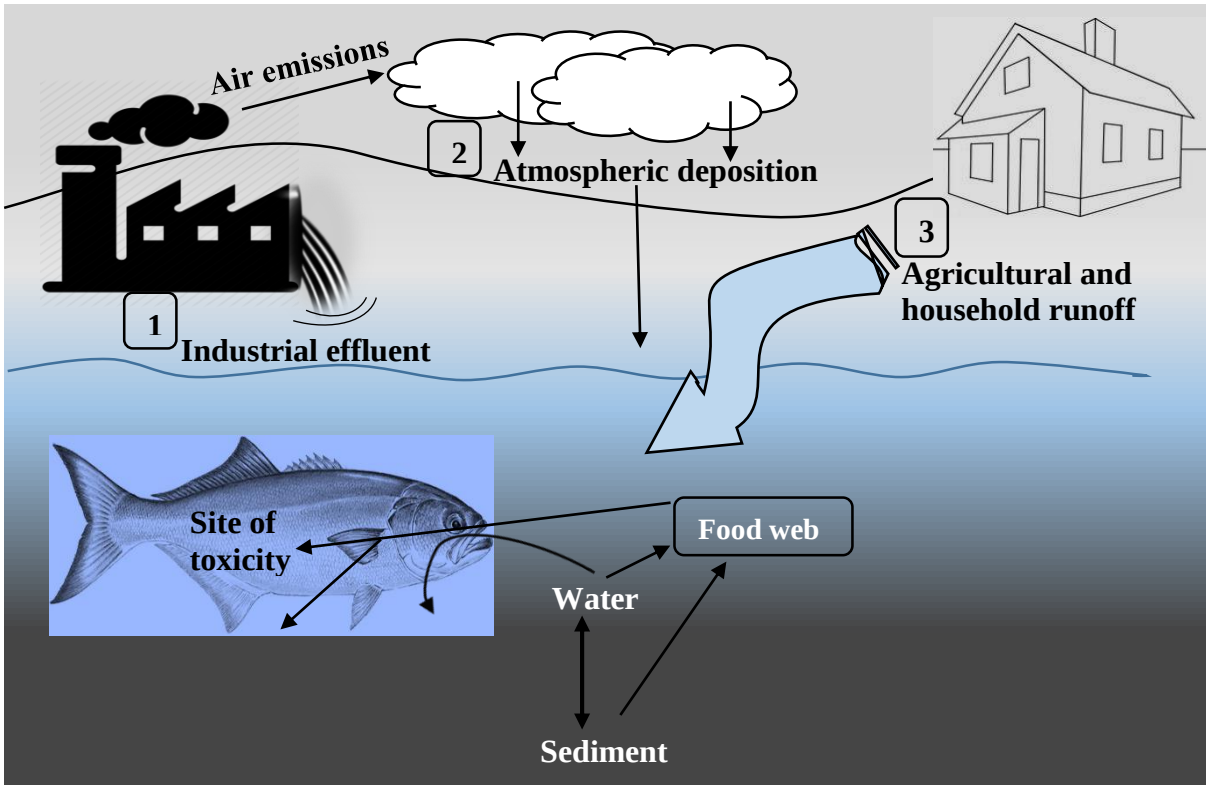

Figure 1-1: Pollutant entrance in to the aquatic systems from different sources and their accumulation in the aquatic organisms, including fish. The possible sources of chemicals may be from industrial effluents (1), atmospheric deposition (2), and surface runoff (3). The aquatic organisms may get these chemicals through ingestion of contaminated food and water; and through contaminated sediment contact. Figure is modified from Erickson, Nichols, Cook, and Ankley (2008).

1.2 Xenobiotic toxicokinetics in fish

It has been stated that "*toxic effects in a biological system is not produced by a chemical agent unless that agent or its metabolic breakdown (biotransformation) products reach appropriate sites in the body at a concentration and for a length of time sufficient to produce a toxic manifestation*" (Eaton and Gilbert, 2013, p. 20). Factors that influence toxicity of a chemical compound include the chemical, physical properties, and biotransformation fate of the compound, the amount of active form that reach the target site(s), and the vulnerability of the exposed organism (Eaton and Gilbert, 2013). Moreover, the toxicity of a compound is significantly determined by both the toxicokinetic (time dependent concentration changes of a compound in the organism) and toxicodynamic (interactions of a toxicant with its biological target molecules and the ultimate downstream biological effects) factors (Figure 1-2) (Boelsterli, 2007). Therefore, in addition to knowing the type of effect produced and the effective dose that a compound can produce, information about the chemical agent, exposure, and its disposition by the exposed organism are also needed to fully characterize the potential hazard of a particular compound (Eaton and Gilbert, 2013). The state of chemical exposure and routes by which chemicals get access into the body of aquatic organisms is different from terrestrial animals. In this regard, aquatic organisms face several challenges from their natural environment. For example, a fish has to breathe approximately 20 times more than its terrestrial counterpart for the same amount of oxygen and required to pass several liters of water through its gills to achieve sufficient oxygen (approximately 48 liters/hour for a 250 g trout). Therefore, the fish gill is exceptionally exposed to complex mixture and specific type of pollutants (Heath, 1995).

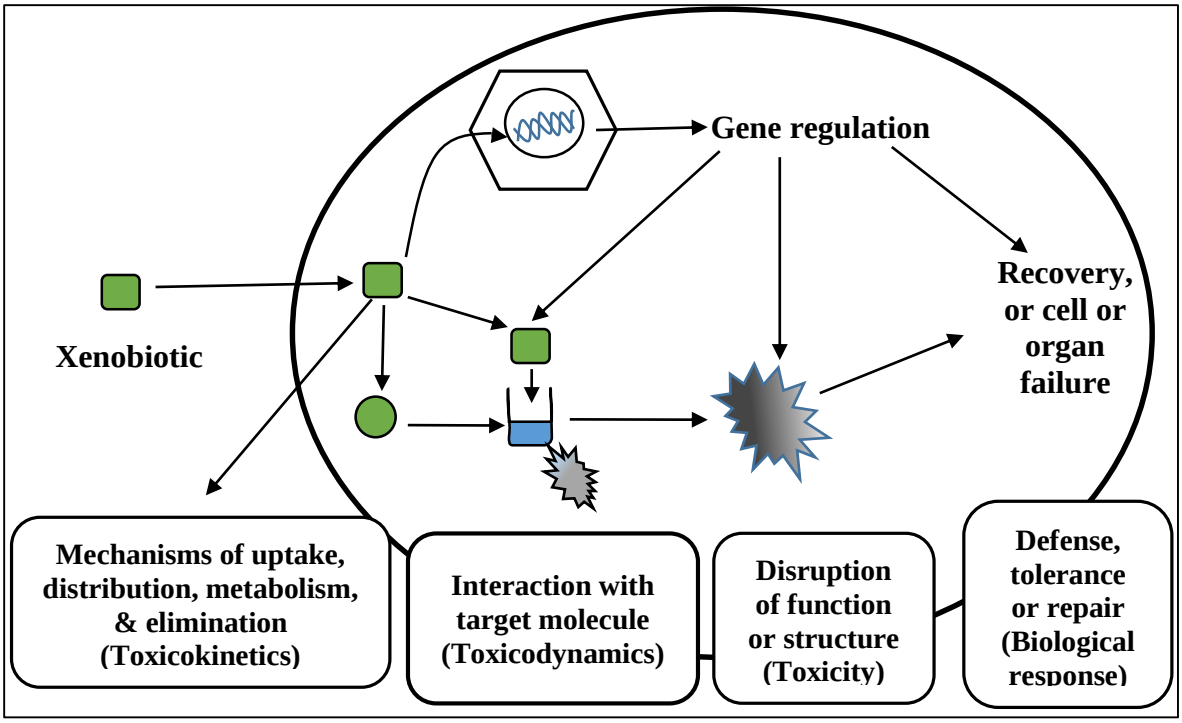

Figure 1-2: Mechanisms of toxicity at different levels following exposure to a potentially toxic compound. After a xenobiotic gets into the body of an organism, it is distributed and subject to possible biotransformation and excretion (the parent molecule or its metabolite). Next, a xenobiotic or its metabolite gets access into the target site and interacts with a target molecule, which ultimately causes a toxic effect. Moreover, the xenobiotic may alter gene expression and cause toxicity. Finally, the cell, organ, or body reacts with a specific response to this offense and gets either recovery, or may result in function failure, cell, or organ failure. Figure is modified from Boelsterli (2007).

1.3 Xenobiotic biotransformation

Detoxification and elimination of xenobiotics are important enzymatic biotransformation processes in both vertebrates and invertebrates (Baldwin and LeBlanc, 1994). These enzymes are virtually found in all animals and are responsible for the metabolic transformation of endogenous compounds, chemotherapeutic and xenobiotics (Livingstone, 1998; Bohne, Hamre, and Arukwe, 2007). They are categorized into two phases, phase I and II pathways with highest concentration and activities in the liver. As a result, the majority of the biotransformation reactions takes place in the liver and associated with the endoplasmic reticulum, while some are non-hepatic and non-microsomal origin. Generally, phase I biotransformation pathways, which include oxidation, reduction, hydration, and hydrolysis are recognized as functionalization (i,e, introduction of -OH, -COOH, -NO_2, among others) or the addition of oxygen to form a reactive site on the exogenous or endogenous compound, and phase II biotransformation enzymes conjugate a large water-soluble group (glutathione, sulfate, glucuronide, amino acid, among others) to the reactive site, thereby creating a more polar and

water-soluble compound (Figure 1-3). Therefore, hydrophobic lipid-soluble organic xenobiotics are converted into more excretable metabolites, which ultimately determine the disposition, residence time, and toxicity (detoxification or activation) of a xenobiotic in an organism (Chambers and Yarbrough, 1976; Bohne *et al*., 2007; Hodges and Minich, 2015; Livingstone, 1998).

Although detoxification and excretion of xenobiotics are the ultimate purpose of biotransformation, the final metabolites produced are sometimes more toxic than the parent compounds as a result of the process called bioactivation. The effective detoxification or eventual bioactivation of xenobiotics is, therefore, dependent on the structure of the chemical and the enzyme involved in the biotransformation reaction, which is responsible for the toxicity and carcinogenicity of many xenobiotics. Generally, production of reactive and more toxic metabolites more often involves phase I biotransformation pathway (Dekant, 2009).

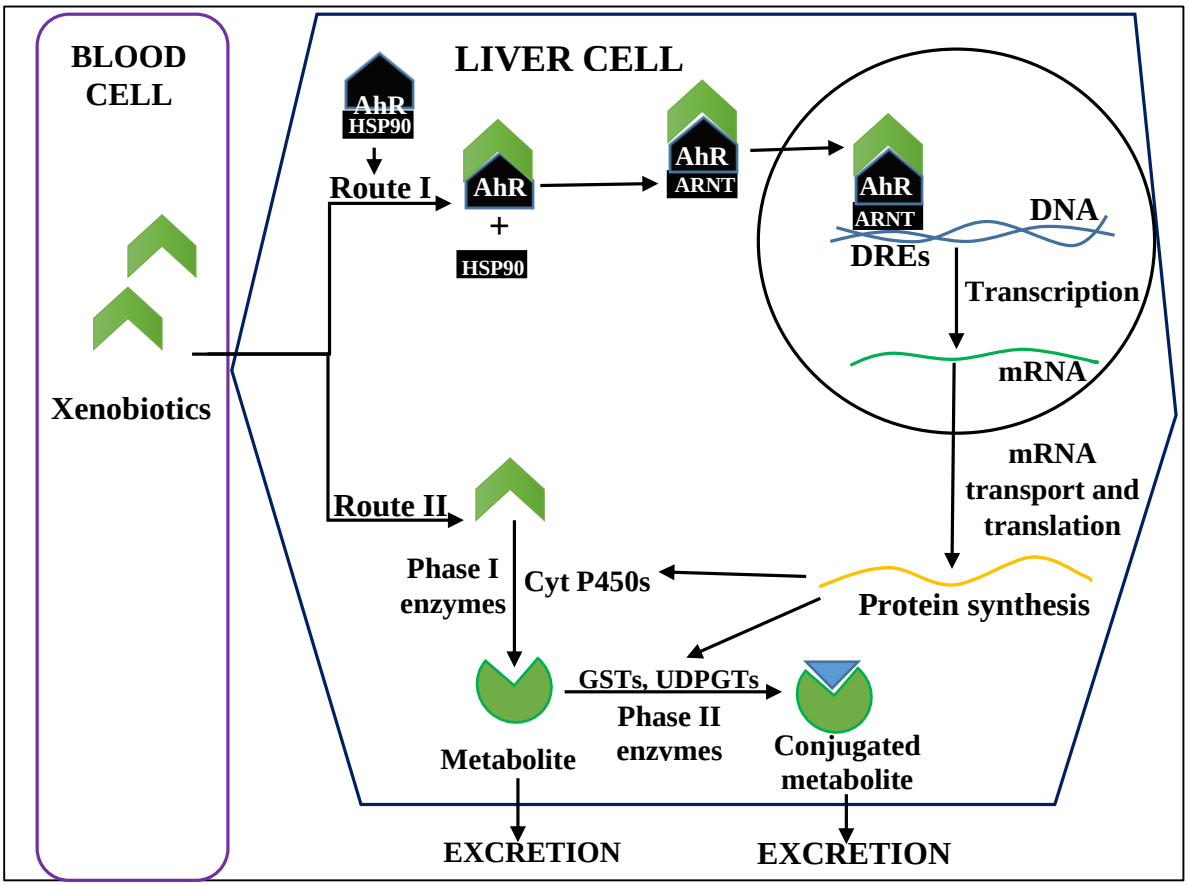

Figure 1-3: Xenobiotic biotransformation in the liver cell. A xenobiotic compound may follow either the detoxification and toxication (Route I) or enzyme induction (Route II). AhR, aryl hydrocarbon receptor; HSP90, 90 kDa heat shock protein; ARNT, AhR nuclear translocator; DREs, dioxin responsive elements; cyt P450s, cytochrome P450 isozymes; GSTs, glutathione S-transferases; UDPGTs, Uridine diphosphate glucuronosyltransferase. Figure is modified from Van der Oost, Beyer, and Vermeulen (2003).

Majority of mammalian biotransformation enzymes have been also found in fish, including phase I (cytochrome P450 dependent monooxygenase or CYP system) and phase II (glutathione *S*-transferase (GST), UDP-glucuronyltransferase (UDPGT), UDP-glucosyltransferase, sulfotransferase, and amino acid conjugases) (Livingstone, 1998; Mortensen and Arukwe, 2007b). The CYP monooxygenase system is best known for the oxidative biotransformation of a variety of exogenous compounds, comprising environmental pollutants, pharmaceuticals and endogenous compounds such as steroid hormones, bile acids, fatty acids and prostaglandins (Arukwe, Celius, Walther, and Goksøyr, 2000).

1.3.1 Phase I enzymes

The CYP constitutes one of the largest gene superfamily coding for microsomal membrane-bound, heme-thiolate enzymes, which are present in all forms of life (plants, bacteria, and mammals) and commonly localized in the endoplasmic reticulum (Hannemann, Bichet, Ewen, and Bernhardt, 2007). They are mainly located in the liver, but also in enterocytes, kidneys, lung, and even the brain (Hodges and Minich, 2015). They catalyze monooxygenase reactions involving molecular oxygen and an equivalent number of electrons. The enzymes are involved in diverse reactions including, hydroxylation of saturated carbon-hydrogen bonds, the epoxidation of double bonds, the oxidation of heteroatoms and aromatics, N-, O- and S-dealkylation, sulfoxidation, deamination, dehalogenation, peroxidation, etc. For this monooxygenase reaction, the enzyme system uses molecular oxygen and transfer one of the oxygen atom into a substrate (RH) and reduce the second oxygen to a water molecule with the help of a reducing agent NAD(P)H, which provides the two electrons (Equation 1) (Bernhardt, 2006; Fröhlich *et al*., 2010; Meunier, De Visser, and Shaik, 2004; Uno, Ishizuka, and Itakura, 2012).

$$RH + NAD(P)H + O_2 + 2e^- + H^+ \longrightarrow ROH + NAD(P)^+ + H_2O \quad (1)$$

Besides the fundamental similar features of monooxygenase reactions shared by all CYP-mediated biotransformations, they demonstrate large differences in both substrate and product compounds (Nebert, Nelson, and Feyereisen, 1989). Some isozymes of different subfamilies are more involved in the biotransformation of xenobiotics, while others are performing more specifically metabolize endogenous substrates. For example, CYP1A subfamily is principally responsive and induced by planar aromatic and chlorinated hydrocarbons, which is observed in all vertebrate species studied (Goksøyr, 1995). In relation to xenobiotics biotransformation, the CYP1, CYP2 and CYP3 enzyme superfamilies specifically metabolize a wide variety of compounds (Mortensen, Letcher, Cangialosi, Chu, and Arukwe, 2011). CYP1A isozyme is among the most studied enzymes in salmonids. The CYP1A gene has been found to be

expressed in liver, kidney, brain, and gill tissues following xenobiotic insult (Finn, 2007). The enzyme induction has been used as a sensitive, convenient, biomarker signal of organic xenobiotics in the aquatic environment. The enzyme system in fish has demonstrated its response to widespread environmental contaminants such as PAH, PCBs, dioxins, oil compounds, pesticides and others (Förlin, Goksøyr, and Husøy, 1994). The planar, aromatic structural nature of these chemical compounds enable them to bind the aryl hydrocarbon (Ah) receptor that leads to the induction of genes causing an increased amount of CYP1A messenger RNA (mRNA), protein, and catalytic activity. Catalytic assays are used in the laboratory to measure the induction of these genes using 7-ethoxyresorufin as substrate in 7-ethoxyresorufin *O*-deethylase (EROD) assay. Additionally, immunochemical techniques such as western blotting and enzyme-linked immunosorbent assay (ELISA) have been routinely used at the protein level using specific fish CYP1A proteins (Nilsen, Berg, and Goksør, 1998).

Expression of CYP1A1 genes is regulated by the ligand activated transcription factor, aryl hydrocarbon receptor (AhR) (Figure 1-3). AhR and its dimerization partner AhR nuclear translocator (Arnt) belong to the basic helix–loop–helix–Per–Arnt–Sim (BHLH–PAS) transcription factor. Upon ligand activation and translocation into the nucleus, AhR dimerizes with its nuclear partner Arnt and then binds to a specific consensus deoxyribonucleic acid (DNA) sequence (5'-GCGTG-3'), xenobiotic responsive element (XRE). Binding of the heterodimer to the DNA sequence leads to transactivation of several genes containing the XRE in their promoter region. Different genes that encode for phase I and II enzymes, contain the XRE, including CYP1A1, CYP1A2 and CYP1B1, UDPGT, GSTA1, AhR repressor (AhRR), and a tumor-specific aldehyde dehydrogenase (ALDH3c). Fortunately, most of the aforementioned enzymes demonstrate a preference for AhR agonists or structurally related compounds as a substrate, which facilitate the elimination of these chemicals and their metabolites and serve as a defense system (Bradshaw, Trapani, Vasselin, and Westwell, 2002; Meucci and Arukwe, 2006; Mortensen and Arukwe, 2008).

In addition to CYP1A, the CYP3A isozymes are also induced following xenobiotic exposure (Finn, 2007). CYP3A4 genes are strongly induced by structurally diverse group of compounds. Many of these compounds are also substrate for the enzyme CYP3A. They are involved in 6β-hydroxylation of steroid and bile acid and oxidation of xenobiotics (Goodwin, Redinbo, and Kliewer, 2002; Kliewer, Goodwin, and Willson, 2002). They are the major CYP isoform present in human, and fish liver and gut with a wide range of substrate specificity, which display sex- and development stage-specific expression and induction (Christen, Oggier, and Fent, 2009; Honkakoski and Negishi, 2000). They are also the most important class of enzymes involved in oxidative metabolism of around 60% of all therapeutic drugs, which

enable them to be the basis for potential life-threatening drug-drug interactions (i.e. where the metabolism of a given drug is accelerated by another drug) (Goodwin *et al*., 2002; Kliewer *et al*., 2002). For instance, a variety of chemicals, including prescription drugs such as rifampicin, dexamethasone, indinavir, paclitaxel, pesticides such as *trans*-nonachlor, chlordane, endocrine disruptors such as phthalic acid, nonylphenol, glucocorticoids and anti-glucocorticoids, barbiturates and other drugs have been found to induce different CYP3A genes in a species-specific manner (Kliewer, 2003).

As a result of their structural similarities, both mammalian and fish CYP3A enzymes demonstrate similar catalytic properties (Christen *et al*., 2009). Thus, the CYP3A gene expression in fish and mammals is regulated by the ligand activated nuclear receptor (NR), pregnane X receptor (PXR) (Kliewer, 2003). Upon ligand activation, PXR hetrodimerizes with the 9-*cis* retinoic acid receptor (RXR) and then binds to XRE in *CYP3A* gene promoters as a transcription factor. Additionally, PXR is also found to control a wide variety of genes encoding for xenobiotic detoxification, including CYP enzymes, aldehyde dehydrogenases, UDPGT, sulfonyltransferases, GSTs and various transporters (Goodwin *et al*., 2002; Kliewer, 2003; Kliewer *et al*., 2002). Generally, the different cytochromes P450 display variations in occurrence and their relative proportion with respect to animal species, strain, sex, age or tissue. Fish from different species and age groups, for example, exhibit marked differences in biotransformation capacity (Buhler and Williams, 1988).

1.3.2 Phase II enzymes

UDPGT and GST are the two major phase II enzymes for the conjugation and ultimate elimination of several endogenous compounds and lipophilic compounds (Figure 1-3) (Mortensen and Arukwe, 2007a, Mortensen *et al*., 2011). In vertebrates, both endogenous and exogenous compounds are metabolized and excreted by glucuronidation pathway. UDPGT transfers the glucuronide moiety of UDP-glucuronic acid (UDPGA) to an aglycone acceptor molecule (Clarke, George, and Burchell, 1992). The enzyme UDPGT family is localized in the endoplasmic reticulum. Upon binding the glucuronide moiety, acceptor compounds are blocked from biological reactivity and thus attain significant polarity which further facilitates their excretion in either urine or bile (Clarke *et al*., 1992; Leaver, Wright, Hodgson, Boukouvala, and George, 2007). The different endogenous substrates for the enzyme UDPGT include steroid hormones, bilirubin, bile acids, 5-hydroxytryptamine, retinol, and thyroxine. While several exogenous xenobiotics become substrate for the enzyme, including pharmaceuticals, agrochemicals, pesticides, and industrial chemicals. Among the xenobiotic substrates are planar and bulky phenols, aromatic amines, which are potential carcinogens (George and Taylor,

2002). Substrate specificity, differential ontogenic pattern and induction, however, exist between animals (Clarke *et al*., 1992).

GSTs enzymes are also involved in the biotransformation of xenobiotics in fish (Bohne *et al*., 2007). The GSTs enzymes help the cellular environment to deal with the detrimental effects of environmental chemicals, anti-tumor drugs and products of reactive stress. They perform detoxification activities by catalytic conjugation of an endogenous tripeptide reduced glutathione (GSH) to a variety of metabolites with electrophilic carbon, nitrogen or sulfur atom, such as epoxides produced by CYP. The GSH conjugated metabolites are further metabolized to mercapturic acid derivatives and excreted in bile or urine. A number of xenobiotics have been known to induce GST, including pesticides, herbicides, pharmaceuticals, industrial intermediates (PCB), metals and PAH. Interestingly, the majority of the chemicals that cause induction of GST are also substrates for the enzyme itself, or can be metabolized by cytochrome P450 enzymes prior to GST conjugation reaction (Bastos *et al*., 2013; Hayes, Flanagan, and Jowsey, 2005; Henson, Stauffer, and Gallagher, 2001, Nimmo, 1987).

Different types of both soluble (cytosolic) and microsomal GSTs are found in fish. Most of them appear to be soluble forms, which have higher activities in the liver and hepatopancreas (Giulio, Benson, Sanders, and Van Veld, 1995). An increased activity of GST toward 1-chloro-2,4-dinitro- benzene (GST-CDNB activity) have been reported from fish exposed to GST inducing agents or fish dwelling polluted environments. However, variations in diet, water temperature, gender, and reproductive cycling in fish may complicate the investigation of GST-CDNB activities. Furthermore, modulation of GST isoforms with specific activity toward environmental toxicants may be overlooked since the GST-CDNB activity denote an integration of the activities of several GST isoforms. As a result, detailed studies of the induction patterns, for example by chromatographic techniques, of the different isoforms in fish may be needed when GST is used as a biomarker (Henson *et al*., 2001; Martinez-Lara, Toribio, Lopez-Barea, and Barcena, 1996; Pérez-López, Nóvoa-Valiñas, and Melgar-Riol, 2002; Schlenk *et al.*, 2008).

1.4 Oxidative stress enzymes in fish

In aerobic life, oxidative stress is inevitable, but biological systems have the mechanism to balance between the production of reactive oxygen species (ROS) and the protection system to remove them (Slaninova, Smutna, Modra, and Svobodova, 2009). Cells and tissues possess a chain of cellular antioxidant protection mechanism to neutralize the toxic effects posed by ROS (Mahboob *et al*., 2014). However, oxidative stress occurs when the production of ROS overwhelms the antioxidant system that neutralize them (Olsvik, Torstensen, Hemre, Sanden,

and Waagbø, 2011). There are several processes within an organism that contribute to the generation of ROS under physiological conditions. These are called endogenous cellular sources and include electron transport chains of both mitochondria and endoplasmatic reticulum, the function of cytochrome P450, auto-oxidation of the key molecules of cellular function (e.g., reduced Flavin adenine dinucleotide (FAD) and Flavin mononucleotide (FMN), glyceraldehyde), and the actuation of oxidative enzymes during catalysis (Livingstone, 2001; Slaninova *et al*., 2009).

Pollutants stimulate the production of ROS by several mechanisms, including disruption of membrane-bound electron transport and continuous buildup of reduced intermediates, autoxidation (e.g., CYP), redox reactions with O2 and ROS (e.g., Co, Cr, Ni, Va), photosensitization, enzyme induction (e.g., CYP, flavoprotein reductases), depletion of antioxidant defenses (e.g., GSH), and depletion of radical scavengers (Kelly, Havrilla, Brady, Abramo, and Levin, 1998; Livingstone, 2001; Slaninova *et al*., 2009).

Fish have a powerful antioxidant protection system to neutralize hostile conditions generated by ROS (Pandey *et al*., 2003; Zhu, Zhu, Lang, and Chen, 2008). The protection mechanism involves numerous low-molecular-weight, nonenzymatic antioxidants (e.g., GSH) and various antioxidant enzymes, such as superoxide dismutase (SOD), which catalyzes the transformation of superoxide radical to H_2O_2, catalase (CAT), which reduces H_2O_2 to water and oxygen, glutathione peroxidase (GPx), which reduces both H_2O_2 and organic peroxides by a glutathione-dependent reaction, and glutathione reductase (GR), which catalyzes the NADPH-dependent regeneration of GSH from the oxidized disulfide form (GSSG) generated by GPx (Figure 1-4) (Mahboob *et al*., 2014; Morales, Perez-Jimenez, Hidalgo, Abellán, and Cardenete, 2004). Both GPx and CAT enzymes catalyze the breakdown of hydrogen peroxide to molecular oxygen and water (Equation 2). In addition to catalyzing the reduction of hydrogen peroxide, GPx enzymes can also reduce other peroxides. CAT enzymes are involved in fatty acid metabolism and are located in the peroxisomes. Since these situations may influence the interpretation of the change in activities of the enzyme, erythrocytes may be used as a reliable

source for CAT activity investigation in vertebrates (Olsvik *et al*., 2011; Van der Oost, Beyer, and Vermeulen, 2003).

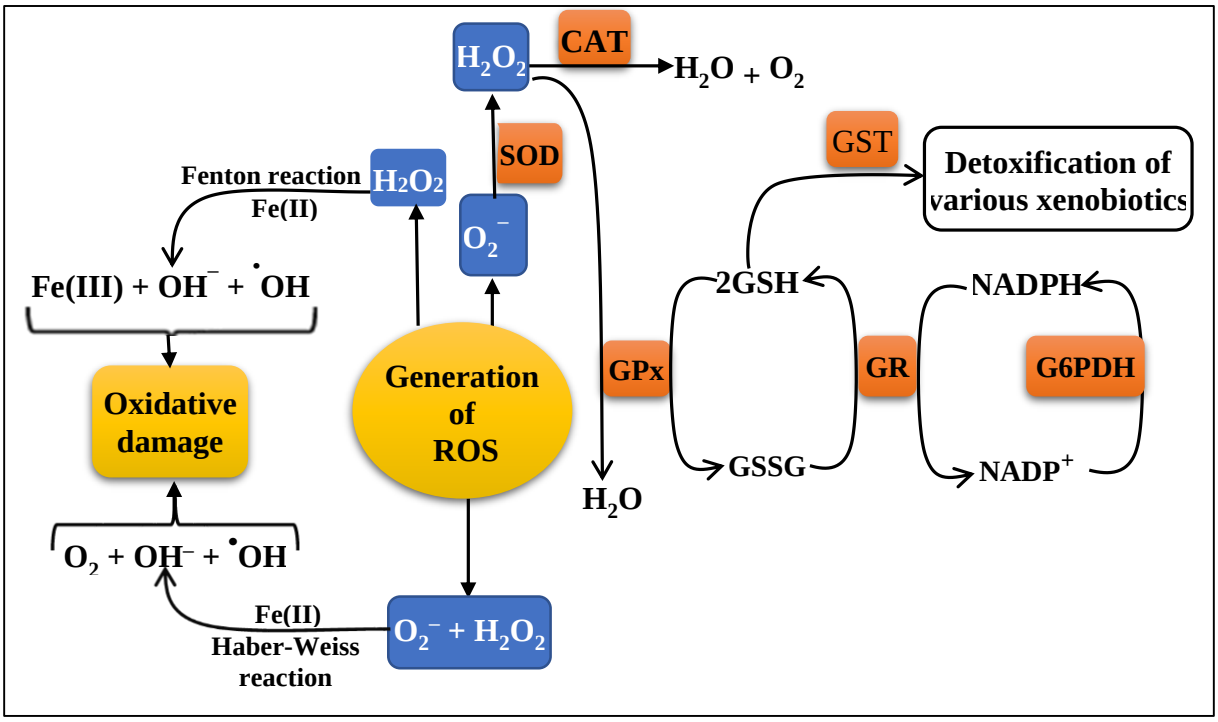

Figure 1-4: Schematic representation of the enzymatic antioxidant defenses system. The enzymes work in concert and provide cell protection mechanism against reactive oxygen species (ROS). The abbreviations SOD, superoxide dismutase; CAT, catalase; GST, glutathione S-transferase; GR, glutathione reductase; GPx, glutathione peroxidase; GSH, reduced glutathione; GSSG, oxidized glutathione, and G6PDH, glucose-6-phosphate dehydrogenase. Figure is modified from Hermes-Lima (2004).

$$2H_2O_2 \rightarrow O_2 + 2H_2O \qquad (2)$$

Peroxides represent enzymes that catalyze the reduction of several peroxides to their corresponding alcohols. Of which GPx are the major enzymes in fish that utilizes GSH as a cofactor. During catalysis, GPx converts hydrogen peroxide to water and oxygen with a simultaneous oxidation of GSH (reduced form) to its oxidized form, GSSG (Van der Oost *et al*., 2003).

GR involvement in oxidative stress is different from the enzymes previously described. Yet, it plays an important role in maintaining GSH:GSSG balance of homeostasis under oxidative stress conditions. It reduces the GSSG to GSH at the expenses of oxidizing NADPH to $NADP^+$, which intern replenished by pentose phosphate pathway (Peña-Llopis, Ferrando, and Peña, 2003; Van der Oost *et al*., 2003).

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O \qquad (3)$$

$$GSSG + NADPH \rightarrow GSH + NADP^{+} \quad (4)$$

Both GPx and GR in combination with GST and glutamate:cysteine ligase (GCL) are among the enzymes that are involved in regulating the level of glutathione. Glutathione is conjugated with reactive electrophiles for easy removal by means of enzyme GST. Additionally, GPx enzymes catalyze oxidation of two molecules of GSH to the oxidized glutathione disulfide form (GSSG) coupled with the removal of ROS (Equation 3). Furthermore, GR reduces GSSG back to the reduced GSH (Equation 4) (Peña-Llopis *et al*., 2003; Piechota-Polanczyk and Fichna, 2014).

1.5 Endocrine disruption and reproduction in fish

The endocrine system is a set of hormonal network that plays a vital role in organizing and regulating many biological processes, which include nutritional, behavioral, growth, organs functioning, and reproductive processes and maintaining homeostasis (Damstra, Barlow, Bergman, Kavlock, and Van Der Kraak, 2002; Tarrant *et al*., 2005). The regulation is coordinated with the nervous and immune systems and involves communication with target tissues via hormones secreted by endocrine glands, which include the pituitary, thyroid, pancreas, adrenal, and the male and female gonads (testes and ovaries). The secreted hormones are then transported via the blood stream in the free state or attached to carrier proteins to exert their effects on distant target tissues or organs (Crisp *et al*., 1998). Upon binding to specialized hormone receptors (these can be either cell surface or nuclear receptors), the released hormone specifically interacts with these target tissues or organs and activates different cell- or organ-specific functions (regulate gene activities or transmit signals to regulate ion channels or enzymes) (Crisp *et al*., 1998; Tarrant *et al*., 2005).

Over the years, the widespread of endocrine-disrupting chemicals (EDCs) and their potential impact in modulating and/or disrupting the endocrine system of human and wildlife have been recognized and documented. The endocrine system has been targeted by a variety of EDCs. These chemicals affect the endocrine system at different levels, including the hypothalamus-pituitary-gonadal axis. Additionally, EDCs have the potential to mimic endogenous hormones and modulate their synthesis, secretion, transport, binding, action, and elimination or alter the signal transduction, or modifying hormone receptor levels in the body (Mills and Chichester, 2005; Tarrant *et al*., 2005). As a result, the EDCs subsequently affects the sensitive hormonal regulation of the reproductive system, which may affect the development and maturation processes or during the sensitive critical periods of life (Arcand-Hoy and Benson, 1998; Mnif, Pillon, Balaguer, and Bartegi, 2007; Patrick *et al*., 2016; Segner *et al*., 2003). A vast majority of anthropogenic chemicals has been reported as hormone mimics

such as OCPs (DDT and its metabolites), PCBs and their hydroxylated metabolites, dioxin-like chemicals (PCDDs and PCDFs), bisphenol-A, alkylphenolic chemicals (industrial surfactants and antioxidants), vinclozolin (a fungicide), tributyltin (TBT: an anti-fouling agent) and some phthalate esters (plasticizers) (Tyler, Jobling, and Sumpter, 1998). However, the complex nature of the system makes the identification of specific mechanisms through which these EDCs produce observed biological responses very difficult (Tarrant *et al*., 2005).

As an ultimate sink for natural and artificial chemicals, the aquatic environment receives large amounts of domestic and industrial wastes from the surrounding environment (Sumpter, 1998). For example, EDCs have been found in freshwater, estuarine, and marine environments, which leads to an increased threat posed by these group of chemicals to organisms living in these aquatic environments (Mills and Chichester, 2005). As a result of their watercourse habitats they reside, the aquatic species are exposed to relatively high concentrations of EDCs from agricultural runoff, domestic and/or industrial effluents either after wastewater treatment or as raw effluents (Tarrant *et al*., 2005). There have been evidences from field studies, particularly on the aquatic environment, that developmental and reproductive alterations in exposed organisms have suggested a relation with exposure to EDCs (Segner *et al*., 2003). For example, high environmental and biota concentrations of EDCs, such as phthalates with developmental abnormalities and reproductive effects (Adeogun *et al*., 2015), intersex and alterations in reproductive development in fish (Adeogun, Ibor, Adeduntan, and Arukwe, 2016), and contaminants with cytotoxic, genotoxic and endocrine disrupting effects in the sediments (Amaeze *et al*., 2015), have been reported in freshwater and marine environments in Nigeria. Like other vertebrates, reproduction in fish is controlled by gonadal steroids and is under the direct control of hypothalamic-pituitary-gonadal axis (HPG) (Figure 1-5) (Scholz and Mayer, 2008). Following perception of environmental signals (photoperiod, temperature, feeding, and social factors) and neural stimulation from the central nervous system, the hypothalamus directs the synthesis and release of hormones and chemical messengers. The hypothalamus then releases gonadotropin-releasing hormone (GnRH), which stimulates the secretion of gonadotropin from the pituitary gland. There are two forms of gonadotropin, termed GtH I and GtH II, isolated from fish, which are found to be mammalian analogous of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), respectively. Similar to other vertebrates, fish GtH I is involved in gametogenesis and steroidogenesis while GtH II is involved in the final maturation stages of gametogenesis. They are both take part in stimulating the synthesis of sex steroids, such as androgens, estrogens, and progestins. The sex steroids,

then interact with the target tissues and control gametogenesis, reproduction, sexual phenotype, and behavioral characteristics (Arcand-Hoy and Benson, 1998; Arukwe, 2001).

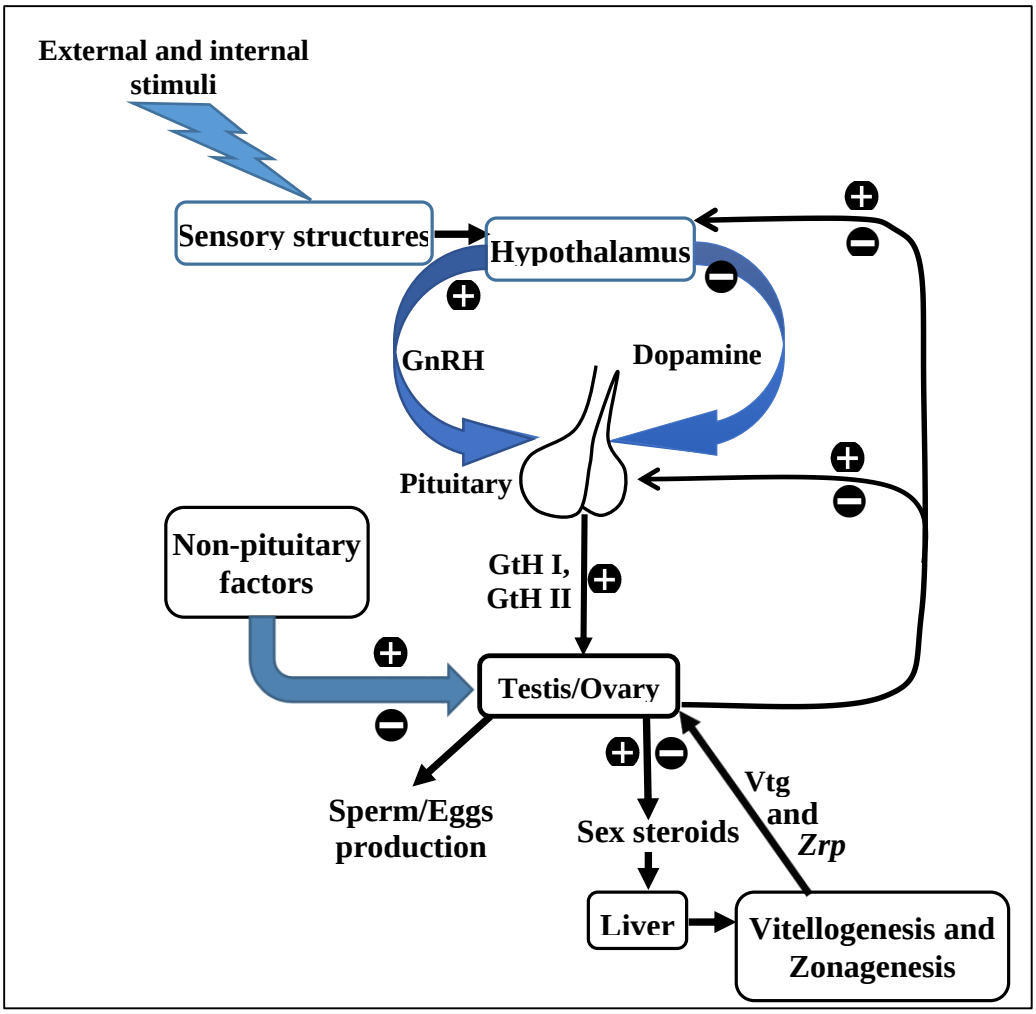

Figure 1-5: Schematic representation of regulation (environmental signals and hormonal) of maturation/reproduction pathways in adult teolest fish. It represents stimulation (+) and inhibition (-), which controls structures or behaviors. GnRH, gonadotropin releasing hormone; GtH, gonadotropin hormone I and II; Vtg and Zrp, vitellogenin and zona radiata protein, respectively. Picture is modified from Arukwe (2001).

In female fish, GtH I is involved in oogenesis development. During early oocyte development, the level of GtH I circulating in the blood increase and then bind to receptors on the thecal and granulosa layer of the follicle. The steroidogenic thecal cells synthesize and provide the androgen substrate, testosterone, to the ovarian granulosa cells that provide aromatase and synthesize estradiol-17β (E2) (Lubzens, Young, Bobe, and Cerdà, 2010). The E2 binds to hormone-binding globulin in the blood and transported to the liver hepatocytes where they interact with a specific steroid-receptor protein, the estrogen receptor (ER) in target cells (Figure 1-6). After binding its ligand, E2 or estrogen mimics, the ER dissociates from heat

shock protein 90 (a protein chaperone that hold ER in its inactive form) (Hara, Hiramatsu and Fujita, 2016). The ER then dimerizes and translocated into the nucleus, where the hormone-receptor complex binds at the estrogen responsive element (ERE) of estrogen responsive genes and induce gene expressions (Arukwe and Goksøyr, 2003).

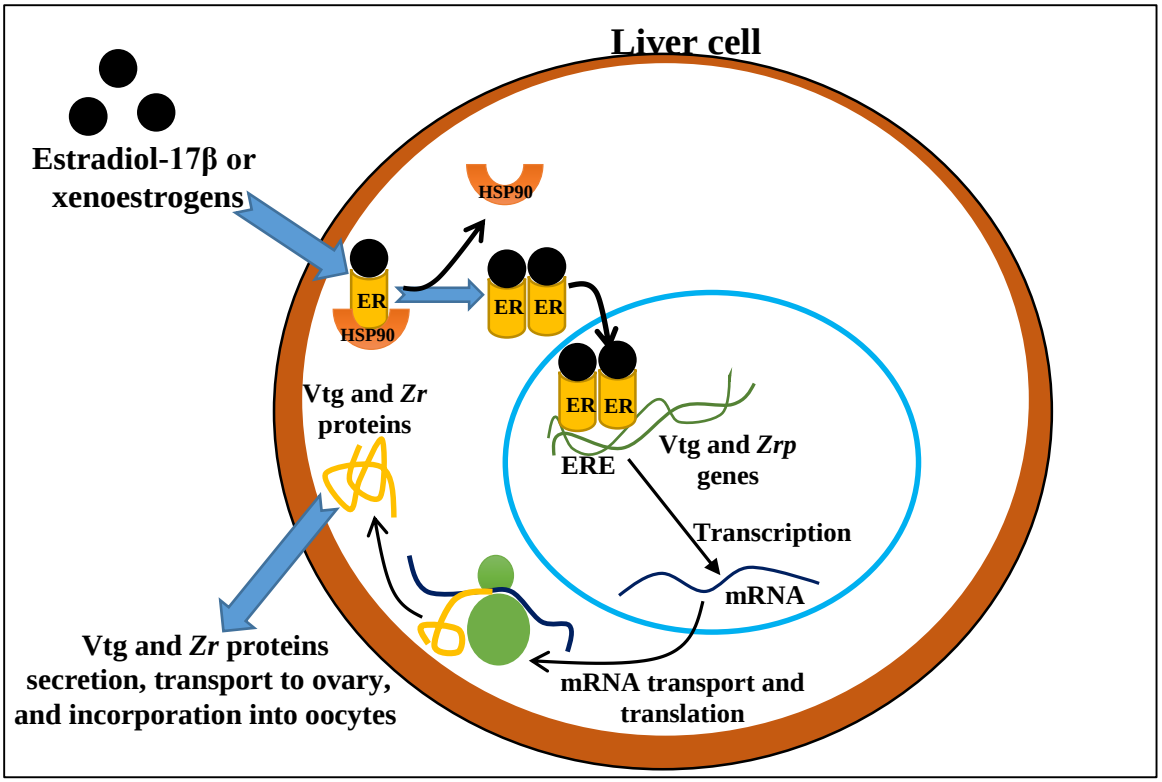

Figure 1-6: Schematic representation of oogenic protein synthesis in hepatocytes. The ligand, Estradiol-17β or estrogen mimics, activation of estrogen receptor (ER) and binding to the estrogen responsive element (ERE) leading to induction of the estrogen responsive gene expressions, which results in vitellogenesis and zonagenesis. Vtg: vitellogenin; Zrp: zona radiata protein; Hsp 90: 90 kDa, heat shock protein. Figure is modified from Arukwe and Goksøyr (2003).

Among other estrogen responsive genes, the egg yolk protein, vitellogenin (Vtg), gene is also transcribed and subsequent increase and stabilization of Vtg mRNA is achieved. The Vtg mRNA then translated and modified in the rough endoplasmic reticulum (RER) and carried by the blood to the ovaries, where it binds to the Vtg receptor and selectively sequestered by receptor mediated endocytosis by the developing oocytes (Arukwe and Goksøyr, 2003; Tyler and Sumpter, 1996). Likewise, the eggshell proteins, termed *zona radiata* protein (*Zrp),* is also produced in the liver under the influence of E2 level and transported by the blood and incorporated in the developing oocytes (Arukwe and Goksøyr, 2003).

Several hormones secretion is controlled through feedback mechanisms with a closed loop and occurs when the response of a signal influences its source. In teleost, the HPG axis is controlled by feedback systems, which have a positive or negative effect depending on the

physiological and reproductive needs of the fish (Thomas, 2008). A change in steroid synthesis could alter the feedback regulation, or vice versa, which eventually affect and damage the reproductive processes (Arcand-Hoy and Benson, 1998; Thomas, 2008). Therefore, environmental pollutants disrupt the endocrine function on multiple targets through a variety of mechanisms. These chemicals: -

- Could interfere with sex hormone actions by binding to their receptors and
- Could act on the hypothalamus and affect neurotransmitter function controlling GnRH secretion or directly interfere with GnRH neurons.
- Alter the secretion of GnRH and regulatory neurotransmitters, which intern affect pituitary GnRH receptor levels and gonadotropin secretion from the pituitary. Additionally, certain chemicals can have a direct impact on the GnRH signaling pathways and influence the gonadotropin secretion.
- Induce alterations in gonadotropin secretion often result in alterations in gonadal function and development, including steroid hormone production and gamete maturation. Some chemicals could directly act on the gonads to alter the activities of gonadotropin and enzymes participating in the synthesis of steroid hormones.
- Changes the production of steroid hormones and perturb steroid levels in the blood, which in turn affect their action at the target tissues (including the gonads, accessory reproductive tissues, liver, and neuroendocrine system). For example, induction of hepatic CYP enzymes by certain chemicals such as PCBs increases the metabolic clearance of steroids and influences the level of circulating steroids.
- Could directly interact with both nuclear and membrane steroid receptors and act as agonists or antagonists of steroid hormone actions. For example, the steroid feedback system could be influenced through changes in the production, clearance, or action of steroids.

In the same manner, direct interactions of chemicals with ERs in the liver could result in induction of vitellogenesis and controls production of Vtg (Jobling and Sumpter, 1993; Thomas, 2008). Additionally, Vtg production could also be influenced by other factors that alter blood E2 levels; or could be cumulative results of several factors acting simultaneously (Thomas, 2008). There are growing evidences of xenoestrogenic endocrine disruption in marine environment fish and to a lesser extent in the estuaries (Oberdörster and Cheek, 2001). Elevated concentrations of blood plasma Vtg level have been found in male flounder (*Platichthys flesus*) caught from industrialized estuaries in the United Kingdom and the Netherlands and large number of different estuarine and coastal fish species, such as marbled sole (*Pleuronectes yokohamae*), common goby (*Acanthogobius flavimanus*), and grey mullet (*Mugil cephalus*) in

estuarine and coastal areas in Japan. Additionally, enhanced production of Vtg and *Zrp* in the liver and/or their presence in the blood has been detected in swordfish (*Xiphias gladius*) from the Mediterranean and off the coast of South Africa (Scott *et al*., 2006).

1.6 Interactions of chemical mixtures

Laboratory tests of toxicity from a single pure chemical undoubtedly contribute to the scientific investigation of toxicants to the aquatic organisms. These organisms, however, are typically exposed to a variety of pollutants with varying concentrations and constituents in their environment as a complex mixture (Faust *et al*., 2003). Thus, interactions of different chemicals in the mixture create unexpected effects, which lead to the development of synergistic, antagonistic or even additive responses. As a result, the effects that a single contaminant alone produced on fish are rather distinct from the collective effects that this contaminant produces in combination with other contaminants (Bizarro, Eide, Hitchcock, Goksøyr, and Ortiz-Zarragoitia, 2016; de Almeida, Rodrigues, and de Oliveira Ribeiro, 2014). For example, according to Tridico *et al*. (2010), the organophosphate pesticide diazinon interferes with the induction of CYP1A by benzo[α]pyrene (BaP) when fish were exposed to the mixtures of both chemicals. The interference occurs via the inactivation of CYP1A after the sulfur atom from the pesticide binds to the catalytic site of the enzyme. Therefore, the observed responses from a mixture of different chemicals do not always reveal the toxic effects of its components (de Almeida *et al*., 2014).

Three models have been used in the determination of effects of chemical mixture. Of which, concentration addition and independent action are collectively grouped under the non-interaction model where components of the mixture do not interact or interfere with each other (Bliss, 1939; Plackett and Hewlett, 1948). The third group, however, occurs when components of the chemical mixture interact with each other and creates synergistic or antagonistic effects (Figure 1-7) (Plackett and Hewlett, 1952). In concentration addition model, the toxicity of the whole mixture is the addition of the toxicity of individual components. Individual chemicals are assumed to have similar mechanism of toxicity (similarly acting chemicals) and common sites of action. Due to this additive effect, individual components that are at sub-threshold level would have contribution to the overall toxicity of the mixture. In relation to this effect, estrogenic compounds have showed concentration addition toxic effects, which were not elicited when these chemicals are applied individually (Heys, Shore, Pereira, Jones, and Martin, 2016). For example, Tinwell and Ashby (2004), performed a series of investigation on mixtures of estrogens in relation to rat uterine weight and found that individual constituents of the mixture that are individually inactive were capable of producing an overall positive response in

combination. In independent action model, also known as response addition, individual components have an independent mechanism of toxicity and target sites. Therefore, toxicity of individual chemicals acts independently from each other and thus do not influence each other (Heys *et al*., 2016; Rider and LeBlanc, 2005).

In relatively few cases, the toxicity of a chemical mixture, however, is somehow different from the aforementioned mode of actions. In these cases, the components of a chemical mixture interact each other and create an overall toxic effect that is either stronger (synergistic, potentiating) or weaker (antagonistic, sub-additive) than expected. Therefore, different factors influence the interactions, which include relative dose levels, the route(s), timing and duration of exposure, and the biological target(s) (European Commission, 2012; Heys *et al*., 2016). In a complex chemical mixture, interactions occur in different manners, which include toxicokinetic interactions (chemicals modifying the absorption of others or competing for active transport mechanisms), metabolic interactions (a chemical in a mixture modifies the metabolism of the other), toxicodynamic interactions (interactions between the biological responses, e.g., ligand-receptor interaction) (European Commission, 2012).

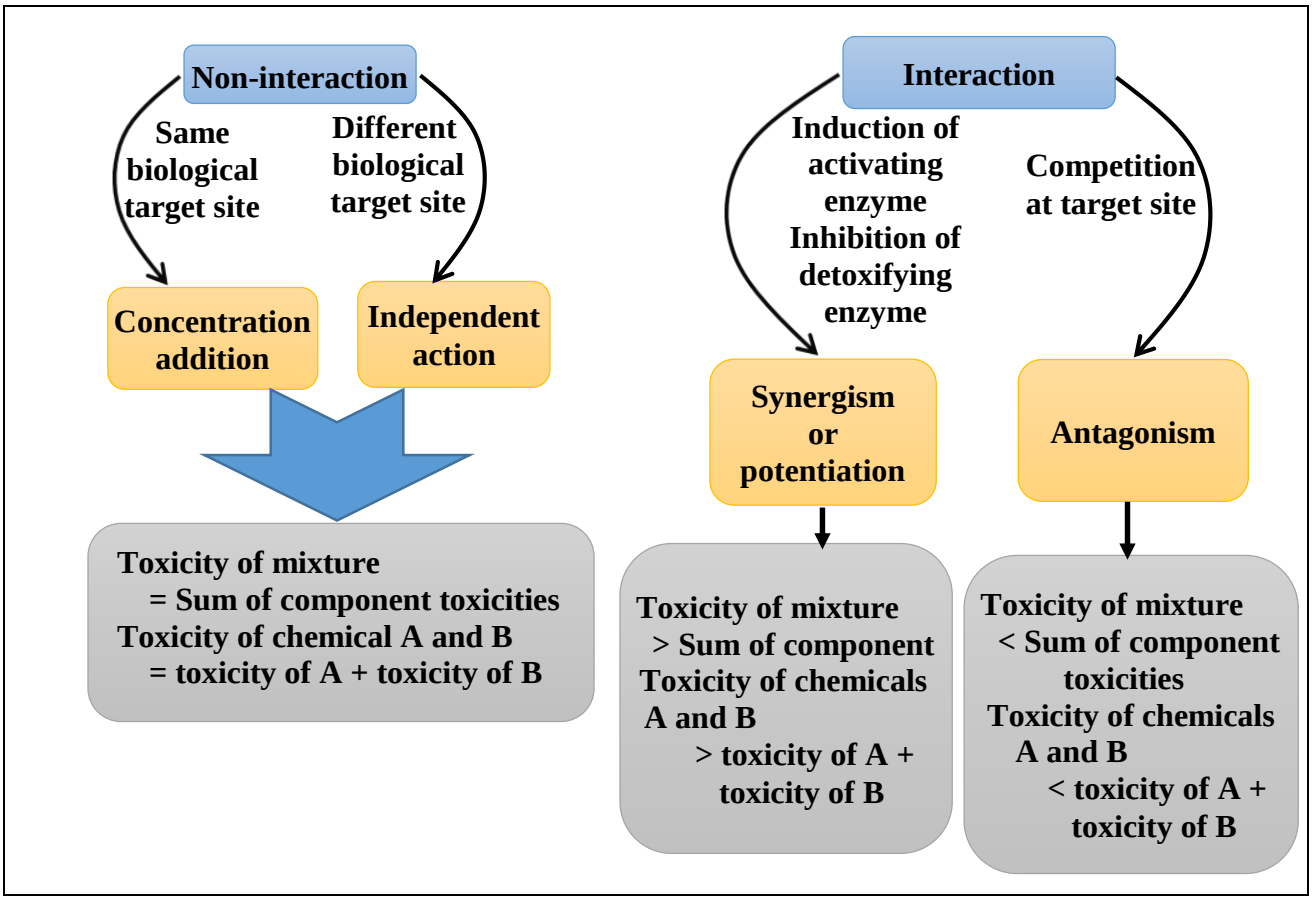

Figure 1-7: Schematic representation of the theoretical models of chemical mixture interactions. Figure is modified from Heys, Shore, Pereira, Jones, and Martin (2016).

Synergistic effects that enhance the chemical uptake are mostly demonstrated by the group of chemicals known as surfactants and additives to pesticides, which are deliberately introduced for the enhanced uptake of the active compounds. Moreover, synergistic effect also observed as a result of alteration of metabolic activity of chemicals. The metabolism of a chemical can be either increased or decreased by another chemical in a mixture and create synergistic effects. When the parent compound is toxic, decreased metabolism will result in increased toxicity. However, increased metabolism creates toxicity when the metabolites are more toxic than the parent compound. Azole fungicide application, for example, interfered metabolism of pesticides by inhibiting cytochrome P450 monooxygenases enzymes. Induction of Vtg was enhanced by a mixture of 1 μM of nonylphenol, octylphenol, o,p'-DDT, Arochlor 1221 and bisphenol-A in cultured rainbow (Sumpter and Jobling, 1995). On the other hand, E2 induced Vtg synthesis appears to be modulated by tamoxifen and cortisol. These chemicals produced reduction of Vtg protein production and show antagonism in Arctic char (Berg, Modig, and Olsson, 2004). Additionally, transcription of Vtg was inhibited, when rainbow trout were exposed to a mixture of E2 and cadmium (Olsson, Kling, Petterson, and Silversand, 1995)

1.7 Molecular biomarkers of pollutant exposure in fish

Over the years, many thousands of organic pollutants have been produced and released to the environment. These chemicals ultimately find their way to the aquatic environment where the different toxic compounds are increasingly overloaded. The presence of these chemical compounds by themselves do not necessarily represent toxic effects unless connections has been made between exposure levels (external concentration), internal levels (tissue concentration) and early adverse effects (Van der Oost *et al*., 2003). Additionally, the adverse effects at the population level take longer time for the effects to be manifested and even the damaging effects may have passed the critical point where remedial actions are still possible (Figure 1-8). However, earlier changes in biological process, which allow the manifestation of early warning signals are always followed by effects at higher hierarchical levels. As a result, it has become highly detrimental to carry out a rapid assessment and establish sensitive early warning signals, or biomarkers, that reflect the hostile biological responses of the environmental pollutants (Sarkar *et al*., 2006; Van der Oost *et al*., 2003).

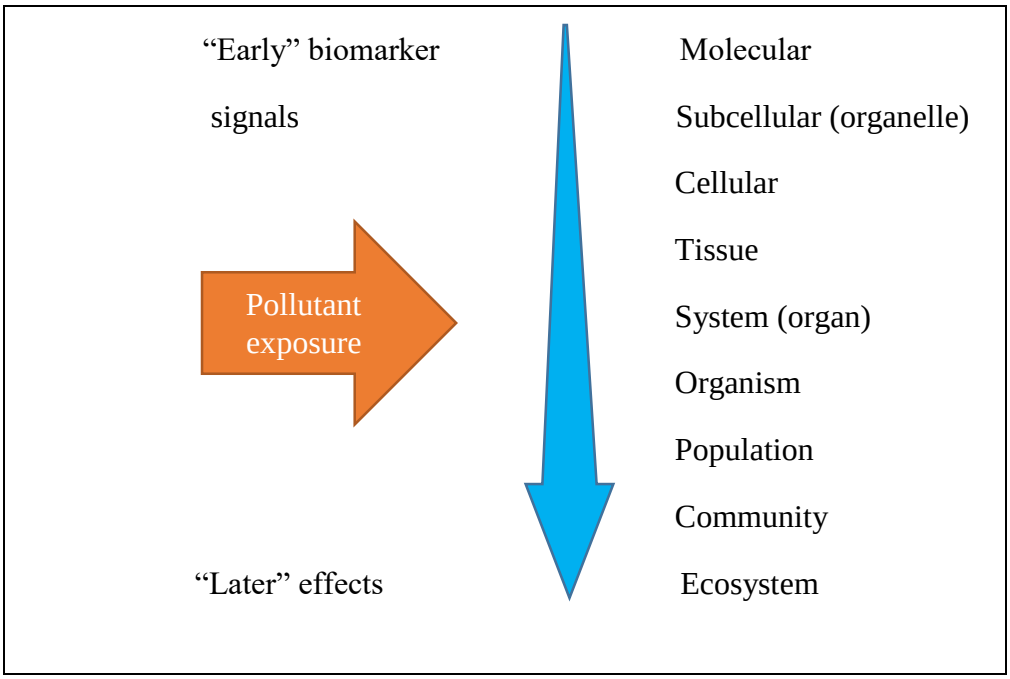

Figure 1-8: Responses to pollutant stress within a biological system and their sequential order. Figure is modified from Van der Oost et al. (2003).

Van der Oost *et al*. (2003) defined biomarkers as "*measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the presence and magnitude of toxicants, or of host response*". According to Sarkar *et al*. (2006) and Van der Oost *et al*. (2003), biomarkers can be expressed as biomarkers of exposure, effect, and susceptibility. A biomarker of exposure is the detection and measurement of exogenous substance or its metabolite or other related products in the biological system. A biomarker of effect is the measurement of endogenous components, including biochemical, physiological or other alterations in the biological system that reflect possible health impairment or disease. A biomarker of susceptibility is the inherent ability of the biological system to respond to a specific challenge of chemical exposure (Sarkar *et al*., 2006; Van der Oost *et al*., 2003).

In order to assess the potential threats in the environment, detection of biochemical responses in the exposed animals, reflecting the impairment of physiological processes has become an important approach for the detection of all toxicants is impractical. In fish, the impact of toxic xenobiotics can be investigated with the help of different biochemical and biological parameters that represent potential biomarkers of exposure or effect. Out of these parameters the following were used in this study; biotransformation enzymes (CYP1A, CYP3A, GST), oxidative stress parameters (GPx, CAT, GR), biotransformation products (protein, RNA, DNA), reproductive and endocrine parameters (Vtg, *Zrp*, and estrogen receptor alpha (ERα)) (Van der Oost *et al*., 2003).

CYP1A in fish as a result of exposure to certain classes of organic contaminants is specifically used as a biomarker (Cajaraville *et al*., 2000). The determination of CYP1A induction via ethoxyresorufin *O*-deethylase (EROD) activity in fish liver is a reputable sensitive biomarker of xenobiotic exposure in fish. Thus, investigation of cytochrome P450 in fish has been used in monitoring environmental pollutants in the aquatic environment (Blahova *et al*., 2009; Havelková, Randák, Blahová, Slatinská, and Svobodová, 2008; Sarkar *et al*., 2006; Uno *et al*., 2012). Since the expression and activity of CYP3A are influenced by xenobiotics, an alteration in the expression of CYP3A could also be used as a biomarker of xenobiotics exposure in fish. Therefore, the enzyme can be investigated by measuring the change in mRNA and CYP3A activity as suitable biomarkers of effects and exposure of xenobiotics, respectively (Christen *et al*., 2009). Among other phase II biotransformation enzymes, GST has gained credibility as biomarkers of environmental pollutant exposure in monitoring pollution of the aquatic environment (Sayeed *et al*., 2003). Further details about GST enzyme can be found in the phase II biotransformation enzymes section.

Additionally, oxidative stress caused by several environmental pollutants has been used for biomarker development. Yet, when compared to phase I and II enzymes, antioxidant enzymes are generally less responsive to pollutants (Kopecka-Pilarczyk and Correia, 2009). Their detoxification activities, however, gains potential application in oxidative stress investigations (Van der Oost *et al*., 2003). In addition, it should be noted that there is no single sensitive and specific biomarkers identified to study oxidative stress. Among the oxidative stress investigation parameters, assaying the activities of antioxidant enzymes have been used as the most sensitive biomarkers to indicate the antioxidant status of the organism. Some of the investigated enzymes include GR, GPx, CAT, and GST (Slaninova *et al*., 2009; Van der Oost *et al*., 2003).

1.7.1 Reproductive parameters biomarkers

It has been reported that the widespread occurrence of a number of xenobiotics to have reproductive effects through endocrine activity that might influence the survival of vulnerable species. Fish have been found demonstrating responses to pollution stress with effects on reproductive competence, including reduced fecundity and long term population decline and extinction. Additionally, it has been documented that pollutants utilize multiple sites to influence the reproductive system. This includes malformations of organs (gonads, pituitary, liver, and brain), inhibition of hormone (hypothalamus, pituitary, and gonads) production and secretion, and altered metabolism in the liver (Van der Oost *et al*., 2003). It was found that induction of hepatic CYP1A activity in xenobiotic exposed fish interfered with the activity of

the general cytochrome P450 enzymes and eventually influence regulation of sex steroids. This situation may be linked to accelerated testosterone clearance rates by cytochrome P450 enzymes and influence plasma E2 level. In addition to this, upon binding to the ER, xenoestrogenic pollutants (e.g., DDTs, HCHs and alkylphenols) cause estrogenic effects (Van der Oost *et al*., 2003).

Even though the latter is more sensitive, the synthesis of both Vtg, the eggyolk precursor protein, and *Zrp*, eggshell protein, in the liver is under the influence of E2. Reduced plasma E2 and Vtg concentration, reduced ovarian growth, and impaired pituitary gland secretion were observed in female Atlantic croaker (*Micropogonias undulatus*) exposed to Arochlor 1254 (Arukwe *et al.*, 2000). Additionally, pollutants such as nonylphenol, bisphenol-A, PCBs and PAHs have also shown an affinity to ER, which might influence Vtg (Van der Oost *et al*., 2003). Induction of Vtg and subsequent feminization of male fish were also reported after exposure to endocrine disrupting chemicals. Therefore, Vtg response in male and juvenile fish may be used as a sensitive biomarker of exposure to estrogenic compounds (Arukwe *et al*., 2000; Van der Oost *et al*., 2003). Induction of *Zrp* has been reported at lower plasma E2 level than Vtg in juvenile Atlantic salmon. Thus, *Zrp* may serve as a sensitive biomarker for environmental estrogens (Arukwe *et al*., 2000). Both proteins can be investigated at the gene expression level by analyzing their mRNA or by quantification of protein levels using antibodies that specifically bind to these proteins (Van der Oost *et al*., 2003).

1.8 Environmental pollution

As the world population is growing and many communities (e.g., South Asia and Sub-Saharan Africa) are facing repeated problems of hunger and malnutrition, fisheries products are extremely needed to meet the parallel demand for nutritious and healthy food (Garcia and Rosenberg, 2010; Golden *et al*., 2016). However, the productivity of the aquatic environment is hugely strained by growing pollution, toxic contamination, coastal degradation, excessive fishing pressure, and climate change (Garcia and Rosenberg, 2010). The massive population growth in many developing countries has been creating a strong expansion of uncontrolled urbanization, agricultural, and industrial land use, which eventually results in a significant increase in pollutant release into waterways (Adeyemo, 2003). In Africa, agricultural products, untreated municipal sewage, and industrial wastewater are the main sources of pollutants that are continuously discharged into the aquatic environments. The situation is even exacerbated by lack of effective environmental pollution control laws or law enforcement and poor waste management system (Adeyemo, 2003; Kivaisi, 2001; Amaeze, Egonmwan, Jolaosho, and Otitoloju, 2012).

The city of Lagos, where the Lagos lagoon (our study site) is situated, is West Africa's "most important" coastal city with a population of more than 10 million and the center of economic and business development in the region, accommodating around 65% of the country's industrial institutions and a home to four of the country's eight seaports (Adelekan, 2010; Adelekan and Asiyanbi, 2016). As a consequence, the city has been experiencing a high level of urbanization and industrialization, which leads to the generation of domestic and industrial wastes, creating biological impacts in the coastal aquatic environment (Okoye, Afolabi, and Ajao, 1991).

Like other coastal aquatic environment, the Lagos lagoon system is receiving effluents from over 2000 medium and large-scale industries in Lagos city and the neighboring Ogun State (Amaeze *et al*., 2012). Both natural and anthropogenic activities such as urbanization and industrial development have created pressure on the lagoon resources. Additionally, onshore and offshore crude oil exploration activities along the coastal environment by various multinational companies have been creating crude oil spillage (leakage from pipelines and accidental spills) into the lagoon system. In connection to this, petroleum hydrocarbons have been reported in the aquatic ecosystems of the Niger Delta, including the surface water, sediment, and local seafood species (Benson, Essien, Asuquo, and Eritobor, 2014). It has been suggested that human activities in and around Lagos lagoon are the major sources of PAHs, which may lead to a huge reduction in fishery resources and threatening their sustainability (Sogbanmu *et al*., 2016). Along with several other fish species, the lagoon system has been dominated by resistant Tilapia species (e.g., *Sarotherodon melanotheron*, Ruppel) and catfish species (e.g., *Chrysichthys nigrodigitatus*, Lacepede) that are tolerant to hypoxic and sometimes polluted water bodies (Ayoola and Kuton, 2009; Emmanuel and Onyema, 2007). This situation may be an indication of higher pollutant level in the lagoon system (Amaeze *et al*., 2012). Previous studies revealed that water samples from the lagoon contain a wide range of OCPs, which are possibly from point sources and through agricultural runoff (Adeyblissemi, Ukpo, Anyakora, and Uyimadu, 2009). Additionally, fish and sediment samples from the lagoon were also found with higher concentration of PCBs (Adeyemi, Anyakora, Ukpo, Adedayo, and Darko, 2011) and heavy metals (Okoye *et al*., 1991). Therefore, sand mining and harbor dredging activities in the Lagos lagoon contribute to the reintroduction of contaminants from the sediments into the aquatic phase by inadvertently resuspending sediment into the water column (Amaeze *et al*., 2015; Eggleton and Thomas, 2004). As a result of these, the aquatic biota near the benthos is continuously exposed to contaminants (Amaeze *et al*., 2015).

In relation to this, Sogbanmu *et al*. (2016) reported that fish exposed to sediment extracts from the Lagos lagoon showed teratogenic, embryotoxic and genotoxic effects, which are

correlated to high molecular weight-PAHs. Moreover, different studies showed that fish species inhabiting the Lagos lagoon system suffer from severe intersex (Adeogun, Ibor, Omiwole, and Arukwe, 2014), developmental and reproductive effects in relation to phthalic esters exposure, which were detected and measured in both water and sediment (Adeogun *et al*., 2015), obesogenic effects of environmental pollutants (PCBs and PAHs) in Tilapia species from Ogun River, which is the major source of water to the lagoon (Adeogun, Ibor, Regoli, and Arukwe, 2016), physiological and other effects. However, more studies to establish the state of pollution in relation to detrimental effects to the health of the ecosystem and humans (due to the consumption of contaminated fish) in the Lagos lagoon was recommended (Sogbanmu *et al*., 2016).

1.9 Study objectives

Thus, the aim of the present study was to further investigate and document whether the overt physiological and health effects observed at the Lagos lagoon were due to accumulated contaminants in sediment samples, using the Atlantic salmon, *Salmo salar*, as model species. Specifically, this project was designed to investigate the molecular and biochemical effects in Atlantic salmon exposed to sediment extracts from Makoko and Ikorodu locations of the Lagos lagoon system by examining:

(a) the main liver enzymatic activities of the biotransformation (CYP1A1, CYP3A, and GST) and antioxidant (GR, GPx, and CAT) systems, and

(b) the expression of estrogenic responses (ERα, Vtg, and *Zrp*) in the liver.

The hypothesis is that exposure of salmon to sediment extracts from Makoko and Ikorodu sites of the Lagos lagoon system will produce molecular and biochemical effects that affect endocrine, biotransformation functions and oxidative stress, which are indicative of potential and adverse health effects.

1.10 The Atlantic salmon as a model organism

The Atlantic salmon, *Salmo salar*, belongs to the family Salmonidae, which also includes whitefish, graylings, trout, and charr (Figure 1-9) (Von Schalburg *et al*., 2008). The Atlantic salmon is an anadromous species, which spawn in freshwater in the fall. The embryo, then grows in the streambeds over the winter and hatched as fry in the spring. The young fish (parr) remain in freshwater and grow for 2 to 4 years and undergo a transformation to seaward-migrating smolts. The smolts then migrate downstream very quickly to sea (Hansen and Quinn, 1998; Metcalfe, Huntingford, and Thorpe, 1988). After smolting in the freshwater and undertaking a long journey in the ocean, adult salmons return to their natal river to spawn with high accuracy. Even though distinct genetic differences between the anadromous species, there

are also some populations that do not migrate to sea (these populations may be landlocked) (Hansen and Quinn, 1998).

Regarding their distribution, Atlantic salmon is native to the temperate and subarctic regions of the North Atlantic Ocean. They occur in the northwest Atlantic stretching from the Ungava Bay of Canada to the New England in the United States, and in the northeast Atlantic, salmon occur from northern Portugal in the south in the Barents and White Sea areas of northern Europe and Russia. They also inhabit rivers in the United Kingdom, Iceland, Ireland and Greenland (only one river) (Thorstad, Whoriskey, Rikardsen, and Aarestrup, 2010).

Figure 1-9: The Atlantic salmon (Salmo salar) that was used as a model organism in this study.

The majority of the salmonid species, including Atlantic salmon are known for their significant economic value through aquaculture, wild stock fisheries, and recreational sport fisheries. They also offer social benefits, for example, as a traditional food source. Furthermore, some of them have environmental importance, which can be used as a sentinel species for monitoring the aquatic environment. For example, salmon and trout have been widely used for ecotoxicology studies. As a result, the salmonid species, therefore, receive a combined commercial and scientific attention (Davidson *et al.*, 2010). Therefore, the ecological and economically important fish species, Atlantic salmon (*Salmo salar*), was used in this study.

2 MATERIALS AND METHODS

2.1 Chemicals and reagents

Dimethyl sulfoxide (DMSO) and 2-propanol were purchased from Merck KGaA Chemicals (Darmstadt, Germany), Ethanol was obtained from VWR BDH Prolabo (Fontenay-sous-Bois, France), 17α-Ethynylestradiol (EE2), dichloromethane (DCM), purpald®, bovine serum albumin (BSA), NADPH, 1-chloro-2,4-dinitrobenzene (CNDB), ethoxyresorufin, methoxyresorufin, and benzyloxyresorufin, were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Direct-zol™ RNA MiniPrep RNA isolation kit was purchased from Zymo Research Corporation (Irvine, CA, USA). iScript cDNA Synthesis Kit and iTaq SYBR Green Supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA). IScript cDNA synthesis kit and iTaq™ universal SYBR® Green supermix with ROX were purchased from Bio-Rad Laboratories (Hercules, CA, USA). All other chemicals were of the highest commercially available grade.

2.2 Study area

The study was conducted in the Lagos lagoon (Longitude 3°23″ and 3°40″E and latitude 6°22″ and 6°38″N). The lagoon is the largest tropical lagoon complex in the Gulf of Guinea, expanding to 250 km from Cotonou in the Republic of Benin to the borders of the Niger Delta (Figure 2-1) (Adeogun *et al*., 2015). The lagoon system links the Atlantic Ocean (in the west and south) and Lekki lagoon (in the east) with an area of 6354.708 km^2 (Alani, Olayinka, and Alo, 2013). It is a brackish water environment that is fed in the north by a number of large rivers, including Ogun, Yewa, Ona, and Osun rivers as well as tidal creeks. Of which, the Ogun river discharges a large volume of water into the lagoon. During the rainy season, the lagoon empties in the south and opens into the Atlantic Ocean via the Lagos harbor (Adeogun *et al*., 2015; Amaeze *et al*., 2015). It serves as a means of livelihood and transport, residential and recreation site for the surrounding Lagosian. It also functions as a dumpsite for residential wastes and receives a discharge from the surrounding industries that account about 80-85% of the industries in Nigeria. As in other developing country, very few industries in Nigeria have treatment plant and most industries discharge mainly untreated effluents (Adeogun *et al*., 2015). The lagoon is inhabited by several marine and freshwater species and serve as breeding and feeding grounds for some of them. Regarding the fish inhabitants of the lagoon, Tilapias (*Oreochromis niloticus*, *Tilapia melanotheron* and *T. zillii*) and Catfishes (*Clarias* and *Chrysichthys* sp) are among the freshwater fish species. Additionally, there are marine fish species, including Mullets (*Mugil* and *Liza* sp), Ten pounder (*Elops lacerta*), Clupeids (*Ilisha africana*, *Ethmalosa fimbriata*) and Sciaenids (*Pseudotolithus typus* and *P. senegalensis*).

Moreover, indigenous species also exist, which include mudskipper (*Periophthalmus papilio*) and gobiid (*Bathygobius soporator*) (Lawson, 2011).

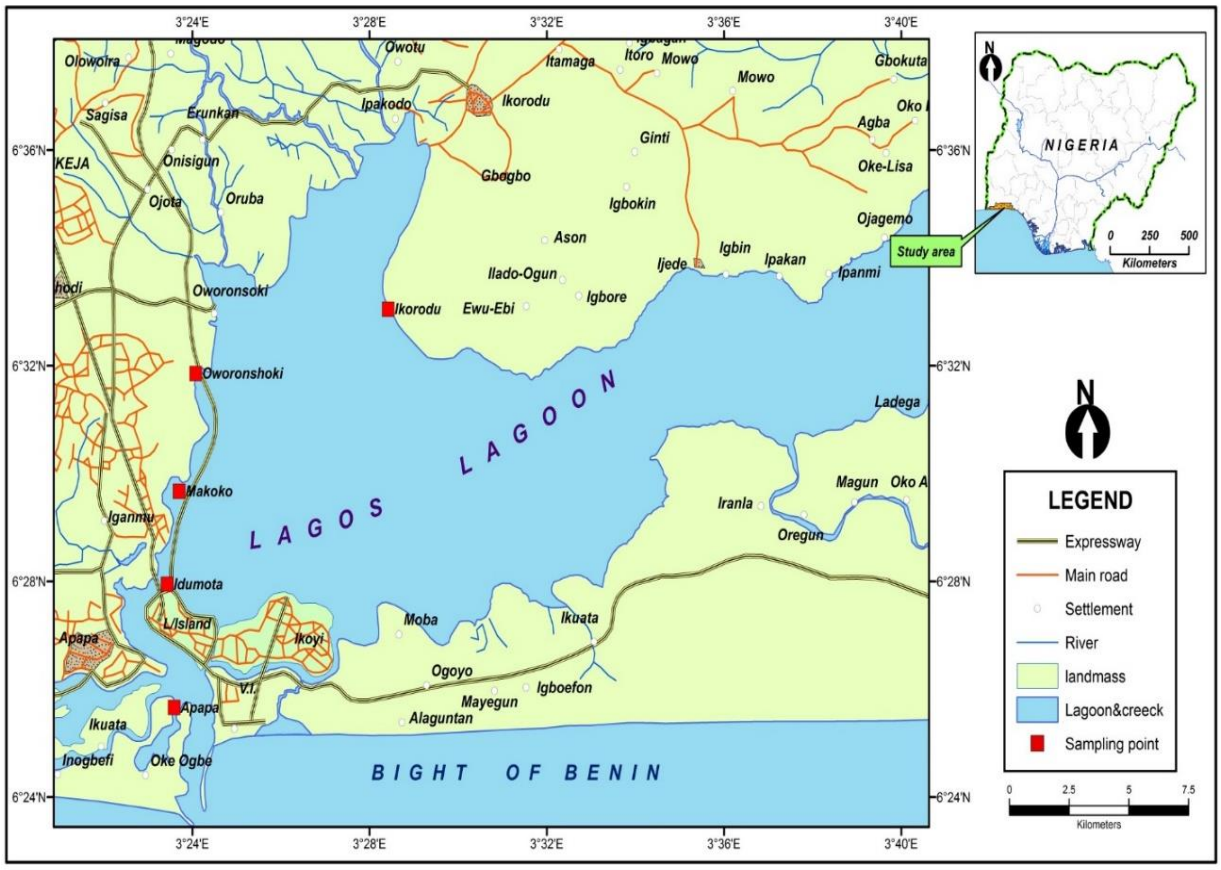

Figure 2-1: *Map of the Lagos lagoon showing the sampling sites where the sediment samples were collected. Out of the collected samples, only Makoko and Ikorodu sediment samples were used due to insufficient sample volume from the other sites (Apapa, Edumota, and Oworonshoki) during pollutant extraction.*

2.3 Sediment sample collection and extraction

2.3.1 Sample collection

The sediment samples were collected by Arukwe research group at the department of biology, NTNU. The sediment samples were collected from five different sampling locations, namely Makoko, Ikorodu, Apapa, Edumota, and Oworonshoki of the Lagos lagoon (Figure 2-1). However, only Makoko and Ikorodu sediment samples were used for this study due to logistic issues. Sediment samples from the two sites were collected, dried and sieved to remove particles and to collect fine sediment samples. The resulting sieved sediment samples were wrapped with aluminum foil and transported to the laboratory in polyethylene bags where they were stored frozen at -20 ^{o}C. The samples were kept in a freeze dark room at -20 ^{o}C before extraction and treatment (Østby, Sundby, and Krøkje, 2006).

2.3.2 Ultrasonic extraction procedure

2.3.2.1 Extraction of polar and non-polar pollutants

Both polar and non-polar pollutants were extracted from the sediment samples using an ultrasonic extraction procedure, which was carried out as previously described by Østby *et al*. (2006) (Figure 2-2). Separate sediment samples and different extraction solvent were used for the polar and non-polar extractions. From each location, 50 g of sediment was distributed into ten clean ultrasonic test tubes each containing 5 g sediment. To each test tube, 30 ml of extraction solvent, either dichloromethane (DCM) for non-polar or isopropanol for polar pollutant extraction, was added and the test tubes were placed in a rack. The rack was then placed in the ultrasonic bath previously filled with distilled water and ultra-sonication was performed using an Ultrasonik 6QT (*Ney, BarkMeyer Division, Yucaipa, CA, USA*). Extraction was performed for 30 minutes at 40 KHertz and then centrifuged at 1000xg for 10 minutes. The supernatant was pipetted out gently into a 500 ml capacity collection Erlenmeyer flask. To the sediment remaining in the ultrasonic tubes, 25 ml DCM or isopropanol was added for second time extraction for another 30 minutes and centrifuged as described above. The supernatant then combined with the first extract and the whole supernatant was filtered through a glass-sinter filter (SF33) and stored at 4 ^{o}C and dark place before drying.

Different drying methods and equipment were used for polar and non-polar pollutants. For the non-polar pollutants, the extract was allocated into 10 ml capacity test tubes. The test tubes were placed in a rack and evaporated to dryness at room temperature under a stream of nitrogen using a Techne sample concentrator and a Techne Dry block, type DB3D (Techne, Cambridge, UK). The concentrator has a gas chamber mounted above a block onto which the rack containing test tubes with the samples are placed. The chamber is also connected to a pure nitrogen cylinder. The gas chamber is fitted with hyperdermic needles which bring the nitrogen down from the chamber into the test tubes. The needle's end was placed into the test tubes above the liquid surface where the flow of gas displaces the evaporated solvent from the extract. After drying, the residue was dissolved by adding DMSO. A 100 µl of stock solution of the sediment extract corresponded to 100 mg of sediment (Østby *et al*. 2006).

For the polar pollutants, the RV® 10 digital rotary evaporator (IKA Works Inc., Germany) was used for drying the extracts (Miranda, Cunha, Dores, and Calheiros, 2008). The evaporator was adjusted to the following conditions: water bath temperature, 43 ^{o}C; pressure, 140 mbar; revolution, 90 rpm within a 4 second interval for 20 to 25 minutes. Then, 30 ml of sample extract was transferred into a round bottom flask (1000 ml evaporator piston) and attached to the rotary head and the collection flask to the condenser using stainless steel clamps. The evaporation system was closed and vacuum condition was created. The rotary head was

lowered to the bottom position until the evaporator flask was surrounded by heated distilled water in the water bath. Finally, the evaporation was completed within 20 to 25 minutes and the rotary head was moved back to upper position. After drying, the extract was resuspended in 1.5 ml mixture of ethanol and water (1:1 ratio). Finally, both redissolved polar and non-polar extracts were stored at 4 ^{o}C in the dark before the exposure experiment.

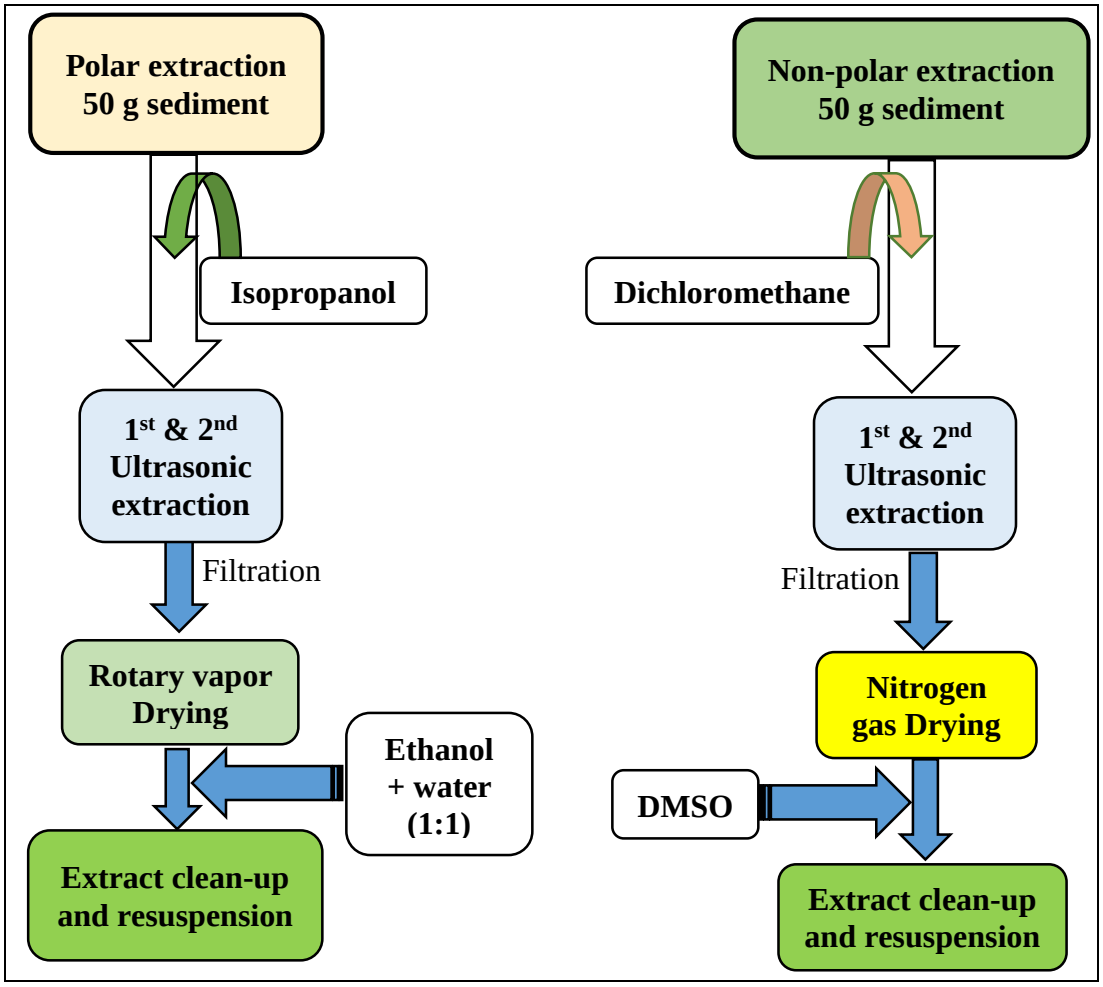

Figure 2-2: Polar and non-polar sediment pollutants extraction procedures that were performed in this study. Both procedures were done for each sampling site and both polar and non-polar sediment extracts were used for the fish exposure experiment.

2.4 Fish exposure experiment

Juvenile Atlantic salmon with mean weight 75±17.5 g and length 21±1.6 cm were purchased from Lundamo hatcheries (Trondheim, Norway) and transported to the Norwegian University of Science and Technology (NTNU) Centre of Fisheries and Aquaculture (SeaLab) animal holding facilities and kept in aerated aquariums at 10 ^{o}C. Before the exposure, the fish were acclimatized to a temperature of 10 ^{o}C and at 12:12 hours light:dark photo period for eight days. In order to investigate molecular and biochemical effects in salmon exposed to sediments from

different locations of the Lagos lagoon system, Nigeria, six groups of (12 fish per group) fish were placed in different aquariums each containing 50 liters tap water at 9.5±0.3 ^{o}C and 79.7±8.8 % dissolved oxygen (Table 2-1). Two groups were exposed to extracts from Ikorodu sediment samples (one polar and one non-polar exposure groups), two groups were exposed to extracts from Makoko sediment samples (one polar and one non-polar exposure groups), one group was exposed to EE2 (2.5 µg/ml), and a control group exposed to the carrier compound, DMSO that assists in the solubilization and dispersion of lipophilic pollutants into the test media (Hallare, Nagel, Köhler, and Triebskorn, 2006). The final concentration of DMSO was kept 160 ppm in all exposure groups. Fish were exposed for five days under aerated condition without water replacement and they were not fed during acclimatization and exposure days. Unfortunately, all the fish of Ikorodu polar and Makoko non-polar exposure groups were found dead on day four and day five (sampling day) respectively. Eight individuals from all exposure and control groups (DMSO) were sacrificed at day five after exposure. Then the fish were stunned by a blow to the head and become unconscious, fish length and weight were recorded and blood samples was collected quickly from the caudal vein using heparinized syringe and transferred into a pre-cooled eppendorf tubes. After sacrifice, whole liver was excised, weighed, and wrapped with aluminum foil. Finally, the liver samples were snap frozen in liquid nitrogen and stored at -80 ^{o}C prior to further analysis. The fish carcasses were wrapped with aluminum foil and stored at -80 ^{o}C.

Table 2-1: Overview of the six exposure regiments and their respective exposure chemical mixtures prepared and executed in this study.

Exposed compound	Mixture components	Remarks
Control (DMSO)	0.016 % DMSO	The true control group
Ethynylestradiol	17α-ethynylestradiol dissolved in DMSO (2.5 µg/ml and 0.016% final concentration in the aquarium, respectively)	Positive control group
Makoko polar extracts	Polar sediment extract dissolved in a clean-up solvent (ethanol in distilled water at 0.08 % final concentration in the aquarium)	
Makoko non-polar extracts	Non-polar sediment extract dissolved in DMSO (0.016 % final concentration in the aquarium)	All fishes were died at day four
Ikorodu polar	Polar sediment extract dissolved in a clean-up solvent (ethanol in distilled water at 0.08 % final concentration in the aquarium)	All fishes were died at day five.
Ikorodu non-polar	Non-polar sediment extract dissolved in DMSO (0.016 % final concentration in the aquarium)	

2.5 Preparation of post-mitochondrial fraction (PMF)

Before PMF preparation, the homogenizing buffer and the pestle of the tissue homogenizer were kept on ice in advance. From the intact liver samples, a volume of 0.3-0.8 g was quickly excised and homogenized in four volumes of 0.1 M homogenizing sodium phosphate buffer (containing 100mM ethylenediaminetetraacetic acid (*EDTA*), 1mM dithiothreitol, and 10% glycerol at pH 7.4) containing 0.15 M KCl using a motorized pre-chilled Glass-Teflon tissue homogenizer as described by Pesonen and Andersson (1987). Homogenate were centrifuged at 12,000 x g for 20 minutes at 4 ^{o}C and the supernatant containing PMF was separated in aliquots and preserved at -80 ^{o}C before further analysis.

2.6 Biochemical analysis

2.6.1 Protein assay

Total protein concentration was determined from the PMF by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as standard. Briefly, 80 mg of Coomassie Serva Blue G was dissolved in 100 ml ethanol (95%) and then mixed with diluted phosphoric acid (17%) separately in a ratio of 1:1 and made a final concentration of 0.004%, 4.75% and 8.5% (W/V) coomassie Serva Blue G, ethanol, and phosphoric acid respectively. After measuring the absorbance of diluted BSA (0.2 mg/ml) at 280 nm and calculated the exact concentration using an extinction coefficient of 0.667 cm^{-1} ml/mg, nine separate serial dilutions were made with different concentrations (0-0.07 mg/ml) in distilled water. A volume of 500 µl of the different BSA dilutions was prepared and 3 ml of the previously made coomassie reagent was added to each dilution. These were used as a standard to determine the protein in unknown samples. The PMF samples were then diluted in a ratio of 1:4 in distilled water. Using ELISA microtiter plate, 50 µl from each diluted sample and 300 µl of coomassie reagent were added into triplicate wells. A volume of 350 µl from the mixture of BSA standards and coomassie reagent were also added into separate wells in parallel with the unknown samples. The absorbance was then measured at 595 nm after 5 minutes with the help of a Synergy^{TM} HT microplate reader (Bio-Tek Instruments, Winnoski, VT, USA). The final concentration of protein in each sample was then calculated based on the plate readers value and expressed as mg protein per ml of BSA standard.

2.6.2 Enzyme assays

The PMF was used to carry out the enzyme assays. Absorbance and fluorescence measurements related to enzyme activities were performed using a Synergy^{TM} HT microplate reader (Bio-Tek Instruments, Winnoski, VT, USA). The reactions were run in triplicates and enzyme activity

values were normalized to protein content in the samples. Unless described, all enzyme activities were assayed at room temperature (25 ^{o}C) (Arukwe and Nordbø, 2008).

2.6.2.1 EROD, MROD, and BROD assays

Enzyme assays were done according to Burke and Mayer (1974). For the analysis of ethoxyresorufin *O*-deethylase (EROD), methoxyresorufin *O*-demethylase (MROD), and benzyloxyresorufin *O*-debenzylase (BROD), 50 µl PMF sample and 100 µl NADPH (0.25 mg/ml in phosphate buffered saline with a pH of 7.3) were added to the wells of 96 well, black, clear bottom fluorometric plate and incubated at 37 ^{o}C for 10 minutes. The reaction was started by adding 1 µl of 250 µM (in DMSO) corresponding substrate: ethoxyresorufin (EROD), benzyloxyresorufin (BROD), and methoxyresorufin (MROD) and fluorescence was measured in a microplate reader (excitation: 535 nm; emission: 590) for 20 minutes. As a positive control, a known sample was run together with unknown samples for each plate to assure the stability of the results (Mortensen and Arukwe, 2007b). Enzyme activities were calculated based on a pre-read standard curve generated from known dilutions of resorufin and expressed as the concentration of resorufin produced per milligram protein per minute (pmol/mg/minute) (Adeogun, Ibor, Onoja, and Arukwe, 2016).

2.6.2.2 Glutathione reductase (GR) assay

GR is an abundant flavoenzyme that catalyzes the NADPH-dependent reduction of oxidized GSSG back to the reduced form GSH. This keeps the high GSH/GSSG ratio in the cell, which is essential for the cell to combat oxidative stress (Kim *et al*., 2010; Saydam *et al*., 1997; Zhu *et al*., 2013). Quantification of GR activity was performed by measuring the oxidation of NADPH, which is used as electron donor during the reduction of GSSG to GSH (Lin and Shiau, 2005; Mourente, Dıaz-Salvago, Bell, and Tocher, 2002).

GR was assayed as described by Willmore and Storey (2007) with modifications. The assay solution contained 50 mM potassium phosphate buffer (1 mM EDTA and pH 7.5), 9.5 mM GSSG, and 1.5 mM NADPH. Background controls that contain the assay buffer instead of the sample were also included. A total volume of 190 µl solution was applied into each well of a 96 well microtiter plate, which contain 100 µl assay buffer, 20 µl diluted PMF sample, 20 µl GSSG, and 50 µl NADPH. The mixture was then mixed carefully and the absorbance was measured at 340 nm at every minute for 5 time points using the NADPH extinction coefficient of 0.00373 μM^{-1*}. After correcting all the samples absorbance by the background absorbance, the change in absorbance values of each sample was used to calculate the GR activity based on

* The actual extinction coefficient for NADPH at 340 nm is 0.00622 µM-1 cm-1. This value was adjusted for the path length of the solution in the well (0.6 cm).

equation 5. One unit is defined as the amount of enzyme that will cause the oxidation of 1.0 nmol of NADPH to NADP+ per minute at 25 °C. Finally, the calculated values of each sample were divided by their respective protein concentration and the GR activity was expressed as nmol NADPH oxidized /minute/mg protein in the graphs (Sandhir and Gill, 1995).

$$GR\ activity = \frac{\frac{\Delta\ Absorbance\ at\ 340\ nm}{minute}}{0.00373\ \mu M^{-1}} * \frac{0.19\ ml}{0.02\ ml} * Sample\ dilution\ factor \qquad (5)$$

Where 0.00373 μM^{-1} represents the adjusted extinction coefficient; 0.19 ml = reaction volume; 0.02 ml = volume of the sample; and GR activities are reported as nmol/minute/mg protein.

2.6.2.3 Glutathione S-Transferase (GST) assay

GST enzymes are an important part of phase II detoxification reactions that conjugate GSH with a wide range of endogenous and exogenous electrophilic compounds and facilitate their excretions in bile or urine (Nimmo, 1987). The enzyme activity is measured by forming GSH-CDNB conjugate and monitoring the change in absorbance at 340 nm (Saydam *et al*., 1997).

The hepatic GST activity was assayed as described by Habig, Pabst, and Jakoby (1974) using CNDB as substrate. The assay solution contained 0.1 M sodium-phosphate homogenizing buffer (pH 7.42) 100 mM CDNB in DMSO, and 1 mM GSH. Background controls that contain the assay buffer instead of the sample were also included. A total volume of 220 µl solution was applied into each well of a 96 well microtiter plate, which contain 10 µl homogenizing buffer, 10 µl PMF sample, and 200 µl of a mixture of CDNB and GSH (this contain 1 ml of 100 mM CDNB, 40 ml homogenizing buffer, and 1 ml of 1mM GSH). The mixture was then mixed carefully and the absorbance was measured at 340 nm at every minute for 5 time points using the CDNB extinction coefficient of 5.76 x 10^{-6} nM^{-1}[†]. After correcting all the samples absorbance by the background absorbance, the change in absorbance values of each sample was used to calculate the GST activity based on equation 6. Finally, the calculated values of each sample were divided by their respective protein concentration and the GST activity was expressed as pmol CDNB conjugate formed/minute/mg protein in the graphs (Shah and Iqbal, 2010).

$$GST\ activity = \frac{\frac{\Delta\ Absorbance\ at\ 340\ nm}{minute}}{5.76\ x\ 10^{-6}\ nM^{-1}} * \frac{0.22\ ml}{0.01\ ml} \qquad (6)$$

Where 5.76 nM^{-1} represents the adjusted extinction coefficient; 0.22 ml = reaction volume; 0.01 ml = volume of the sample; and GST activities are reported as pmol/minute/mg protein.

[†] The actual extinction coefficient for CDNB at 340 nm is 9. 6 x 10^{-6} nM^{-1} cm^{-1}. This value was adjusted for the path length of the solution in the well (0.6 cm).

2.6.2.4 Glutathione peroxidase (GPx) assay

GPx catalyzes the detoxification of hydroperoxides at the expense of GSH (Equation 8). Particularly, the enzyme plays an important role in the cytosol by reducing hydrogen peroxide to water and oxygen (Olsvik *et al*., 2005; Thomas, Maiorino, Ursini, and Girotti, 1990). The GPx activity was assayed as described by Velma and Tchounwou (2011). The enzyme activity was assayed by a coupled enzymatic system where NADPH is oxidized by GR to convert the GSSG to its reduced form, GSH (Equation 9), which is then used by GPx as a substrate to detoxify hydroperoxides (Equation 8). Therefore, the GPx activity is measured indirectly from the absorbance reduction of NADPH at 340 nm. The assay solution contained 50 mM Tris-HCl assay buffer (5mM EDTA and pH 7.6), 10 mM GSH, 1.5 mM NADPH, 2.4 U/ml glutathione reductase, and 15 mM hydrogen peroxide. Background controls that contain the assay buffer instead of the sample were also included. A total volume of 200 µl solution was applied into each well of a 96 well microtiter plate, which contain 100 µl assay buffer, 20 µl diluted PMF sample, and 20 µl of GSH, 20 µl GSSG, 20 µl of NADPH, and 20 µl of a reaction starter hydrogen peroxide. The mixture was then mixed carefully and the absorbance was measured at 340 nm at every minute for 5 time points using the NADPH extinction coefficient of 0.00373 μM^{-1}. After correcting all the samples absorbance by the background absorbance, the change in absorbance values of each sample was used to calculate the GPx activity based on equation 7. Finally, the calculated values of each sample were normalized by dividing with their respective protein concentration and the GPx activity was expressed as nmol NADPH oxidized /minute/mg protein in the graphs (Sandhir and Gill, 1995).

$$\text{GPx activity} = \frac{\Delta\, Absorbance\ at\ 340/minute}{0.003730 \mu M^{-1}} * \frac{0.2\text{ml}}{0.02\text{ml}} * \text{sample dilution factor} \quad (7)$$

Where 0.00373 μM^{-1} represents the adjusted extinction coefficient; 0.2 ml = reaction volume; 0.02 ml = volume of the sample; and GPx activities are reported as nmol/minute/mg protein.

$$ROOH + 2\ GSH \xrightarrow{GPx} ROH + GSSG + H_2O \quad (8)$$

$$NADPH + H^+ + GSSG \xrightarrow{GR} NADP^+ + 2\ GSH \quad (9)$$

2.6.2.5 Catalase (CAT) assay

The enzyme catalase, which is mainly situated in peroxisomes catalyzes the decomposition of hydrogen peroxide to water and oxygen (Weydert and Cullen, 2010). The hepatic catalase activity was assayed as described by Velma and Tchounwou (2011). First, a 20 µl from each PMF sample and formaldehyde standard solutions were added into separate wells of a 96 well

microtiter plate and then 100 µl of 100 mM potassium phosphate assay buffer (pH 7.0) and 30 µl methanol were separately added to all wells. The reaction was started with 20 µl of 35 mM hydrogen peroxide and the mixture was incubated on an orbital shaker IKA®-Schüttler MTS 4 (IKA Labortechnik, Staufen, Germany) for 20 minutes shaking at 500 rpm at room temperature. The reaction was then terminated by adding 30 µl of 10 M potassium hydroxide. Thirty µl of 34.2 mM 4-amino-3-hydrazino-5-mercapto-1, 2, 4-triazole (purpald) was added into all wells and incubated on the IKA®-Schüttler MTS 4 shaker for 10 minutes shaking at 500 rpm at room temperature. After incubation, 10 µl of 0.065 M potassium periodate was added to all wells and incubated on the IKA®-Schüttler MTS 4 shaker for 5 minutes shaking at 500 rpm at room temperature. Finally, the absorbance of each reaction mixture was immediately measured at 540 nm.

Table 2-2: Preparation of the formaldehyde standard solutions for the catalase assay. The different concentrations were prepared in separate test tubes from a formaldehyde stock solution with 4.25 mM, which was prepared in 25 mM potassium phosphate-sodium hydroxide sample buffer (1mM EDTA, 1% BSA, and pH 7.5).

Test tube label	Formaldehyde stock solution (µl)	Sample buffer (µl)	Final formaldehyde concentration (µM)
A	0	1000	0
B	10	990	5
C	30	970	15
D	60	940	30
E	90	910	45
F	120	880	60
G	150	850	75

The standard samples comprised 0-75 µM formaldehyde solutions in 25 mM potassium phosphate-sodium hydroxide sample buffer (1mM EDTA, 1% BSA, and pH 7.5) (Table 2-1). The standard solution with 0 % concentration, which contained only the sample buffer was used as a blank. After correcting all the samples absorbance by the background absorbance, the absorbance values were converted to concentrations using an equation generated from the standard curve. Finally, the calculated values of each sample were normalized by dividing with their respective protein concentration and the catalase activity was expressed as nmol/minute/mg protein in the graphs.

2.7 Chemical analyses

Trace metals, aliphatic hydrocarbons (C10-C40), PAHs, OCPs, phenolic compounds, and organotin were analyzed in the sediment samples. The chemical analyses were determined according to previous methods with slight modifications (Bocchetti *et al*., 2008; Fattorini *et al*., 2008; Piva *et al*., 2011; Regoli *et al*., 2014) (see Appendix A for the detailed chemical analyses). Trace metals were analyzed by atomic absorption spectrophotometry, with flame (Varian, SpectrAA 220FS) and flameless atomization (Varian SpectrAA 240Z). The aliphatic hydrocarbons (C10-C40) were analyzed with a gas chromatograph (Perkin Elmer) equipped with an Elite-5 capillary column (30 m × 0.32 mm ID × 0.25 μm-df) and a flame ionization detector. The low molecular weight (LMW) and high molecular weight (HMW) PAHs analyses were carried out by high-performance liquid chromatography (HPLC) in a water and acetonitrile gradient by fluorimetric and diode array detection. Additionally, OCPs, phenolic compounds, and organotin were determined according to United States Environmental Protection Agency (EPA) methods 1614, 3550B, 3665A, 3630C, 8020, 8081B, 8082A with slight modifications. The water pollutant content in sediment was determined for all the samples, and the concentrations were expressed as ng/g dry weight (dw) for PAHs, while as μg/g dry weight (dw) for OCPs, phenolic compounds, organotin, trace metals and aliphatic hydrocarbons (C10-C40).

2.8 Gene expression analysis

In RNA-based gene expression analysis, accurate interpretation is influenced by the quantity and quality of starting RNA. Since RNA is prone to degradation, errors during RNA processing that appear to compromise the quality of RNA may strongly affect the downstream outcomes. The quality of RNA is influenced by contaminants, such as RNases, proteins, and genomic DNA. Therefore, good tissue handling and storage practices are paramount in order to maintain the quality of RNA and improve the relevance of the outcome (Fleige and Pfaffl, 2006).

2.8.1 RNA isolation

2.8.1.1 Sample preparation and RNA purification

Total RNA was isolated using the Direct-zol™ RNA MiniPrep RNA isolation kit (Zymo Research) according to the manufacturer's RNA isolation protocol. Briefly, all the samples and buffers were kept on ice before the isolation. From the intact liver samples, a volume of 30-50 mg was quickly excised and homogenized into 600 μl of TRI reagent using polytron homogenizer. The pistil was then washed with distilled water, 70 % ethanol and DNase/RNase free water (i.e. diethyl pyrocarbonate (DEPC) water) between each sample. The homogenized mixture was centrifuged at 13,000 x g for 1 minute and the supernatant was then transferred

into a new RNase-free tube. The same volume of 95-100 % ethanol as volume of sample was added into the supernatant (at 1:1 ratio of ethanol to supernatant) and mixed carefully by inverting the tube a few times. The mixture then transferred into a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 13,000 x g for 1 minute at 4 ºC. The column was transferred into a new collection tube and the flow-through was discarded.

For the washing procedure, 400 µl RNA Wash buffer was added to the column, centrifuged at 13,000 x g for 1 minute at 4 ºC and the flow-through was discarded. The column was then transferred to a new collection tube. From the previously prepared DNase I cocktail (Table 2-2), about 80 µl was directly added to the matrix of the column containing the RNA. The column with the RNA was then kept in the collection tube and incubated at 35ºC for 15 minutes. After incubation, the mixture was centrifuged at 13,000 x g for 30 seconds at 4 ºC.

Washing was then carried out; 400 µl Direct-zol™ RNA PreWash buffer was added to the column, centrifuged at 13,000 x g for 1 minute at 4ºC and the flow-through was discarded (this step was repeated once). For the last washing, 700 µl RNA Wash buffer was added to the column, centrifuge at 13,000 x g for 2 minutes at 4 ºC and the flow-through was discarded. The column then transferred to a new collection tube and centrifuged at 13,000 x g for 2 minutes at 4 ºC. Finally, the column which contains the RNA was carefully transferred to an RNase-free tube.

The RNA elution was finally carried out; 50 µl of DNase/RNase-Free Water was directly added to the column matrix and centrifuge at 13,000 x g for 30 seconds at 4 ºC. The eluted RNA then stored at – 80 ºC prior to next use.

Table 2-3: Compositions of DNase I preparation cocktail for RNA isolation.

Reagents	Volume per reaction (µl)
DNase I (1U/µl)	5
10 x Reaction buffer	8
DNase/RNase free water	3
RNA wash buffer	64

2.8.1.2 RNA concentration and quality verification

The purity and integrity of RNA can be influenced by several factors. Of which the ribonucleases (RNases) create major problems, as they are ubiquitous and present in both prokaryotes and eukaryotes. In this regard, the RNases may be introduced during sample handling and processing. Therefore, the quality and quantity of isolated RNA samples have been rigorously evaluated prior to downstream quantification assays (Aranda, LaJoie, and Jorcyk, 2012; Fleige and Pfaffl, 2006).

The difference in absorption of the different compounds provides the basis for the spectrophotometer RNA quality measurement. Even though both nucleic acids and proteins absorb at different wavelength, the maximum absorption for both DNA and RNA is at 260 nm, while most proteins absorb maximally at 280 nm. Therefore, protein contamination can be verified by measuring the absorbance at 260 nm and at 280 nm. As a result, ratios of absorbance at 260 and 280 nm (A260:A280) above 1.8 are considered as 'pure' samples; whereas samples with ratio values below 1.8 are considered as an indication of protein contamination. However, this ratio value doesn't indicate the presence of phenol and other organic contaminants, which have a maximum absorption at 360 nm. So that, RNA samples with a A260:A230 ratio value greater than 1.8 are considered as 'pure' (Fleige *et al*., 2006; Imbeaud *et al*., 2005; Teare *et al*., 1997).

The purity and concentration of the isolated RNA were primarily assessed by using a NanoDrop® ND-2000c UV-visible spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). To meet the measurement requirement, RNA samples with a concentration that exceeded 1000 ng/µl were diluted to this concentration with DEPC treated Milli-Q water. Therefore, only samples with a ratio value above 1.8 were used for further analysis.

2.8.1.3 RNA integrity analysis

The use of intact RNA is vital for the successful quantitative reverse transcription polymerase chain reaction (qRT-PCR) experiments, thus total RNA integrity is usually performed by inspection of the 28S and 18S ribosomal RNA (rRNA) bands using agarose gel electrophoresis (Bustin and Nolan, 2004**;** Fleige and Pfaffl, 2006; Imbeaud *et al*., 2005). Of the total cellular RNA, around 80% is composed of rRNA while the mRNA fraction is only 2–5%. As a consequence, the denaturing agarose gel displays the two ribosomal fragments, 18S and 28S, as distinct bands in eukaryotes (Aranda *et al*., 2012; Sigma-Aldrich, n.d.; Zekan and LaRiviere, 2016).

For the RNA integrity assessment, some samples were randomly selected and checked by formaldehyde gel electrophoresis (Figure 2-3). Briefly, 5 µl (200 ng/µl) from the isolated and diluted RNA was mixed with 10 µl sample buffer (see Appendix B for the RNA agarose gel preparations). The mixture then heated at 65 ºC for 10 minutes and placed immediately on ice. About 14 µl of the mixture was applied into the wells of GelRed®/ GelRed™ stained agarose gel covered with running buffer and run at 75 V for 10 minutes and then at 55 V for 2 hours. After UV light exposure, the integrity of the RNA samples was assessed by visual inspection of the 18S and 28S rRNA subunits (Figure 2-3).

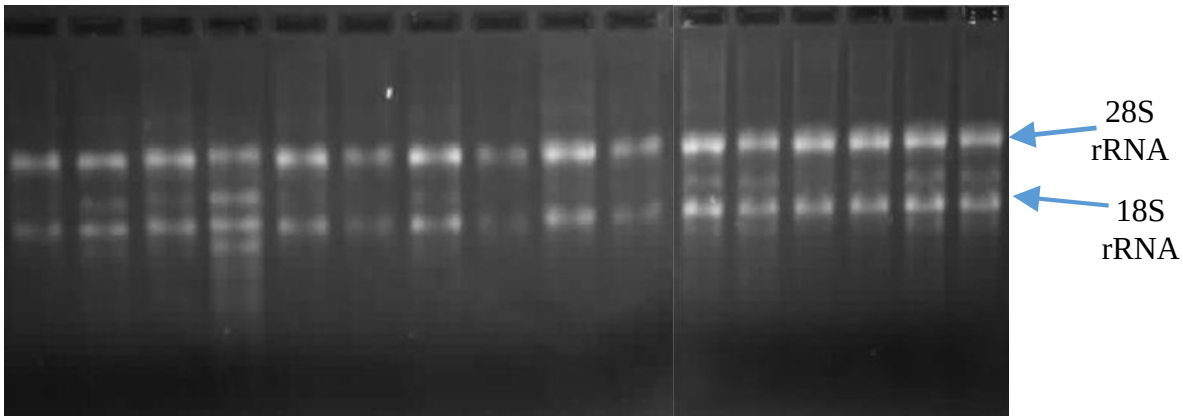

Figure 2-3: Total RNA integrity assessment using 1% formaldehyde agarose gel electrophoresis. The two distinct bands are visualized representing the 18S and 28S rRNA subunits.

2.8.2 Complimentary DNA (cDNA) synthesis

Since the polymerase chain reaction (PCR) uses only cDNA as a template instead of RNA, the reverse transcription of the isolated RNA into cDNA is the first step in a reverse transcription-polymerase chain reaction (RT-PCR) assay (Bustin, 2000). The RT assay usually generates the cDNA transcript using the RNA-dependent DNA polymerase enzyme (reverse transcriptase, RT). The RT-PCR assay can be carried out either as a one-step process that combines the RT and PCR reaction in one tube or a two-step process that execute the RT and PCR reactions separately in two tubes (Bustin, 2000; Nolan, Hands, and Bustin, 2006; Wacker and Godard, 2005). The one-step RT-PCR process is faster to set up, less expensive, and required less sample handling that potentially reduce errors and contamination. However, the RT and PCR reactions performed in one reaction tube may not allow to amplify other genes of interest for later analysis. Since the RT reaction is separated from the PCR reaction in the two-step RT-PCR, the cDNA generated from the RT reaction, however, can be stored and used at a later time to amplify other genes of interest (Wacker and Godard, 2005).

The total RNA reverse transcription to cDNA was performed separately from the PCR reaction using the iScript cDNA synthesis kit, following the manufacturer's protocol (Bio-Rad, n.d.-a). The iScript uses a modified Moloney murine leukemia virus (MMLV)-derived reverse transcriptase. The enzyme has an RNase H+ endonuclease activity that specifically degrades only the RNA in RNA:DNA hybrids with no effects on pure RNA, which provides greater sensitivity in quantitative PCR. First, all the necessary equipment and the bench area were cleaned with a 10 % chlorine and 70 % ethanol. Additionally, the iScript reaction mix consists of a mixture of oligo(dT) and random hexamer primers that can be used for a wide variety of targets and optimized for targets with a length less than 1kb (Bio-Rad). The RNA samples were thawed on ice and the concentration of each sample was adjusted to 1 µg using nuclease free water in total 5 µl final volume based on their a NanoDrop® UV-visible spectrophotometer

measurements. From each diluted RNA sample, 5 µl was applied into the different wells of the 96 well plate and 15 µl from the 5x iScript reaction mix was also added to each well (Table 2-3). No template control (NTC) that contain all components except the RNA as a template was also included. The plate was then incubated in T100^{TM} Thermal cycler (Bio-rad, Singapore) set at 25 ºC for 5 minutes, 42 ºC for 30 minutes, and 85 ºC for 5 minutes. To minimize the risk of contamination, all reactions were set up on the ice. The plate with the synthesized cDNA was then stored at -20 ºC.

Table 2-4: Components of the reaction setup for a single cDNA synthesis reaction.

Components	Volume per reaction
5x iScript reaction mix	4 µl
iScript reverse transcriptase	1 µl
Nuclease-free water	10 µl
RNA template (diluted to 1 µg)	5 µl
Total volume	20 µl

2.8.3 Quantitative (real-time) polymerase chain reaction

Quantitative real-time PCR is the most common and reliable measurement method for characterizing gene expression patterns and comparing mRNA levels in different samples. The amount of gene expression is measured by the polymerase chain reaction (PCR) process that generates products in each cycle, which are directly proportional to the starting amount of templates in each sample before the PCR process (Bustin, 2002; Ginzinger, 2002). During the PCR reaction, the copies of DNA template are generated exponentially and is measured based on the amount of emitted fluorescence (Ginzinger, 2002; Klein, 2002). The iTaq™ universal SYBR® Green supermix with ROX (Bio-Rad) was used in this study, which contains hot-start iTaq DNA polymerase and SYBR® Green I dye with other components. The *Thermus aquaticus* (Taq) DNA polymerase has significantly simplified the PCR process as it doesn't need to reload enzyme after each PCR cycle. The enzyme's ability to function within a wide annealing and extension temperature range (optimum centered around 75 ºC) makes it unique and improves the specificity of the enzyme, which intern increases the sensitivity of PCR for detecting rare target templates (Innis, Myambo, Gelfand, and Brow, 1988). On the other hand, the evident of combining PCR with fluorescent techniques have made the introduction of quantitative or real-time PCR possible (Ramakers, Ruijter, Deprez, and Moorman, 2003). The dye SYBR® Green I specifically bind to the double stranded DNA strongly that results in an

increase in the fluorescence intensity during the polymerization step, which allows to quantify the starting amount of cDNA in real-time (Bustin, 2000; Ramakers *et al*., 2003). During the amplification reaction, the fluorescence values that are recorded from each cycle represent the amount of cDNA amplified. Those samples that have more template at the beginning of the reaction take fewer number of cycles to reach a point above the background in which a statistically significant fluorescent signal is recorded. This point always occurs during the exponential phase of the PCR amplification and termed as threshold cycle (Ct). Finally, quantification of mRNA is performed by either relative or absolute means. The relative quantification is performed using a calibrator (nucleic acid) of known length and concentration. Then, the Ct values of the target gene and the calibrator is compared and verified as having either higher or lower mRNA expression level (Bustin, 2000). In absolute quantification, however the precise copy concentration of the target gene is quantified by relating the Ct value to a standard curve that is prepared from a dilution series of control template of known concentration (Lee, Kim, Shin, and Hwang, 2006).

Table 2-5: Primer pair sequences, amplicon size, and annealing temperature of the investigated genes in this study.

Target gene	Primer sequence (sequences are displayed in the 5'-3' direction)		Amplicon size (nucleotides)	Annealing T (**°C**)
	Forward primer	**Reverse primer**		
CYP1A1	GAGTTTGGGCAGGTGGTG	TGGTGCGGTTTGGTAGGT	76	60
CYP3A	ACTAGAGAGGGTCGCCAAGA	TACTGAACCGCTCTGGTTTG	146	60
Vitellogenin	AAGCCACCTCCAATGTCATC	GGGAGTCTGTCCCAAGACAA	391	60
Zr-protein	TGACGAAGGTCCTCAGGG	AGGGTTTGGGGTTGTGGT	113	60
ERα	TCCAGGAGCTGTCTCTCCAT	GATCTCAGCCATACCCTCCA	173	60

2.8.3.1 Quantitative real-time PCR protocol

The gene expression profiles of the selected genes were performed using absolute quantitative real-time PCR, which was carried out based on the manufacturer's (Bio-Rad, n.d.-b) manual with gene specific primer pairs (Table 2-4). Briefly, all the primers, cDNA, and iTaq Universal SYBR Green supermix were thawed on ice. The cDNA was diluted in 1:6 using a DEPC treated, cold Milli-Q water. The primers and iTaq Universal SYBR Green supermix for each target gene were prepared as a master mix (Table 2-5). By using 96 well qPCR plates, 15 µl from the super mix and 5 µl of diluted cDNA (1:6) were added to each well, sealed with plastic and spin down for a few seconds. The PCR reaction was facilitated by Mx3000P™ Real-time PCR System

(Stratagene, La Jolla, CA, USA) and was adjusted at 95 °C for 3 minutes (enzyme activation step) and 40 cycles of 95 °C for 15 seconds, 60 °C for 15 seconds (annealing temperature), and 72 °C for 15 seconds (extension step). Finally, a melting analysis was performed at 95 °C for 1 minute, 65 °C for 30 seconds, and 95 °C for 30 seconds. As described by Arukwe (2005) negative template controls (without cDNA template) were also incorporated to determine the specificity of target cDNA amplification. After the Ct values plotted against the log copy number of the standard curve, the Ct values obtained were converted into mRNA copy number. Due to their inconsistency with exposure conditions and time, the so-called housekeeping genes are not used in our laboratory and for this study (Arukwe, 2006). Instead, serial dilution of known amounts of plasmid containing the amplicon of interest were used to generate the standard plots for each target sequence. Finally, the mRNA copy number values obtained from the standard curve were expressed as percentage of controls in the graphs using the value from the DMSO (carrier chemical) exposed group as a control.

Table 2-6: Components of the reaction setup for a single quantitative real-time PCR reaction.

Components	Volume per reaction
iTaq™ Universal SYBR® Green supermix (2x)	10 µl
Forward primer	0.5 µl
Reverse primer	0.5 µl
Autoclaved water	4 µl
cDNA (1:6)	5 µl
Total volume	20 µl

2.9 Statistical analysis

Statistical analysis was performed using IBM SPSS® statistics, version 23 software. Data normality was evaluated using the Shapiro-Wilk test. As a requirement for further statistical analysis, datasets that are significantly different from normal distribution was transformed to a normal distribution with the help of natural logarithms (Ln). Normally distributed data was further checked for homogeneity of variance using a Levene's test. When datasets are normally distributed and qualify homogeneity of variance test, significant differences between groups were analyzed using One-way ANOVA followed by Tukey's post hoc multiple comparison test.

On the other hand, when datasets were normally distributed, but failed to qualify homogeneity of variance and/or unequal sample size, significant differences between groups were analyzed using the robust Welch test followed by Games-Howell post hoc test. Datasets

that are not normal and unable to attain normal distribution after transformation were analyzed using the nonparametric Kruskal-Wallis one-way analysis of variance followed by Bonferroni post hoc test. The level of significance was set to 0.05 for all tests. During statistical tests, outliers were approached by manual observation of the boxplot together with Grubbs tests. Thus, potential outliers were removed from some datasets.

All the graphs preparation was done using sigma plot, version 13.

3 RESULTS

3.1 Experimental design

At the beginning of this study, five sediment samples from five sampling sites, Makoko, Ikorodu, Apapa, Idumota, and Oworonshoki, of the Lagos lagoon system were chosen as the study samples. Due to the need for separate sediment samples for polar and non-polar pollutant extraction procedures from each sample site, only Makoko and Ikorodu sediment samples were found with sufficient sample volume for the chemical extraction. Thus, the number of sediment samples for this study was reduced to two, namely Makoo and Ikorodu.

On the other hand, the exposure experiment was planned to last after ten days of fish exposure to the different pollutants with sampling on days 5, 7, and 10. However, all the fishes of Ikorodu polar and Makoko non-polar exposure groups were found dead on days 4 and 5, respectively. As a result, the sediment samples were considered as highly contaminated with different pollutants and only the primary xenobiotic target, the liver, and blood was sampled. Moreover, we also planned to change the aquarium water every 5 days and resuspend the different exposure chemicals twice, at day 0 and 5. However, due to the aforementioned fish mortality at day 4 and 5 and after the aquarium water of polar pollutant groups were found more turbid, the aquarium water was changed at day 4 and the different exposure chemicals was resuspend into the aquarium water once on day 0.

3.2 Chemical levels

The pollutant levels of the sediment samples are illustrated in Table 3-1 and 3-2. In all chemical analysis, the Makoko sampling site showed higher contaminant levels compared to the Ikorodu sampling site, except for some PAHs. The total concentration of aliphatic hydrocarbons at the Makoko sampling site is 3.6 times higher than Ikorodu sampling site. Conversely, the concentration of low and high molecular weight PAHs at the Ikorodu sampling site was respective 5 and 2 times, higher than the concentrations at the Makoko sampling site. Higher concentration of pyrene, benzo[a]anthracene, 7,12-Dimethylbenzo[a]anthracene, and benzo[b]fluoranthene were measured on Ikorodu sampling site, while concentrations of all these chemicals were below the detection limit at the Makoko sampling site. Moreover, a 10 times higher concentration of benzo[a]pyrene was recorded on Ikorodu sampling site, compared with the Makoko sampling site. On the contrary, higher concentration of acenaphthylene, acenaphthene, fluoranthene were measured on Makoko sampling site.

Both sampling sites exhibited concentration of lindane (an organochlorine pesticide) that is, below the detection limit. They also showed nearly equal concentration of 4-iso-

nonylphonol. While the Makoko sampling site showed 4 μg/g, Ikorodu sampling site exhibited concentration of monobutyltin that is below the detection limit. Among the analyzed trace metals, higher concentration of barium, iron, manganese, and zinc were found at the Makoko sampling site. However, nearly equal concentration of iron was measured at both sites.

Table 3-1: Organochlorine pestcide, phenolic compound, organotin and trace metal levels in the sediment sampled at the Makoko and Ikorodu sampling sites of the Lagos lagoon. Values are given as mean ± standard deviation (mean ± SD). ND, not detected.∗

Chemicals	**Sediment samples**	
	Ikorodu	**Makoko**
Organochlorine Pesticides (μg/g)		
Lindane (γ-HCH)	ND	ND
Dieldrin	0.03 ± 0.00	0.12 ± 0.04
Phenolic compounds (μg/g)		
4-iso-Nonyphenol	0.11 ± 0.02	0.17 ± 0.01
4-t-Octylphenol	ND	**0.00 ± 0.00**
Organotin (μg/g)		
Monobutyltin	ND	4.05 ± 0.05
Trace metals (μg/g)		
As	0.25 ± 0.04	0.27 ± 0.11
Ba	83.15 ± 5.73	439.60 ± 53.74
Cd	0.02 ± 0.00	0.36 ± 0.10
Cr	2.95 ± 0.87	11.94 ± 1.23
Cu	4.83 ± 0.06	18.89 ± 2.73
Fe	1277 ± 114.55	1305.5 ± 81.31
Hg	0.09 ± 0.02	0.32 ± 0.01
Mn	64.25 ± 2.76	666.90 ± 52.89
Ni	0.61 ± 0.48	2.39 ± 0.14
Pb	8.24 ± 0.02	24.46 ± 2.74
V	11.03 ± 0.68	24.68 ± 0.30
Zn	5.57 ± 1.79	141.15 ± 20.77

∗ all the concentrations were expressed as μg/g dry weight (dw) of the sediment samples.

Table 3-2: Aliphatic hydrocarbon and polyaromatic hydrocarbon levels in the sediment sampled at the Makoko and Ikorodu sampling sites of the Lagos lagoon. Values are given as mean ± standard deviation (mean ± SD). ND, not detected.∗

Chemicals	**Sediment samples**	
	Ikorodu	**Makoko**
Aliphatic hydrocarbons (µg/gm)		
Total aliphatic hydrocarbons (C10-C40)	25.03 ± 6.48	91.55 ± 12.64
Polycyclic aromatic hydrocarbons (ng/gm)		
Naphthalene	70.68 ± 19.38	ND
Acenaphthylene	ND	78.10 ± 3.33
1-Methylnaphthalene	128.77 ± 34.08	ND
2-Methylnaphthalene	518.15 ± 48.67	ND
Acenaphthene	ND	50.68 ± 25.33
Fluorene	16.16 ± 4.95	7.20 ± 2.98
Phenanthrene	62.27 ± 12.71	ND
Anthracene	14.68 ± 1.99	19.21 ± 3.71
Fluoranthene	131.57 ± 27.10	285.07 ± 74.94
Pyrene	80.14 ± 7.28	ND
Benzo[a]anthracene	27.53 ± 2.76	ND
Chrysene	0.06 ± 0.01	ND
7,12-Dimethylbenzo[a]anthracene	162.75 ± 3.05	ND
Benzo[b]fluoranthene	106.89 ± 9.36	ND
Benzo[k]fluoranthene	65.87 ± 47.56	44.60 ± 11.46
Benzo[a]pyrene	62.51 ± 2.69	0.62 ± 0.66
Dibenzo[a,h]anthracene	32.73 ± 0.42	32.10 ± 2.57
Benzo[g,h,i]perylene	11.06 ± 1.01	0.65 ± 0.27
Indeno[1,2,3,c,d]pyrene	2.91 ± 0.52	2.85 ± 0.37
Low MW PAHs	810.79 ± 83.01	155.39 ± 27.94
High MW PAHs	684.00 ± 100.93	366.14 ± 88.20
Total polycyclic aromatic hydrocarbons	1494.79 ± 183.94	521.53 ± 60.26

∗ the concentrations were expressed as ng/g dry weight (dw) for polycyclic aromatic hydrocarbons and as µg/g dry weight (dw) for aliphatic hydrocarbons (C10-C40).

3.3 Modulation of xenobiotic biotransformation system

Modulation of xenobiotic biotransformation was evaluated in juvenile Atlantic salmon after exposure to polar pollutant sediment extracts from the Makoko sampling site and non-polar pollutant sediment extract from the Ikorodu sampling site (Lagos lagoon, Nigeria), as well as EE2, and the carrier solvent (DMSO) using catalytic EROD, BROD, and MROD assays. In both EROD and BROD activities, the Makoko polar exposure group showed higher enzymatic activities that are significantly higher ($p<0.05$ and 0.01 respectively) than the control group (Figure 3-1A and B). Additionally, Makoko polar exposure also produced a non-significant higher MROD enzymatic activities (Figure 3-1C). On the other hand, Ikorodu non-polar exposure produced significantly higher ($p<0.01$) BROD enzymatic activities and EROD activity that was nearly identical to the control group. Moreover, Ikorodu non-polar exposure produced similar MROD activity as the EE2 exposure group (Figure 3-1C). In all assays, higher levels of enzymatic activities were recorded from the Makoko polar exposure group, compared to Ikorodu non-polar exposure group. Conversely, EE2 exposure significantly inhibited EROD activities below the control group. Furthermore, non-significant reduction of BROD and MROD activities below the control group were recorded by EE2 exposure.

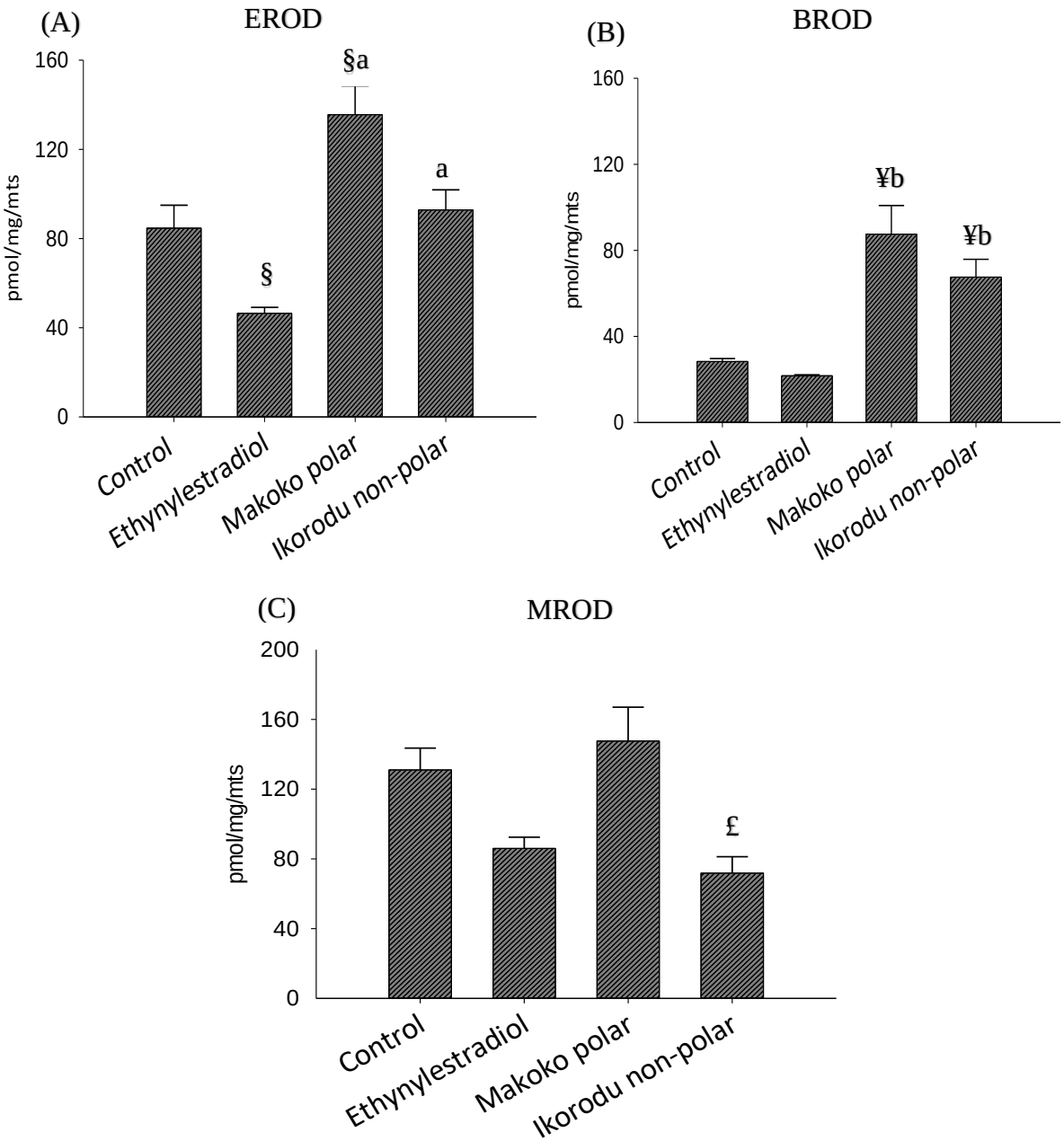

Figure 3-1: Determination of CYP1A-dependent hepatic ethoxyresorufin O-deethylase (EROD: A), benzyloxyresorufin O-deethylase (BROD: B), and methoxyresorufin O-deethylase (MROD: C) activities in juvenile Atlantic salmon after exposure to polar sediment extract from Makoko and non-polar sediment extract from Ikorodu sampling sites of Lagos lagoon (Nigeria), 17α-ethynylestradiol (EE2: 2.5 µg/ml) and the carrier solvent control (0.016 % DMSO). Fish was sampled at day 5 after exposure. All values represent the mean ± standard error of the mean (SEM) of n=6-8. Symbols denote exposure groups that are significantly different from the control group, analyzed by One-way ANOVA followed by Tukey HSD post hoc test (EROD), Kruskal wallis followed by Bonferroni post hoc test (BROD), and Welch test followed by Games-Howell post hoc test (MROD).

3.4 Oxidative stress responses

Catalytic activities of hepatic oxidative stress responses (GR, GST, CAT, and GPx) were evaluated in juvenile Atlantic salmon exposed to polar and non-polar sediment extracts from Lagos lagoon. Generally, all the exposure groups exhibited glutathione reductase activities that were below the control group (Figure 3-2A). Both Ikorodu non-polar extract and EE2 exposure generated nearly similar GR activities. Additionally, Ikorodu non-polar extract and EE2 exposure produced significant reductions in GR activities, compared to the control and Makoko exposure groups (Figure 3-2A).

Unlike the GR activities, all the exposure groups showed increased GST catalytic activity above the control group (Figure 3-2B). EE2 and Ikorodu non-polar exposure resulted in a significant increase of GST activity, compared to the control group. However, non-significant but higher GST activity above the control group was also recorded with the Makoko polar group. Moreover, nearly similar levels of GST activities were displayed between Makoko polar and EE2 exposure groups. For CAT and GPx, all exposure groups did not generally produce any significant difference compared with the control group (Figure 3-3A and B). However, slight increase in catalase and decrease in GPx activities were observed in an apparent exposure dependent manner including the EE2, Makoko polar, and Ikorodu non-polar (Figure 3-3A and B, respectively).

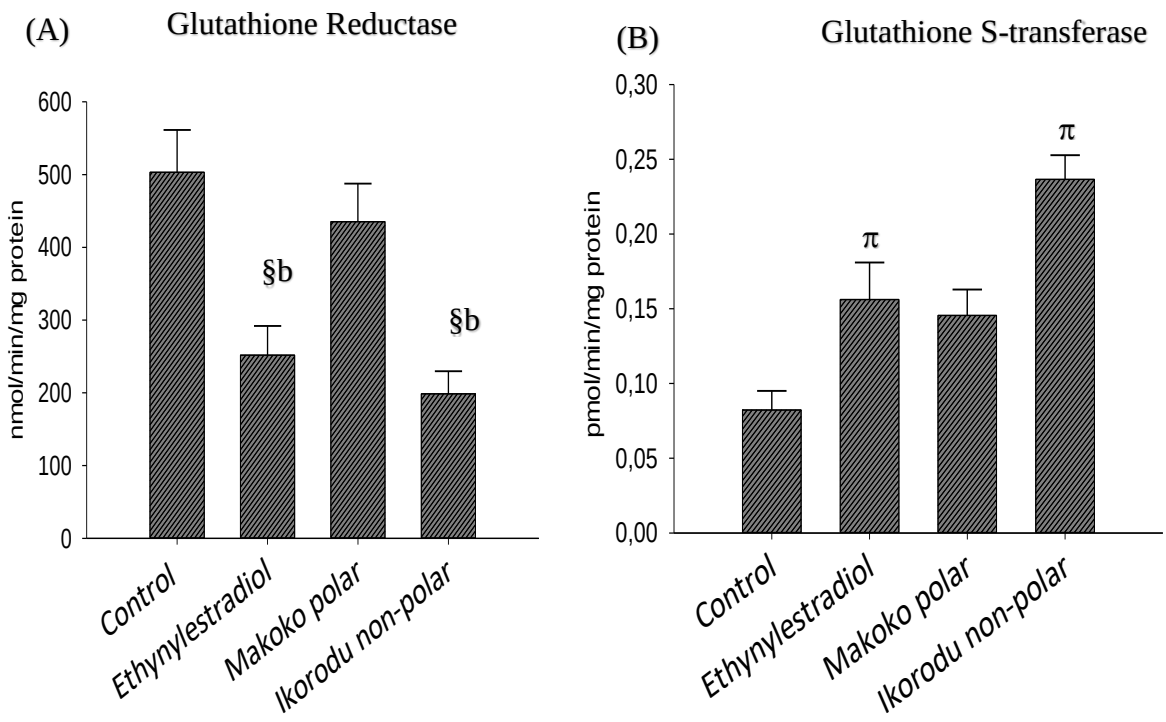

Figure 3-2: Determination of hepatic activities of glutathione reductase (GR; nmol/minute/mg protein) (A) and glutathione s-transferase (GST; pmol/minute/mg protein) (B) in juvenile Atlantic salmon after exposure to polar sediment extract from Makoko and non-polar pollutant sediment extract from Ikorodu sampling sites of Lagos lagoon (Nigeria), 17α-ethynylestradiol (EE2: 2.5 µg/ml) and the carrier solvent control (0.016 % DMSO). Fish was sampled at day 5 after exposure. All values represent the mean ± standard error of the mean (SEM) of n=7-8. Symbols denote exposure groups that are significantly different from the control group and letters denote exposure groups that are significantly different from the Makoko polar group, analyzed by One-way ANOVA followed by Tukey HSD post hoc test.

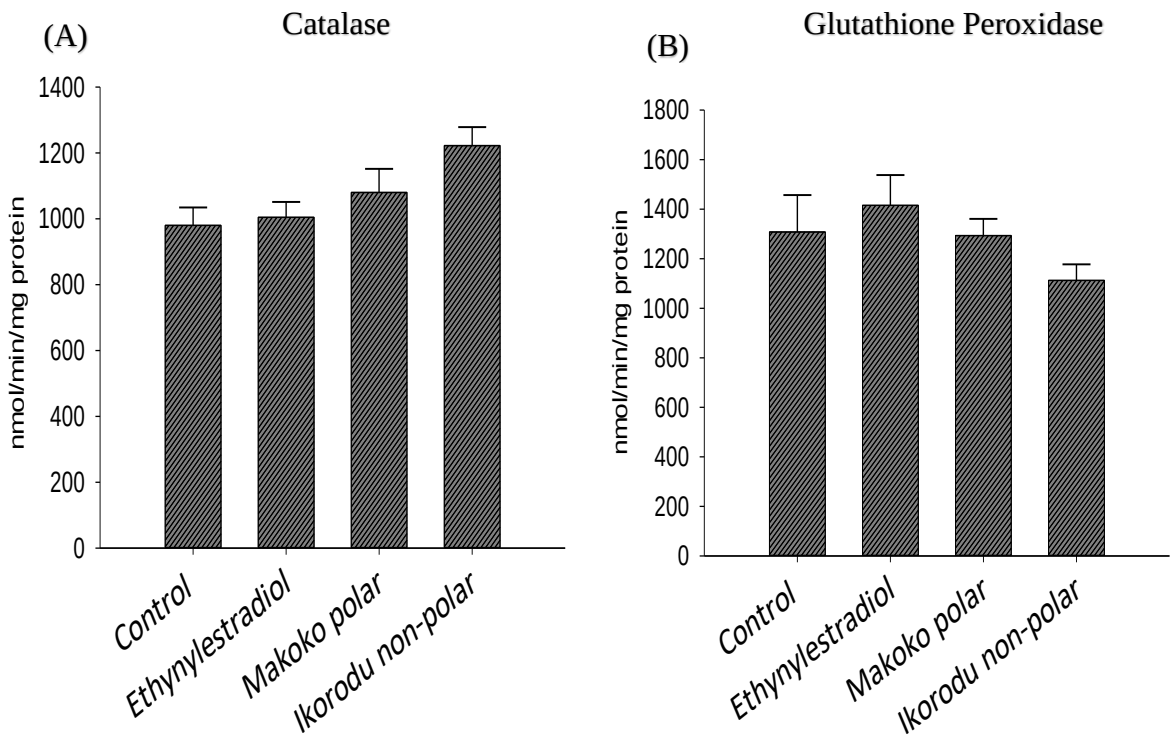

Figure 3-3: Determination of hepatic activities of catalase (CAT; nmol/minute/mg protein) (A) and glutathione peroxidase (GPx; pmol/minute/mg protein) (B) in juvenile Atlantic salmon after exposure to polar sediment extract from Makoko and non-polar pollutant sediment extract from Ikorodu sampling sites of Lagos lagoon (Nigeria), 17α-ethynylestradiol (EE2: 2.5 µg/ml) and the carrier solvent control (0.016 % DMSO). Fish was sampled at day 5 after exposure. All values represent the mean (n=8) ± standard error of the mean (SEM). Symbols denote exposure groups that are significantly different from the control group and letters denote exposure groups that are significantly different from the Makoko polar group, analyzed by One-way ANOVA followed by Tukey HSD post hoc test (CAT) and Welch with Games-Howell post hoc test (GPx).

3.5 Gene expression

3.5.1 CYP1A1 and CYP3A

The gene expression patterns of hepatic CYP1A1 and CYP3A mRNA were evaluated in juvenile Atlantic salmon after exposure to polar sediment extracts from the Makoko and non-polar sediments extract from the Ikorodu sampling sites of the Lagos lagoon (Nigeria), EE2 (2.5 µg/ml), and the carrier solvent (0.016 % DMSO). Largely, the gene expression patterns of all the exposure groups related to modulation of xenobiotic biotransformation system didn't show any significant difference from the control group (Figure 3-4A and B). Consequently, reduction of CYP1A1 and CYP3A mRNA levels were demonstrated by all the exposure groups,

which were below the control group mRNA level. Among which, the EE2 exposure showed a significant reduction in the expression of CYP1A1 mRNA, which is consistent with the EROD activities. Makoko polar exposure also produced the highest non-significant reduction in CYP3A mRNA expression. Despite the repression of gene expression, Ikorodu non-polar exposure produced a slight increase in CYP1A1 and CYP3A mRNA expression level relative to the control group.

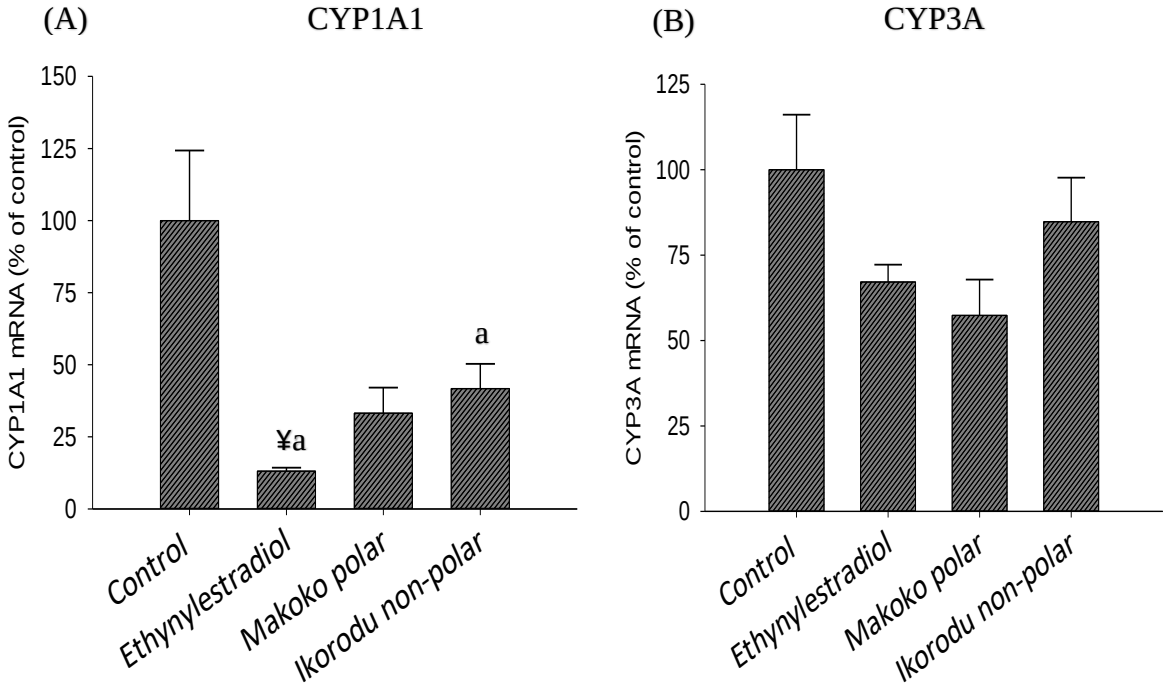

Figure 3-4: Modulation of CYP1A1 (A) and CYP3A (B) mRNA levels in juvenile Atlantic salmon after exposure to polar sediment extract from Makoko and non-polar pollutant sediment extract from Ikorodu sampling sites of Lagos lagoon (Nigeria), 17α-ethynylestradiol (EE2: 2.5 µg/ml) and the carrier solvent control (0.016 % DMSO). Fish was sampled at day 5 after exposure. Messenger RNA (mRNA) levels were quantified using quantitative (real-time) polymerase chain reaction (PCR) with gene specific primer pairs. All values represent percent (%) of control (n=7) ± standard error of the mean (SEM). Symbols denote exposure groups that are significantly different from the control group and letters denote exposure groups that are significantly different from the EE2 group, analyzed by Welch with Games-Howell post hoc test (CYP1A1) and One-way ANOVA followed by Tukey HSD post hoc test (CYP3A).

3.5.2 Estrogenic responses

The gene expression patterns of hepatic Vtg, *Zrp*, and ERα mRNA were evaluated in juvenile Atlantic salmon after exposure to polar sediment extracts from the Makoko and non-polar sediments extract from the Ikorodu sampling sites of the Lagos lagoon (Nigeria), EE2 (2.5 µg/ml), and the carrier solvent (0.016 % DMSO). Salmon exposure to EE2 in this study produced significantly higher levels of Vtg, *Zrp*, and ERα mRNA expression, which were extremely higher and different from all the other groups (Figure 3-5A, B and C). On the other hand, exposure to Makoko polar extract produced a nearly similar expression of Vtg and *Zrp* mRNA as the control group and a non-significant increased expression of ERα mRNA above the control group. Moreover, Ikorodu non-polar extract created a non-significant higher expression of Vtg mRNA above the control group and nearly similar expression of *Zrp* mRNA as the control and Makoko polar groups. Unlike the Makoko polar group, Ikorodu non-polar group, however, produced a reduction in expression of ERα mRNA.

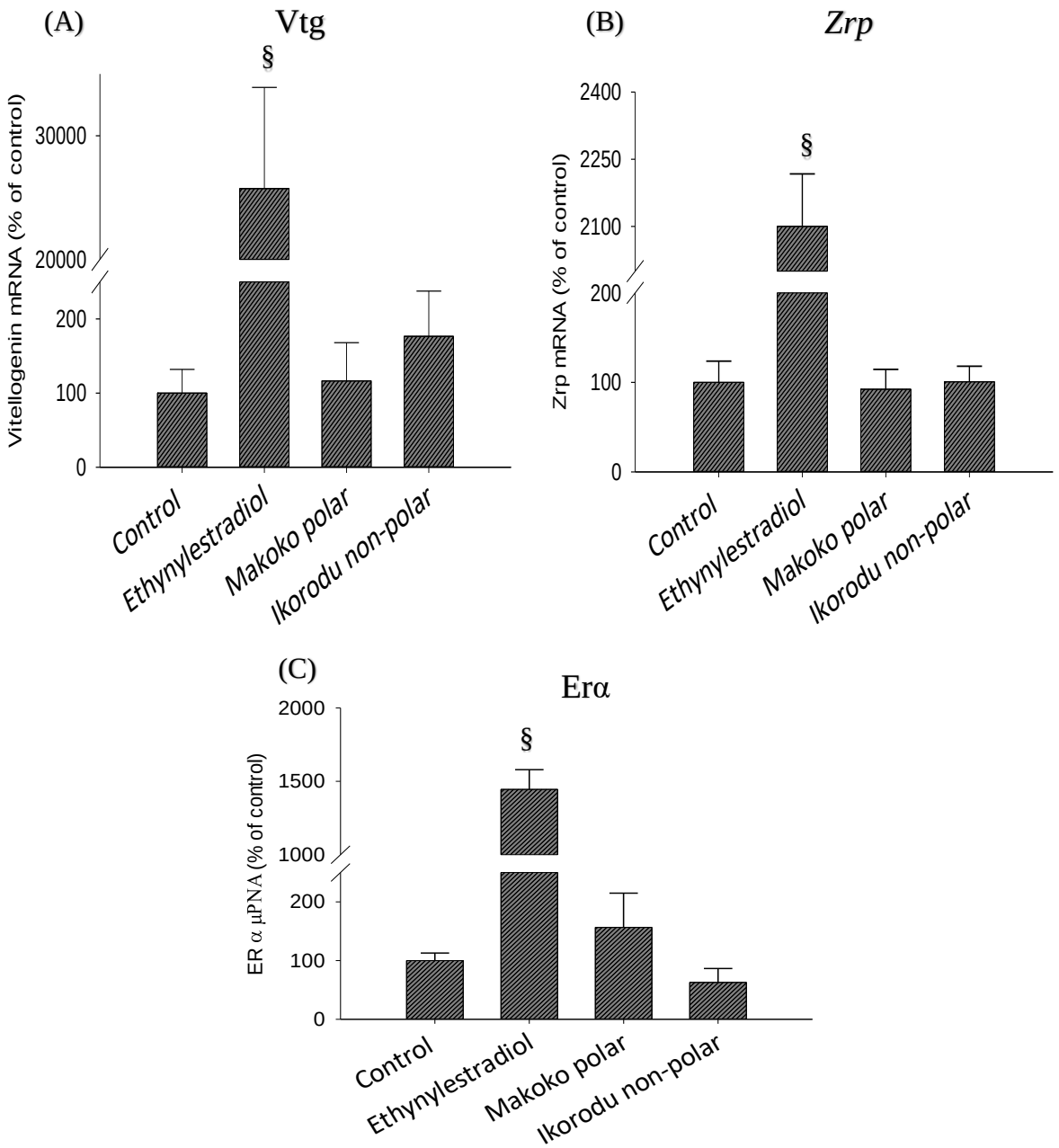

Figure 3-5: Modulation of vitellogenin (Vtg: A) eggshell zona radiata protein (Zrp: B) and estrogen receptor alpha (Erα: C) mRNA levels in juvenile Atlantic salmon after exposure to polar sediment extract from Makoko and non-polar pollutant sediment extract from Ikorodu sampling sites of Lagos lagoon (Nigeria), 17α-ethynylestradiol (EE2: 2.5 µg/ml) and the carrier solvent control (0.016 % DMSO). Fish was sampled at day 5 after exposure. Messenger RNA (mRNA) levels were quantified using quantitative (real-time) polymerase chain reaction (PCR) with gene specific primer pairs. All values represent the mean (n=7) ± standard error of the mean (SEM). Symbols denote exposure groups that are significantly different from the control group, analyzed using Kruskal Wallis with Bonferroni post hoc test.

4 Discussion

Aquatic sediments are known sink for pollutants of low and medium polarity, which accumulate complex contaminants at extremely higher concentrations than the overlying water. As a result of activities, such as sand mining and harbor dredging, resuspension of these pollutants into the water column serve as secondary sources of a continuous pollutant exposure route for benthic organisms and aquatic populations. Among the pollutants associated with sediments and suspended particles that are predominantly dangerous for aquatic populations include dioxins and dioxin-like pollutants (e.g., planar halogenated aromatic hydrocarbons and PAHs), pesticides, PCBs and trace metals (Amaeze *et al*., 2015; Schiwy, Bräunig, Alert, Hollert, and Keiter, 2015; Sutherland, 2000). Close contact between these organic pollutants and the marine organisms facilitate the free uptake and their deposition into the lipid rich tissues (Livingstone *et al*., 1993).

In the Lagos lagoon system, there have been reports of teratogenic, embryogenic, and genotoxic properties of sediment extracts and some of these pollutants are known to cause cancer or are alleged to be carcinogens in humans (Sogbanmu *et al*., 2016). Generally, research resources are inadequate with limited materials and financial support in developing countries (Harris, 2004). Thus, existing reports regarding the Lagos lagoon have mostly focused on measuring environmental and biota heavy metal levels in relation to physico-chemical parameters (Adeniyi, Yusuf, and Okedeyi, 2008; Aderinola, Clarke, Olarinmoye, Kusemiju, and Anatekhai, 2009; Jaji, Bamgbose, Odukoya, and Arowolo, 2007; Okoye *et al*., 1991). On the other hand, like in other developing countries, environmental laws in Nigeria are poorly imposed, which results in the release of industrial, agricultural and urban related contaminants into the aquatic ecosystem (Adeogun, Ibor, Onoja *et al*., 2016). Therefore, it is important to investigate the molecular and biochemical effects of contaminants accumulated in the sediments and to relate the observed physiological and health effects in the organisms of the Lagos lagoon system. Thus, it can further facilitate the gap in the knowledge between contaminant load and their impact. In the present study, the main enzymatic activities of the biotransformation (CYP1A1, CYP3A, and GST) and antioxidant (GR, GPx, and Catalase) pathways and the expression of biotransformation (CYP1A1, CYP3A) and estrogenic (ERα, Vtg, and *Zrp*) pathways were investigated in the liver of juvenile Atlantic salmon after the exposure of extracts from contaminated sediments of the Lagos lagoon, Nigeria.

4.1 Experimental design and pollutant extraction

At the beginning, we planned to perform whole sediment exposure. However, some of the sediment samples were dusty with very fine particles that may suffocate the gills and interrupt the normal breathing of fish and consequently suspected to cause fish mortality. Therefore, we

designed to carry out chemical extraction of the pollutants from the sediment samples. During pollutant extraction procedure, a total of 100 g of sediment sample was required from each site. Out of the five sediment samples collected from five sites of the Lagos lagoon system, three of the sampling sites had insufficient sample volume. In order to normalize the data, only sediment samples with sufficient volume (Makoko and Ikorodu sampling sites) were used for further pollutant extraction. Therefore, the analyzed sample size was reduced from five to two.

During chemical extraction, ultrasound-assisted extraction procedure was used for both polar and non-polar pollutant extractions from the sediment samples. The ultra-sonication has high extractive power with a combination of high temperatures (which increase solubility and diffusivity of the pollutants) and pressure (which favor penetration and transport) at the interface between an organic solvent and a solid matrix (the sediment samples) (Luque-Garcıa and De Castro, 2003). In order to obtain an optimal extraction process, choosing the right solvent is very crucial (Björklund, Nilsson, and Bøwadt, 2000). Therefore, DCM that is suitable for hydrophobic compounds elution was chosen as an extraction solvent for the non-polar pollutants (Di Giorgio, Malleret, Gueydon-Morin, Rigaud, and De Méo, 2011; Ma *et al*., 2010; Sajid and Basheer, 2016). Moreover, because of its application for extracting polar compounds from sediment samples in pressurized liquid extraction (coupled with solid-phase extraction cleanup), isopropanol was chosen as an extraction solvent for the polar pollutants (Burkhardt, ReVello, Smith, and Zaugg, 2005; Di Giorgio *et al*., 2011).

4.2 Exposure

Despite the presence of reports on the exposure of pollutants from sediment extract to different cell lines and exposure of whole sediments to the experimental animals, reports that have addressed the effects of exposure of sediment extracts to the experimental animals are generally limited. As a consequence, finding reports to refer that would have a positive contribution to this study was a great challenge. However, the limited reports and other related resources were used to address the challenges. Even with all the challenges, maximum efforts have been made to reduce any possible experimental failure. For example, all the aquaria were covered by metal mesh so that the fish were kept safe and prevented from skipping. Moreover, the temperature, dissolved oxygen, and pH of the aquaria were also checked daily. Moreover, the fish were not fed during the exposure time that could potentially avoid the loss of any dissolved oxygen needed for the decaying food leftovers and feces. However, maintaining the optimal dissolved oxygen level and keeping a continuous air pumping was among the great challenges during the exposure experiment. Despite all the efforts, unexpected results were recorded, such as fish mortality that consequently forced us to change the exposure plan and reduce the exposure days

from 10 days to 5 days. Moreover, implementing the original plan to sample fish at day 5, 7, and 10 was changed and all the fish were sampled at day 5 once.

On the other hand, after the death of all fish of the Makoko non-polar and Ikorodu polar exposure groups, only the Makoko polar and Ikorodu non-polar exposure groups were left. As a consequence, despite these two sediment extract exposures are polar and non-polar and their result comparison seems inappropriate. However, since the sediments in the Lagos lagoon system has been reported as contaminated with different pollutants, the results that were obtained from the two sediment sites of the Lagos lagoon system were compared.

Wildlife species have been exposed to fluctuating mixtures of environmental contaminants. Among these mixtures, there are chemical contaminants in the effluents that act as ER agonists, including EE2 and other natural and synthetic steroids (Liney *et al*., 2006). EE2, the active ingredient in the contraceptive pills, is known to be present in the aquatic environment at biologically active concentrations and is known as a potent endocrine modulator (Milla, Depiereux, and Kestemont, 2011; Mortensen and Arukwe, 2007a). On the other hand, sediment-bound pollutant exposures are used to investigate the biological risk of a given exposure. However, the efforts do not allow to indicating the effect to specific pollutant in the sample (Hecker and Giesy, 2011). Therefore, since some reports are available concerning exposure of EE2 to fish as potent EDC, in the present study an EE2 exposure group was also included in the Makoko polar and Ikorodu non-polar exposure groups, as a positive control to investigate estrogenic effects of pollutants from the sampled sediments.

4.3 RNA isolation

The primary activity to be performed on attaining meaningful data gene expression is maintaining RNA quality. In order to reduce RNA contamination and its ultimate degradation, several protection mechanisms were employed. All the activities of fish handling, dissection, and organ and blood sampling were performed under optimal laboratory conditions. In this regard, wearing a hand glove and a lab coat, performing all the preparations on ice, and snapping all the sampled tissues in liquid nitrogen immediately after dissection. Moreover, TRI reagent was also used during RNA isolation, which protect RNA degradation. Therefore, the possible exogenous and endogenous RNA degrading enzymes (notably RNases) were minimized. Therefore, the quality and integrity analysis of randomly selected RNA samples didn’t show any sign of degradation (Figure 2-3) and got the assurance to be used as a template for cDNA preparation in the RT-PCR.

4.4 Gene primers

The optimal primer pair concentrations, specificity, and their optimal annealing temperatures need to be verified with some random samples prior to the PCR reaction (Mortensen and

Arukwe, 2007a). While too low primer concentration has less problem, higher concentration of primer above the optimal level may create mispriming and increased non-specific product (Bustin, 2000). So that, the investigated gene expressions for this study were performed based on the optimal conditions verified. However, the gene expression analysis of CYP1A1, CYP3A, Vtg, *Zrp*, and ERα has been continuously used in our lab. Thus, the specificity, concentrations and their annealing temperature of the primer pairs of the abovementioned genes have already been verified. Moreover, the melting curves of all the investigated genes showed single peak that are indicative of specific amplifications (see Appendix C). Therefore, the already optimized and routinely used primers were used for this study.

4.5 Normalization and quantification

When gene expression patterns between different individuals are compared, data normalization with a valid reference is the most challenging task. Moreover, none of the proposed solutions are ideal and the problem becomes serious when dealing with *in vivo* samples (Bustin, 2002). Therefore, during mRNA transcript quantification using RT-PCR, utilization of an appropriate normalization method and normalization of data values are vital in order to minimize errors produced by sample-to-sample variations and in order to achieve reliable results. This situation is especially evident when samples have been taken from different individuals, which may lead to misinterpretation of the expression profiles of the genes of interest. Thus, there must be an internal reference that has to be amplified simultaneously with the target and ultimately used to normalize other RNA values. In this regard, housekeeping genes that are used to perform basic cellular functions in normal cells, has been used by some researchers to normalize the gene expression patterns of different target genes. Thus, the housekeeping genes are expected to be expressed at a constant level in different tissues, and need to be unaffected by the experimental treatment (Arukwe, 2006; Bustin, 2000).

Due to their considerable variability in different experimental conditions and the ultimate misinterpretation of the expression profile of a target gene, housekeeping genes are not used in our laboratory (Arukwe, 2006). In this study, normalization of each target gene was performed using a standard curve prepared with serial dilutions of known concentration solution of plasmid containing the gene of interest (Mortensen and Arukwe, 2008).

4.6 Statistical analysis

Regarding statistical analysis, the common challenges in this study were data failure to attain normality. Despite the parametric tests such as analysis of variance (ANOVA) are suited for data that are normally distributed and whose variances are homogeneous (‘‘equal’’ variance), data that comply with this assumption are, however, not common in biology. Thus, about two third of the data in this study don’t attain normal distribution. However, whenever the

assumptions are met, the parametric test was preferred and performed over the non-parametric test, due to their ease of use and because they are often considered to be more sensitive than non-parametric tests (Wang and Riffel, 2011). To this effect, the normal Q-Q plot and the boxplot were also checked to back Shapiro-Wilk test for data normality test. But, when the data violated the normality assumption, data transformation is needed (Zuur, Ieno, and Elphick, 2010).

The other statistical challenge was dealing with the outliers. Outliers may arise as a consequence of poor experimental design; in such case it is practical to drop the observation. It is a valid justification for dropping observations when outliers arise due to measurement or researcher errors. However, a more refined approach may be required for outliers in the response variable, especially when they represent genuine variation in the variable being measured (Zuur *et al*., 2010). Therefore, outlier's detections were performed by visual observation boxplot and Grubbs' test. Additionally, experimental notes were taken when unusual events occur, so that information will be used to re-examine outliers.

On the other hand, even if the level of significance was set to 0.05 for all tests, results with p-values nearly 0.05 were also recorded. Therefore, non-significant values of groups that exhibited higher or lower values than the control group were also discussed, and thus clearly identified by the term "non-significant".

4.7 Experimental data

4.7.1 Chemical analysis

Although the different pollutants are present in the aquatic ecosystem, the main distress in environmental science is the investigation of bioavailable toxic potential of contaminated sediments (Schiwy *et al*., 2015). The abundant environmental contaminants, including dioxins and dioxin-like compounds (such as PAHs), are strongly associated with suspended particles and consequently with the sediments.

In this study, the hydrocarbon content analysis of the sediment samples has been used to identify the potential sites of oil contamination in the Lagos lagoon. From the chemical analysis result, when compared to the Makoko sediment samples, the Ikorodu sediment samples showed higher concentration of the high molecular weight PAHs and some low molecular weight PAHs. While the Makoko sampling site exhibited highest concentration of all the analyzed aliphatic hydrocarbons and some of the low molecular weight PAHs, including acenaphthylene, acenaphthene, and anthracene, and a high molecular weight fluoranthene. The Ikorodu sampling site has been described as a site where domestic and agricultural effluents are discharged and sand mining and construction activities are carried out (Adeogun, Ibor, Regoli *et al*., 2016). On the other hand, the Makoko sampling site was described as a place of direct

domestic and municipal waste discharge, solid waste discharge, high boat transport density, wooden residential buildings, and fishing activities (Amaeze *et al*., 2012; Amaeze *et al*., 2015). In connection to these, onshore and offshore crude oil exploration activities by multinational companies are prevalent, creating crude oil spillage (leakage from pipelines and accidental spills) and considered to be sources of PAHs discharge into the lagoon system (Benson *et al*., 2014). Additionally, the PAH concentration has been suggested to increase as a result of anthropogenic activities in and around the Lagos lagoon and produced a reduction of fishery resources (Adeogun *et al*., 2015). However, most organisms are able to metabolize PAHs and therefore, the PAHs exposure and the parent PAHs in animal tissues are not the same (Le Dû-Lacoste *et al*., 2013). Moreover, as a result of complex chemical mixtures present in the sediment samples and the possible additive, synergistic or antagonistic interactions between components of the pollutants, it is challenging to determine the toxic effect of PAHs in the contaminated sediment (Sogbanmu *et al*., 2016)

Regarding heavy metal concentration, the Makoko sediment sample again showed higher concentrations than the Ikorodu sediment sample. Metal is reported to be well concentrated in the sediments and water (Aderinola *et al*., 2009; Lawson, 2011). Sediment samples from coastal aquatic regions that are in close proximity to large industrial and urban sites are typically polluted by heavy metals (Ajao and Fagade, 1990). Metal ions are also known to stimulate reactive oxygen species and cause to induce oxidative stress in fish (Padmini, Rani, and Geetha, 2009; Stohs and Bagchi, 1995).

4.7.2 Biotransformation responses

In mechanistic toxicology, cytochrome P450 is used as one of the most vital enzyme families (Boelsterli, 2007). As representatives of the cytochrome P450 and their role in xenobiotic biotransformation, CYP1A1 and CYP3A were selected for this study (Hegelund, Ottosson, Rådinger, Tomberg, and Celander, 2004). Regarding investigations of animal's pollutant exposure, xenobiotic biotransformation enzymes (phase I and phase II enzymes), including members of the CYP1A, CYP3A, and GST are among the most studied and play important role in the metabolism of a variety of exogenous and endogenous compounds (Meucci and Arukwe, 2006; Mortensen and Arukwe, 2007a). The expression of CYP1A1 and phase II enzyme GST is regulated by the ligand-dependent basic helix–loop–helix–Per–Arnt–Sim (bHLH–PAS) transcription factor, AhR, whereas the expression of CYP3A is regulated by the nuclear receptor, PXR (Bradshaw *et al*., 2002; Kliewer *et al*., 2002). As a result, the modulation of these receptors through their agonists can create an altered gene expression and toxicity (Meucci and Arukwe, 2006; Mortensen and Arukwe, 2007a). The induction of these xenobiotic biotransformation enzymes in fish to different classes of contaminants are normally used as

early biochemical responses that may be used as biomarkers of environmental pollution of the aquatic ecosystem (Arukwe, Nordtug, Kortner, Mortensen, and Brakstad, 2008; Krøvel, Søfteland, Torstensen, and Olsvik, 2008). The enzyme system in fish has demonstrated its response to widespread environmental contaminants such as PAH, PCBs, dioxins, oil compounds, pesticides (Förlin *et al*., 1994). The planar, the aromatic structural nature of these chemical compounds enable them to bind the AhR that leads to the induction of genes causing an increased amount of CYP1A mRNA, protein, and catalytic activity (Nilsen *et al*., 1998). The amount of CYP1A induction in wild fish is shown to be strongly associated with the degree of contamination by different contaminants, including PCBs and PAHs in sediment at the same place (Goksøyr *et al*., 1994; Goksøyr and Förlin, 1992). Hepatic CYP1A induction in fish has been evaluated by a number of mechanisms whose enzyme activity levels can be performed by determining EROD activity (Rees and Li, 2004). In fish, some studies showed that EROD and MROD activities show strong similarities, which indicate the involvement of a single CYP1A isozyme for both activities, whereas other studies revealed CYP2A-like isozyme induction in connection with MROD activities in several fish species (Arukwe, *et al*., 2008). However, there is a general association between induction of CYP1, 2 and 3 families and xenobiotic biotransformation in fish (Mortensen and Arukwe, 2007a; Olufsen and Arukwe, 2015).

From this study, exposure of polar sediment extracts from the Makoko site to juvenile Atlantic salmon produced significantly higher hepatic EROD and BROD activities and a non-significant higher MROD activity. On the other hand, the Ikorodu non-polar group showed a non-significant EROD activity that was a bit higher than the control group and significantly higher BROD activity, while exhibiting a significant reduction in MROD activity. Thus, such higher catalytic activities may be a response to AhR ligand exposure and an indication of toxic planar compound uptake in fish (Whyte, Jung, Schmitt, and Tillitt, 2008). Similarly, induction of EROD activity was recorded in Crab (*Carcinus maenas)* and Clam (*Ruditapes philippinarum)* exposed to sediments containing high concentrations of PCBs and PAHs (Martín-Díaz, Blasco, Sales, and DelValls, 2007). Whenever higher catalytic activities are recorded, it is usually confirmed by gene expression analysis. Therefore, the higher catalytic activities of both Makoko and Ikorodu exposure groups created the motivation to analyze the mRNA expression levels of CYP1A1 and CYP3A. Unexpectedly, the CYP1A1 and CYP3A mRNA levels of all the sediment extracts were below the control group. As a result, the respective polar and non-polar sediment extracts from Makoko and Ikorodu sampling sites, produced a reduction of CYP1A1 and CYP3A mRNA levels, which were below the gene expression level of the control group. Whenever the sampled sediments have environmental contaminant load, one would expect that the gene expression responses from the exposed fish

should paralleled the higher catalytic activities that are observed. These are indications of the differences between mRNA level and enzyme activities of both CYP1A1 and CYP3A. These may be related to the temporal patterns of EROD activity, CYP1A protein concentration and CYP1A mRNA levels. In this regard, exposure to easily metabolized inducers keep elevated CYP1A protein concentration and EROD activity for a longer period than the mRNA levels. Therefore, there may be cases of CYP1A mRNA clearance after the inducer(s) is/are metabolized following a period of CYP1A induction.

For example, rainbow trout treated with β-naphthoflavone (BNF) showed a rapid induction and clearance of CYP1A mRNA (Whyte *et al*., 2008). In laboratory-exposed organisms, there have also been reports of different time course responses due to shorter half-life of mRNA compared to protein. For example, when compared to fish, acutely exposed to single chemicals, the European flounder (*Platicthys flesus*) that chronically exposed to polluted estuarine sediments showed a different pattern of hepatic mRNA expression of genes (Regoli, Giuliani, Benedetti, and Arukwe, 2011). In this regard, it would have been good if sampling day earlier than 5 was included in order to see the temporal patterns of enzyme activities and gene expression patterns. Moreover, because of their relatively less hydrophobic nature, estradiol-17β (E2), estrone, and nonylphenol were found in sediments (Houtman *et al*., 2006). Therefore, the polar pollutant extraction protocol favors the extraction of polar pollutants, and thus the polar sediment extract from Makoko site is expected to contain the phenolic compounds such as 4-iso-nonyphenol that were analyzed for this study. Due to their estrogenic effect, these compounds have been shown to produce an initial significant decrease in CYP1A1 mRNA level after exposure and a significant increase over the normal amount in the latter days. It was suggested that the CYP1A1 mRNA level increased as a result of nonylphenol metabolism by the fish or bacteria, which tremendously diminished this compound and allowed the transcription of CYP1A1 mRNA (Meucci and Arukwe, 2006). However, there have been reports of significant increase in mRNA expression of CYP1 isoforms, which was observed in fish from Ogun River, which is the major source of water to the Lagos lagoon and these results paralleled data on PCBs and PAHs tissue concentrations (Adeogun, Ibor, Regoli *et al*., 2016). Therefore, there may be other possible factor(s) contributing to the reduction of mRNA expression level for CYP1A1 and CYP3A in the present study. The other possible factor may be failure during gene expression analysis, particularly during PCR analysis. In this respect, errors during PCR can be introduced from several sources such as assay design, PCR reagents, PCR equipment, and human error.

Particularly, during assay design, the specificity and stability of the primers as well as PCR product size are very crucial to obtain an accurate result (Wong and Medrano, 2005). Since

all the primers used in the gene expression analysis of this study have been routinely applied in our laboratory and they have already been optimized for their respective gene analysis, this problem may not be the cause for the aforementioned problem. On the other hand, copy numbers derived from standards are also prone to error. Additive errors due to various factors such as calculation of molecular weight, pipetting and spectrophotometry errors cause copy numbers to be an approximation rather than an absolute unit. Moreover, differences in RNA concentration, RNA quality, and reverse transcription rates may also produce incomparable copy numbers among experimental samples. As a result, samples with partially degraded RNA will produce a lower copy number, whereas samples that undergo effective reverse transcription will contain higher copy numbers (Peirson, Butler, and Foster, 2003). Due to inexperience in the gene expression analysis experiment, the cumulative effects of experimental and human errors may possibly play a role in the problem incurred in the gene expression analysis in this project. On the other hand, due to the complex contaminants mixture present in the sediment extracts, interactions between components may create antagonistic effects (Sogbanmu *et al*., 2016). For example, the organophosphate pesticide diazinon interferes with the induction of CYP1A by benzo[α]pyrene (BaP) when fish were exposed to the mixtures of both chemicals (Tridico *et al*., 2010). The interference occurs via the inactivation of CYP1A after the sulfur atom from the pesticide binds to the catalytic site of the enzyme. Therefore, the observed responses from a mixture of different chemicals do not always reveal the toxic effects of its components (de Almeida *et al*., 2014; Tridico *et al*., 2010). In general, the above-mentioned factors may individually and/or collectively play role(s) for the inconsistency of the enzyme activities with the gene expression levels.

Sex-specific expression of several CYPs has been observed in fish, rats and mice liver, which is an indication of sex steroids regulatory role in the expression of several CYPs in fish. In the present study, exposure of juvenile Atlantic salmon to EE2 (2.5 µg/ml) was also included as a positive control. As expected, the fish exposure to EE2 in this study produced a significant reduction of EROD activity and non-significant reduction of BROD, and MROD activities below the control group. Moreover, it also showed down-regulation of CYP1A1 and CYP3A mRNA levels. This result is in accordance with a previous study showing that EE2 produced suppression of EROD activity and expression of CYP1A1 mRNA level in Atlantic salmon in *in vivo* experiment (Mortensen and Arukwe, 2007a). It has been established in several *in vivo* and in vitro experiments that estrogenic compounds are known to decrease hepatic CYP1A1 transcriptional levels and its subsequent reduction in EROD activity and protein levels. Several reasons have been hypothesized and explained by the hormone-mediated reduction of CYP1A1, which includes inhibition of the CYP1A1 protein catalytic activity proceeded by the binding of

steroid hormones and/or metabolites to the CYP1A1 protein (Mortensen and Arukwe, 2007a; Arukwe *et al*., 2015). It has been hypothesized that the inhibitory effect of E2 could, in some way, be produced by the interference of the CYP1A1 gene with the hepatic ER-E2 complex. Generally, the results obtained from EE2 exposure are in accordance with previous studies revealing that a 2.5 µg/ml concentration of EE2 showed significant suppression of hepatic CYP1A1 mRNA levels and EROD activity in Atlantic salmon (Mortensen and Arukwe, 2007a).

As a phase II biotransformation enzyme, GST is involved in the conjugation and elimination of compounds having electrophilic groups such as organic xenobiotics and hydrocarbons. The detoxification reaction involves catalytic conjugation of the reduced glutathione (GSH) and oxidant reduction (Henson *et al*., 2001). However, since the relationship between pollution and enzyme induction in aquatic species has not always been observed, the GST responses following pollutant exposure are conflicting (Cossu *et al*., 1997). Generally, both the EE2 exposure and the Ikorodu non-polar exposure groups showed significantly higher catalytic activities of GST, compared to the control group and the Makoko polar group also showed a non-significant increase in GST catalytic activity above the control group, which was more or less similar with the EE2 exposure group. As a result of the important role that GST plays in the detoxification of PAH epoxides (Henson *et al*., 2001), the GST catalytic result of the Ikorodu non-polar group may be a reflection of the amount of analyzed PAHs at the two sediment sampling sites, where the Ikorodu sediment sample contains higher values of PAHs than the Makoko sediment samples. Benzo[a]pyrene, for example, which is found in higher concentration in the Ikorodu sediment sample can be metabolized to benzo[a]pyrene diones by CYP1A, which eventually generate ROS (Lushchak, 2011). Moreover, the extraction solvent, DCM, and the pollutant extraction protocol for the Ikorodu non-polar sediment would also favor the extraction of non-polar pollutants, including PAHs (Ma *et al*., 2010). Thus, the Ikorodu sediments that had higher concentrations of the analyzed PAHs exhibited higher GST, whereas the Makoko sediments with relatively lower concentration levels of the analyzed PAHs showed GST activities lower than the Ikorodu sediment samples.

On the other hand, the polar sediment extract from the Makoko site showed nearly similar GST catalytic activities to that exhibited by the EE2 exposure. Similarly, Houtman *et al*. (2006) and Viganò *et al*. (2008) found that polar fractions of sediment extracts exhibited highest estrogenic activity. Therefore, the similar activity of the polar sediment extract and the EE2 exposure in this study may be an indication of the presence of estrogenic compounds in the polar sediment extract of the Makoko exposure group, and thus revealed similar induction of the GST enzyme.

4.7.3 Oxidative responses

As previously described, cells and tissues possess a chain of cellular antioxidant protection mechanism to neutralize the toxic effects posed by ROS (Mahboob *et al*., 2014). However, oxidative stress occurs when the production of ROS overwhelms the antioxidant system that neutralize them (Olsvik *et al*., 2011). Pollutant in the aquatic ecosystem, however, causes redox cycling of pollution, which is the main contributor to oxidative stress in fish. Moreover, even without generation of pollution, xenobiotic metabolism also yields the continuous production of ROS (Mahboob *et al*., 2014). Many organic and metallic pollutants, including PAHs, OCPs (DDT, dieldrin), PCDDs and PCDFs, nitroaromatic and other heterocyclic, organophosphate fertilizers, estrogenic compounds (alkylphenol ethoxylate surfactant) and many metals, including cadmium, chromium, copper, iron, mercury, zinc, lead, and silver are powerful oxidants (Livingstone, 2001). These pollutants are taken up by the aquatic animals from sediment, suspended particulate materials, water-column, and food-sources, which consequently accumulate in the tissues of fish and generate ROS, which may lead to environmental oxidative stress (Livingstone, 2001; Mahboob *et al*., 2014). From the pollutant extract analysis result, when compared to the Ikorodu sediment samples, the Makoko sediment samples showed higher concentration of the abovementioned metals (particularly, chromium, copper, and mercury) with relatively higher concentration and are mentioned as powerful oxidants (Martinez-Alvarez, Morales, and Sanz, 2005). However, the non-polar sediment extracts from the Ikorodu sampling site is expected to contain these metallic oxidants than the Makoko sediment polar extracts. These differences may, therefore, contribute to the higher GST catalytic activities of the Ikorodu non-polar groups.

Fish have a powerful antioxidant protection system to counteract hostile conditions generated by ROS (Pandey *et al*., 2003; Zhu *et al*., 2008). The protection mechanism involves numerous low-molecular-weight, nonenzymatic antioxidants, GSH and various antioxidant enzymes, such as SOD, CAT, GPX, and GR, which catalyze the different reactions to maintain the homeostasis condition inside the cell (Mahboob *et al*., 2014; Morales *et al*., 2004). Therefore, the induction of these enzymes following the occurrence of oxidative stress by several environmental pollutants has been used for biomarker development. Yet, when compared to phase I and II enzymes, antioxidant enzymes are generally less responsive to pollutants (Kopecka-Pilarczyk and Correia, 2009). However, their detoxification activities, have gained potential application in oxidative stress investigations (Van der Oost *et al*., 2003).

During investigation of the effects of the different classes of contaminants, most studies have focused on either the gene expression or biochemical responses of biotransformation and antioxidant enzymes in fish. Moreover, the transcriptional expression responses have been

found inconsistent with the catalytic responses of the antioxidant system (Regoli *et al*., 2011). Therefore, only the enzymes catalytic activities were performed in this study.

Previous studies have reported that sediment extracts from the Lagos lagoon system showed oxidative DNA damage on Zebrafish embryo (Sogbanmu *et al*., 2016) and RTgill-W1 fish cells (Amaeze *et al*., 2015). There have been also reports of heavy metal in the sediments, water (Don-Pedro, Oyewo, and Otitoloju, 2004; Okoye *et al*., 1991), and in the tissues of fish (Okoye, 1991) in the Lagos lagoon system. Thus, heavy metal accumulation in the tissues of fish may promote chemical reactions that ultimately produce ROS and cause environmental oxidative stress (Mahboob *et al*., 2014). In this study, oxidative stress results of the different enzyme catalytic activities investigated and involved in the oxidative stress didn't generally show any significant change from the control group, except for the GR and GST. As a member of phase II enzyme, the enzyme GST is already discussed in the last part of the biotransformation responses. On the other hand, all the exposure groups exhibited reduced GR catalytic activities as opposed to the GST responses. EE2 and Ikorodu non-polar sediment extract exposure produced significant reduction of GR activity. During oxidative stress, the increased production of ROS initiates the induction of antioxidant enzymes. However, deficiency of the system resulted in inhibition of the antioxidant enzymes (Ameur *et al*., 2012). Therefore, the reduction of the enzymatic activities of the two exposure groups may be an indication of deficiency of the system as a result of high levels of pollutant exposure. These two exposure groups (EE2 and Ikorodu non-polar sediment extract) were demonstrated higher GST catalytic activities. Therefore, the GR enzyme was expected to convert the GSSG to GSH and supplement the GST enzymes with the GSH for the conjugation and elimination of electrophilic compounds, such as organic xenobiotics and hydrocarbons. However, the reduction of the GR enzymatic activity might be an indication of a failure in antioxidant defenses (Morales *et al*., 2004). Moreover, all the exposure groups didn't generally show any remarkable catalase and GPx activities compared to the control group. This is, therefore, further strengthen that the deficiency of the antioxidant system. In accordance to the present study, there have been reports of lower antioxidant enzymes activities in the liver of *Mugil cephalus* from polluted environment of Perama Bay, which was mentioned to be associated with high levels of pollutant exposure (Tsangaris, Vergolyas, Fountoulaki, and Nizheradze, 2011). Therefore, the reduced antioxidant enzymes activities and higher GST activity in the present study may indicate that GST might function more as a detoxification activity than as an antioxidant defense activity.

4.7.4 Estrogenic responses

In fish liver, ERs respond to estrogen by synthesizing the egg yolk protein (Vtg) and eggshell protein (*Zrp*). Even though male, female, and juvenile fish have such receptors in their liver, only female fish are expected to have sufficient estrogen to produce Vtg and *Zrp* (Arukwe *et al*., 2001). Therefore, a form of endocrine disruption demonstrated by estrogenic responses in male and juvenile Atlantic salmon exposed to environmental contaminants is the unfortunate production of Vtg and *Zrp* and have been used as a biomarker for assessing estrogen mimicking xenobiotics (Arukwe *et al*., 2000; Mcardle, Mcelroy, and Elskus, 2004).

The potential adverse effects of sediment bound EDCs have been reported by several researchers in aquatic wildlife using *in vitro* and *in vivo* assays (Hecker and Hollert, 2011). Moreover, effluent and sludge from municipal sewage treatment plant is one of the most important sources of EDCs, which includes natural and man-made estrogens, and alkylphenol ethoxylates (Mcardle *et al*., 2004). In connection to this, different studies showed that fish species inhabiting the Lagos lagoon system suffer from severe intersex (Adeogun *et al*., 2014), developmental and reproductive effects in relation to phthalic esters exposure, which were detected and measured in both water and sediment (Adeogun *et al*., 2015). Therefore, we exposed Atlantic salmon to polar and non-polar sediment extracts from Makoko and Ikorodu sites, respectively in order to investigate their estrogenic effects in fish. In this study, both polar and non-polar sediment extracts exposure to juvenile Atlantic salmon showed non-significant small increase and decrease of Vtg, *Zrp*, and ERα gene expressions compared to the control group. Specifically, the polar sediment extract from the Makoko site produced a non-significant increased expression of ERα mRNA and nearly similar expressions of Vtg and *Zrp* mRNA as the control group. Despite the involvement of ER for the expression of Vtg and *Zrp* mRNA, the higher expression of ER mRNA in this study didn't parallel Vtg and *Zrp* mRNA expressions. It has even been reported that the basal ER levels were enough to produce an increased expression of Vtg and *Zrp* mRNA (Mortensen and Arukwe, 2007a). Moreover, the extraction procedure for the polar sediment extract is expected to favors relatively polar estrogenic compounds (Schmitt, Reifferscheid, Claus, Schlüsener, and Buchinger, 2012; Viganò *et al*., 2008). Due to the aforementioned reasons, the Makoko polar extract was expected to show estrogenic responses. In accordance with this result, Mcardle *et al*. (2004) also reported that the fish (*Fundulus heteroclitus*) exposure to sediment extracts did not induce Vtg, but did induce CYP1A. Similarly, the Makoko polar exposure group showed higher CYP1A1 activities. Therefore, the unaffected estrogenic responses may indicate that the estrogenic compounds in the sampled sediment may not be found at the levels that produce estrogenic effects or other

contaminant in the sediments may antagonize and reduce the estrogenic effects (Mcardle *et al*., 2004).

On the other hand, the non-polar sediment extract from the Ikorodu site showed a non-significant increased expression of Vtg mRNA, while exhibiting expression of *Zrp* mRNA more or less similar to the control group and a small reduction of the ERα mRNA level. This finding is, therefore, in accordance with the previous study that Houtman *et al*. (2006) also found compounds with estrogenic activities in the non-polar portion of sediment extract. Mortensen and Arukwe (2008) previously reported that PCB126 produced a time- and concentration-specific increase of ERα and Vtg expressions in salmon primary hepatocytes. Even though PCBs were not included in the chemical analysis of this study, due to their non-polar nature, PCBs may be present in the Ikorodu non-polar sediment extract. Moreover, fish and sediment samples from the Lagos lagoon were also found with higher concentration of PCBs (Adeyemi *et al*., 2011). Therefore, the non-significant higher expression of Vtg mRNA in the Ikorodu non-polar exposure group may be produced by non-polar and known AhR agonists (such as PCB126), which were also produced higher CYP1A1 catalytic activity in this exposure group. These AhR agonists have also showed reduced Vtg synthesis in both *in vivo* and in vitro studies. This condition is observed in the absence of ER agonists and is described as the ER–AhR crosstalk or estrogen receptor hijacking. Through a direct receptor interactions or crosstalk with other nuclear receptors, several environmental pollutants are known or assumed to negatively affect the estrogen-signaling pathway. Therefore, reduced Vtg synthesis or impaired gonadal development have been reported in *in vivo* and in vitro and could be associated with exposure to AhR agonists in teleost (Gjernes, Schlenk, and Arukwe, 2012).

Regarding the positive control, EE2 produced significantly higher expressions of Vtg, *Zrp*, and ERα that were extremely higher than the control group. Similarly, a time- and concentration-specific decreased transcription of ER isoforms (ERα and ERβ) and increased expression of Vtg and *Zrp* mRNA level was also reported in the Atlantic salmon liver at day 3 after exposing to EE2 at lower concentration than the present study (Mortensen and Arukwe, 2007a). Therefore, the presence of pharmaceutical EDCs, such as EE2 in the aquatic environment could have been interfering with hormonal regulation in fish. This ultimately affects the sensitive seasonal variation in plasma sex steroids and cause impaired reproductive system in fish (Milla *et al*., 2011). However, a more serious consequence above the investigated endpoints (Vtg, *Zrp*, and ER) might have been expected from the combined effect of EDCs on hormonal pathways.

4.8 Conclusion and future direction

In evaluating the toxic potential of the sediment extracts to living organisms, hydrophobic organic contaminants are usually identified and quantified. This effort is, however, inadequate because of the possible additive, synergistic or antagonistic interactions between different contaminants that create unexpected effects. As a result, the effects that a single contaminant alone produced on fish are rather distinct from the collective effects that this contaminant produces in combination with other contaminants (de Almeida *et al*., 2014; Bizarro *et al*., 2016). Therefore, this study showed that the sediment samples from the Lagos lagoon produced higher EROD, BROD and MROD activities in salmon, which are biochemical biomarkers of CYP1A1 catalytic activity induced as a result of diverse groups of xenobiotic exposure (such as AhR agonists). Moreover, both sampled sediment also produced higher GST catalytic activities as a phase II conjugating enzyme, which further strengthen the possible presence of xenobiotics in the sediment samples.

Regarding oxidative stress, both sediment extracts produced a reduction of GR activities, which might be an indication of failure in antioxidant system due to prolonged higher pollutant load that further suggests that GST might function more as a detoxification activity than as an antioxidant defense activity. Finally, both sediment extracts didn't influence the gene expression of estrogenic responses (Vtg and *Zrp* mRNA level), which may indicate that the estrogenic compounds in the sampled sediments may not be found at the levels that produce estrogenic effects or other contaminants in the sediments may antagonize and reduce the estrogenic effects.

Therefore, the exposure of salmon to sediment extracts from Makoko and Ikorodu sites of the Lagos lagoon system showed biotransformation of xenobiotics, failure in antioxidant defense system and unaffected estrogenic responses, which may be an indicative of potential and adverse health effects. However, a more comprehensive study that covers more target organs (such as kidney and gills), earlier sampling days (such as 0 and 3), and more endpoints might be a future directive in order to fully investigate the potential and adverse health effects of sediments in the Lagos lagoon system.

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Appendix

Appendix A: Detailed method of chemical analysis

Chemical analysis in sediment have been determined according to previous methods with slight modifications (Bocchetti *et al*., 2008; Fattorini *et al*., 2008; Piva *et al*., 2011; Regoli *et al*., 2014).

For trace metals, sediments were dried to constant weight at 60°C and digested under pressure with nitric acid and hydrogen peroxide (7:1) with microwave (Fattorini *et al*., 2008). Arsenic, cadmium, chromium, copper, iron, manganese, nickel, lead, vanadium, zinc were analyzed by atomic absorption spectrophotometry, with flame (Varian, SpectrAA 220FS) and flameless atomization (Varian SpectrAA 240Z) (Fattorini *et al*., 2008).

Aliphatic hydrocarbons were extracted treating sediment (about 3 g, wet weight) with hexane:acetone (2:1) in a microwave (110 °C for 25 minutes, 800 Watt) (Mars CEM, CEM Corporation, Matthews NC). After centrifugation at 3.000 × *g* for 10 minutes, the supernatants were purified with solid-phase extraction (Phenomenex Strata-X, 500 mg × 6 ml plus Phenomenex Strata-FL, 1000 mg × 6 ml) and then concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dryness. Samples were finally recovered with 1 ml of pure, analytical GC grade *n*-hexane and analyzed with a gas chromatograph (Perkin Elmer) equipped with an Elite-5 capillary column (30 m × 0.32 mm ID × 0.25 μm-df) and a flame ionization detector. For quantitative determination, the system was calibrated with an unsaturated pair *n*-alkane standard mixture according to EN ISO 9377-3 (Fluka 68281).

For analysis of polycyclic aromatic hydrocarbons (PAHs), about 3 g (wet weight) of sediment was extracted in 10 ml of 0.5 M potassium hydroxide in methanol with microwave at 55 °C for 20 minutes (800 Watt) (CEM, Mars System). After centrifugation at 3.000 × *g* for 10 minutes, the methanolic solutions were concentrated using a SpeedVac and purified with solid-phase extraction (Octadecyl C18, 500 mg × 6 ml, Bakerbond). A final volume of 1 ml was recovered with pure, analytical HPLC gradient grade acetonitrile, and HPLC analyses were carried out in a water and acetonitrile gradient by fluorimetric and diode array detection. The PAHs were identified according to the retention times of an appropriate pure standards solution (EPA 610 Polynuclear Aromatic Hydrocarbons Mix), and classified as low molecular weight (LMW: naphthalene, acenaphthylene, 1-methyl naphthalene, 2-methyl naphthalene, acenaphthene, fluorene, phenanthrene, anthracene) or high molecular weight (HMW: fluoranthene, pyrene, benzo[a]antrhacene, chrysene, 7,12-dimethyl benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, benzo[a]pyrene, dibenzo[a,h]anthracene, benzo[g,h,i]perylene, indeno[1,2,3,c,d]pyrene.

Organo-halogenated pesticides (OCPs), phenolic compounds, and organotin have been determined according to EPA methods 1614, 3550B, 3665A, 3630C, 8020, 8081B, 8082A with slight modifications. About 3 g of wet sediment was extracted with hexane:acetone (2:1) solutions in a microwave (110 °C for 25 minutes, 800 Watt) (Mars CEM, CEM Corporation). After centrifugation at 3.000 × g for 10 minutes, the supernatants were purified with solid-phase extraction (Phenomenex Strata-X, 500mg, 6mL and Phenomenex Strata-FL, 1000mg, 6 mL), using an automated SPE system (Gilson Aspec GX271, Gilson Middleton, WI, USA) and then concentrated using a SpeedVac (RC1009; Jouan, Nantes, France) to dryness. Samples were finally recovered with 1 mL of pure, analytical GC grade n-hexane and analyzed with a GC-MS system (Varian Saturn 2000 ion trap, Agilent Technologies, Santa Clara, CA, USA) using a GC capillary column Zebron (Zebron ZB-5MS, 30m, 0.25mmID, 0.25µ, Phenomenex), applying three different spit-splitless and oven temperature ramp methods for OCPs (lindane and dieldrin), phenolic compounds (4-iso-nonyphenol, 4-t-octylphenol), and organotin (monobutyltin).

For all of the chemical analyses, quality assurance and quality control were assured and monitored by processing blank and reference standard materials (NIST-2977, National Institute of Standards and Technology, USA); concentrations obtained from these SRM analyses were always within the 95 % confidence intervals of the certified values. The water content in sediment was determined for all the samples, and the concentrations were expressed as ng/gm dry weight (dw) for PAHs, while as µg/gm dry weight (dw) for OCPs, phenolic compounds, organotin, trace metals and aliphatic hydrocarbons (C10-C40).

Appendix B: Preparation of RNA agarose gel

Before preparation of the agarose gel, the different compositions were prepared in advance (Table 1B-3B). All the Gel electrophoresis equipment then cleaned with 10% NaOH and rinsed with DEPC treated Milli-Q water. The appropriate amount of powder agarose was mixed with the DEPC treated Milli-Q water (Table 4B), microwaved until completely dissolved and cooled down to 50 ^{o}C. Finally, the appropriate amounts of formaldehyde and GelRed were added into the agarose gel solution and then poured immediately. After solidification, the agarose gel was used.

Table 1B: Preparation of 10X 3-(N-Morpholino) propanesulfonic acid (10X MOPS)

10X MOPS[§]	Volume per reaction
MOPS	41.86 g
Sodium acetate	4.10 g
EDTA (0.2 M)	25 ml
DEPC treated Milli-Q water*	475 ml

§ After mixing all the components and stirring, the pH of the solution was adjusted at 7 and finally autoclaved at 121 ^{o}C for 20 minutes.

** DEPC (Diethyl pyrocarbonate) treated Milli-Q water (0.1%) is prepared by mixing 1 ml of DEPC in 1 liter of Milli-Q water and autoclaved at 121 ^{o}C for 20 minutes.*

Table 2B: Compositions for the running buffer preparation

1X MOPS running buffer	Volume per reaction
10X MOPS	20 ml
37% Formaldehyde*	5 ml
DEPC treated Milli-Q water	225 ml

**work in the hood*

Table 3B: Compositions for the electrophoresis sample buffer preparation

Sample buffer *	Volume per reaction
Deionized Formamide	250 µl
10X MOPS	50 µl
37% Formaldehyde	83 µl
DEPC treated Milli-Q water (0.1%)	57 µl
Glycerol	50 µl
Bromophenol blue (2.5%)	10 µl

**stored at -20°C*

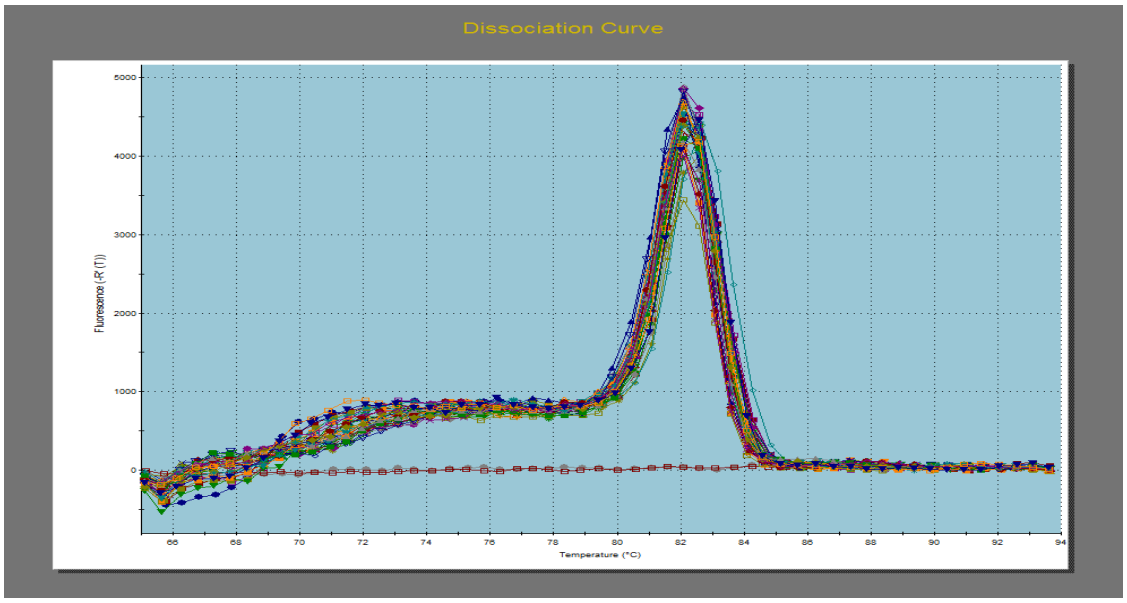
Table 4B: Compositions for formaldehyde agarose gel preparation

Formaldehyde agarose gel (100 ml)	Volume per reaction
Agarose	1.214 g
10x MOPS	10 ml
DEPC treated Milli-Q water	88.2 ml
37% Formaldehyde	1.8 ml
GelRed	10 µl

Appendix C: Melting curves

Some selected dissociation curves of the analyzed genes for as a quality control for the specificity of the PCR amplification (Figure 1A and B).

A



B

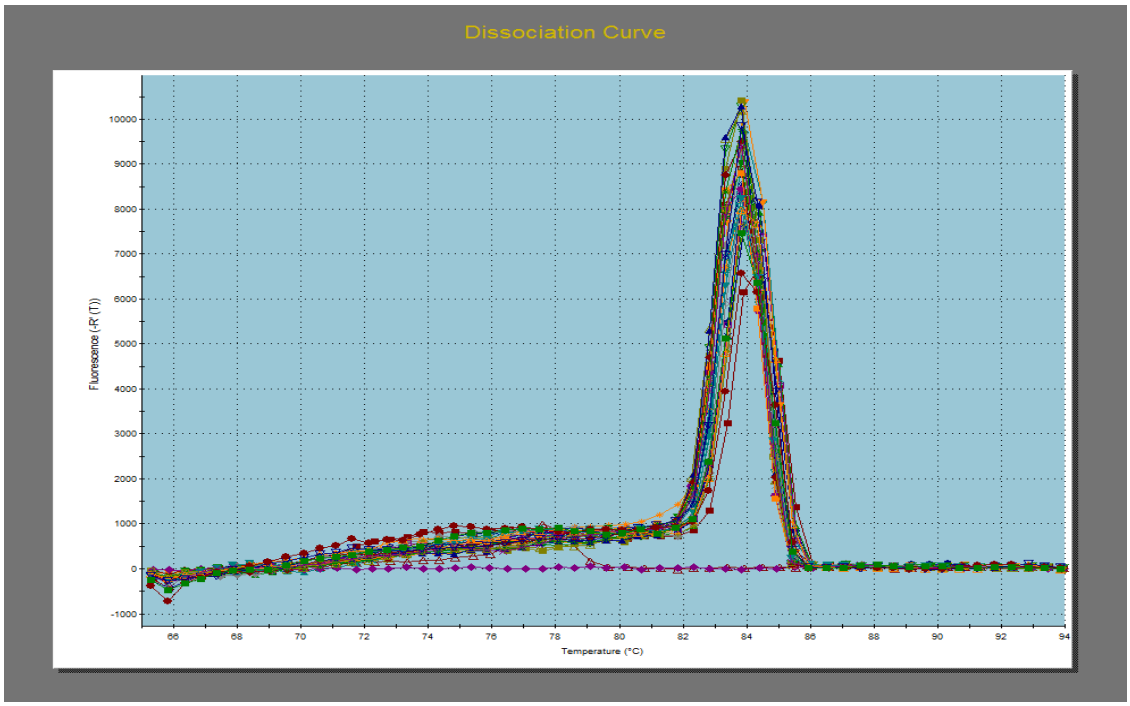


Figure 1C: Dissociation curves of CYP1A1 (A) and Vtg (B) as representatives of the analyzed genes. Both showing a single peak at around 82 °C with almost non primer dimer peaks that indicates specific amplification of the target genes.