GENOTOXIC EFFECT AND VITELLOGENIN GENE EXPRESSION IN *OREOCHROMISNILOTICUS* ON EXPOSURE TO ENDOCRINE DISRUPTOR CHEMICALS (EDC)

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ABSTRACT

This study was conducted to evaluate the genotoxicity of atrazine and endosulfan as a potential endocrine disruptor chemicals (EDCs) in *Oreochromis niloticus* by using micronucleus test and RAPD assays, as well as to determine vitellogenin gene expression in male *O. niloticus*for 96 hours exposure period. *O. niloticus* was exposed to atrazine at concentration of 0.50 mg/L, 2.50 mg/L, and 4.75 mg/L, and to endosulfan concentration of 1.50 µg/L, 3.60 µg/L, and 7.00 µg/L. In the micronucleus test, athigh concentration of atrazine and endosulfan, there was a significant increase of the frequency of micronucleus and nuclear abnormalites. These results showed that exposure to atrazine and endosulfan significantly increased genetic toxicity in *O. niloticus* blood cells. A subsequent RAPD assay also showed that atrazine and endosulfan induced genotoxic effect in *O. niloticus*by changing the DNA profiling as compared to control test. The five RAPD primers tested produced unique polymorphic band patterns and generated RAPD profile variations that displayed the disappearance of bands and appearance of new bands of amplified DNA in the atrazine and endosulfan-treated genomic DNA. There was also induction of vitellogenin transcript observed in male *O. niloticus* upon treatment to all atrazine concentrations and high concentrations of endosulfan. These results suggested that atrazine and endosulfan are potentially harmful to fishes as EDCs as the chemicals have been shown to cause genotoxicity effect and vitellogenin expression in male *O. niloticus*.

ABSTRAK

Kajian ini telah dijalankan untuk menilai kesan genotoksik atrazine dan endosulfan yang berpotensi sebagai bahan kimia mengganggu endokrin (EDCs) ke atas *Oreochromis niloticus* dengan menggunakan ujian mikronukleus dan RAPD, dan mengenalpasti ekspresi gen vitellogenin pada *O. niloticus* jantan selepas didedahkan dengan bahan-bahan kimia ini selama 96 jam.*O. niloticus* didedahkan kepada atrazine pada kepekatan 0.50 mg/L, 2.50 mg/L, dan 4.75 mg/L, serta kepada endosulfan pada kepekatan 1.50 µg/L, 3.60 µg/L, dan 7.00 µg/L. Di dalam ujian mikronukleus, terdapat peningkatan secara signifikan terhadap frekuensi mikronukleus dan keabnormalan nukleus apabila ikan didedahkan pada kepekatan atrazine dan endosulfan yang tinggi.Hasil ujian ini menunjukkan pendedahan ikan kepada atrazine dan endosulfan telah menyebabkan peningkatan ketoksikan genetik secara signifikan di dalam sel-sel darah *O. niloticus*.Ujian RAPD juga menunjukkan bahawa atrazine dan endosulfan mampu untuk mendorong kesan genotoksik untuk berlaku ke atas *O. niloticus* dengan melihat kepada perubahan profil DNA berbanding dengan ujian kawalan.Lima primer RAPD yang digunakan menghasilkan jalur-jalur polimorfik yang unik, dan variasi pada profil RAPD memaparkan kehilangan jalur dan kehadiran jalur baru yang terhasil daripada amplifikasi DNA genomik ikan yang terdedah kepada atrazine dan endosulfan.Hasil kajian juga menunjukkan terdapatnya penghasilan transkrip vitellogenin di dalam ikan jantan apabila ikan didedahkan kepada kesemua kepekatan atrazine dan pada kepekatan tinggi endosulfan.Hasil-hasil ujian mencadangkan bahawa atrazine dan endosulfan berpotensi untuk memberikan bahaya kepada ikan sebagai EDCs, memandangkan kedua-dua bahan kimia ini telah menunjukkan kesan genotoksik dan ekspresi vitellogenin di dalam *O. niloticus* jantan.

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LIST OF SYMBOLS AND ABBREVIATIONS

Symbols

bp	Base pair
cm	Centimeter
°C	Degree celcius
g	Gram
kb	Kilobase
LC_{50}	Lethal concentration that results in 50% death
µg/L	Microgram per liter
µL	Miroliters
mg/kg	Miligram per kilogram
mg/L	Milligram per liter
mL	Mililiters
ng	Nanograms
nm	Nanometers
%	Percentage
x g	Relative centrifugal force
C_T	Threshold cycles

V	Volt
v/v	Volume over volume
w/v	Weight over volume

Abbreviations

E_2	17β-estradiol
BPA	Bisphenol A
cDNA	Complementary DNA
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleotide triphosphatase
ELISA	Enzyme-linked immunosorbent assay
EDC	Endocrine disruptor chemical
EDTA	Ethylenediaminetetraacetic acid
ER	Estrogen receptor
EtBr	Ethidium bromide
GTS	Genomic template stability
MALDI-TOF	Matrix-assisted laser desorption/ionization-Time of flight
mRNA	Messenger RNA

MN	Micronucleus
NA	Nuclear abnormality
O. niloticus	*Oreochromis niloticus*
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
qPCR	Quantitative real time PCR
RAPD	Random amplified polymorphic DNA
RT	Reverse transcription
RNA	Ribonucleic acid
RNase	Ribonuclease
NaOH	Sodium hydroxide
TBE	Tris-borate-EDTA
Vtg	Vitellogenin

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CHAPTER 1

INTRODUCTION

Chemicals that can either mimic endogenous hormones, interfere with pharmacokinetics, or act by other mechanisms are referred to endocrine disruptor chemicals (EDCs). The term “endocrine disruptors” is used because the substance is not naturally produced in the body, but it can mimic or antagonize natural hormones which can alter the normal endocrine system (Larkin et al., 2003; Shammas, 2007). EDCs have the potential to be harmful not only toanimalsbut also humans. It is thought that EDCs are responsible for endocrine disruption in wildlife as seen in fish (Al-Sabti and Metcalfe, 1995; Ayllon and Garcia-Vazquez, 2001), and amphibians (Miyahara et al., 2003), besides causingsome reproductive problems in both human and rodents (Fan et al., 2007), and also increase the frequency of occurrence of certain types of cancer in human (Brusick, 1994; Fan et al., 2007). Therefore, it has becomea major concern to the public nowadays, since people started to be aware about the effect of such compound to their health.A lot of researches have been done to study the effect of EDCs on human and animal. Because hormone receptor system in human is similar with animals, thus effects observed in wildlife can be extrapolated to humans (Shammas, 2007).

Exposure of toxic substances can cause DNA damages,such as DNA base modifications, strand breaks, depurination and cross-linkages (Frenzilli et al., 2004; Sayed et al., 2013), in living cells. A growing interest to detect genotoxicity caused by genotoxic agents, such as EDCs, has led to the development of sister chromatid exchange,

chromosomal aberration, comet assay, micronucleus (MN) test, and nuclear abnormalities (NA) to detect genotoxicity directly (Al-Sabti and Metcalfe, 1995; Ayllon and Garciz-Vazquez, 2001; Mohanty et al., 2011), or by using molecular approaches especially polymerase chain reaction (PCR) basedtechniques such as random amplified polymorphic DNA (RAPD), denaturing gradient gel electrophoresis (DGGE), and single stranded conformational polymorphisms (SSCP)(Tice et al., 2000; Cajaraville et al., 2003; Atienzar and Jha, 2006).

MN test and comet assay are among the tests which have been used widely since these tests give reliable results, simple and sensitive (Ayllon and Garcia-Vazquez, 2001). The presence of MN in cells reflects structural and/or numerical chromosomal aberrations during mitosis, thus indicate genotoxic effect of the toxic agents (Ventura et al., 2008). Genotoxicity study in fish using MN test and NA is a suitable measure to detect the presence and absence of genotoxins in water, as well as to assess the water quality (Talapatra and Banerjee, 2007). Another reliable technique to assess genotoxicity caused by EDCs is using RAPD. This technique is a modification of conventional PCR. RAPD offers great scope to detect and compare changes between normal and genotoxicant exposed group of animals in studies related to genotoxicity (Mohanty et al., 2011). The genoxicity in exposed group of test studies to genotoxicants is determined by the gain of new bands and loss of bands (Atienzar and Jha, 2006). The advantage of RAPD in genotoxicity studies includes ease, speed and low cost of experiment when the focus is to detect genomic mutations (Mohanty et al., 2011).

It has been demonstrated that most EDCs act as xenoestrogens, which can mimic, alter, or antagonize the action of endogenous estrogen, and thus disrupt the reproductive capacities of various animals (Livingstone et al., 2000; Marin and Matozzo, 2004; Huang et al., 2010). In fish, the synthesis of vitellogenin (Vtg), which is the female-specific egg-yolk proteinscan be stimulated in male fish when exposed to EDCs (Sabo-Attwood et al., 2007). In reproductively active female fish, endogenous 17β-estradiol (E_2)stimulates the liver to produce Vtg. While in male fish and immature females, there are necessary E_2receptors and genetic machinery to produce vitellogenin (Sumpter and Jobling, 1995). Therefore, Vtg induction in male and immature fish has been used as biomarker both in laboratory and field studies to detect the estrogenic activity of chemicals suspected as being endocrine active (Arukwe and Goksøyr, 2003; Ortiz-Zarragoitia and Cajaraville, 2005).

The study of EDCs in aquatic environment especially in fish is important because fish is one of major protein consumed by human. Possibility for human to require the effects of toxic chemical can be achieved through the contaminated fish. There was a study done by Skerfving and colleagues (1974), which indicated the induction of chromosomal damage in lymphocyte of persons after consuming contaminated fish. Such cause is due to the exposure of methyl mercury from the fish. There are a lot of studies which utilized fishes as models to study aquatic toxicology because they responded similarly to higher vertebrate when exposed to toxicity by chemical contaminants and they also are good bio-indicators for pollutant effects (Ventura et al., 2008).

Oreochromis niloticus, which is commonly known as the Nile tilapia, have been used in many researches to study genotoxicity. The fish can be found in estuaries in most part of the world (Vijayan et al, 1996). In Malaysia, the fish have been crossed among*Oreochromis sp.*(Musa et al., 2009).The increasing demand in market and its economical value makes it worth to use this fish as a model of study in aquatic toxicology, particularly to study EDCs. The advantages of using *O. niloticus* as a biological model in toxicology studies are due to its sensibility to environment alteration, high growth rate, its ability to adapt to diverse diets and it has high resistance towards diseases (Ventura et al., 2008).

1.1 Objectives of Research

The objective of this research is to determine genotoxic effects of selected chemicals and their potential to affect endocrine system in the fish. In order to achieve the main objective, the measurable objectives of this study are stated as follow:

i. To observe the genotoxic effect through the occurrence of MN and NA in the blood cells of *O. niloticus*after exposure to EDCs.
ii. To determinechanges in RAPD banding pattern in *O. niloticus* after exposure to EDCs.
iii. To study the expression of vitellogenin after exposure to EDCs, especially in male*O. niloticus*.

CHAPTER 2

LITERATURE REVIEW

2.1 Endocrine Disruptor Chemicals (EDCs)

The endocrine system consists of glands, hormones and receptors. The glands produce hormones, such as adrenocorticotropic hormone, coricostreoid, adrenalin, estrogen, testosterone, androgen, insulin, triiodothyronine, and thyroxin (Shammas, 2007). These hormones regulate about every biological functions in the body which include; (1) reproduction and embryo development, (2) growth and maturation, (3) energy production, use and storage, (4) electrolytes regulation to control the balance and maintenance of water and salt, (5) reaction to stimuli, such as fright and excitement, and (6) behavior of human being and animals (US EPA, 2001; Larkin et al., 2003; Pait and Nelson, 2003). Receptors in endocrine system recognize and response to the hormones. Receptors regulate the response so that the biological functions work properly. Any disruption to the balance can cause change in the reaction as well as unwanted harm to the body. The disruption may be caused by certain chemicals and they are known as endocrine disruptor chemicals (EDCs) (Larkin et al., 2003). The term “endocrine disruptors” is used to describe substances that are not produced in the body and which can mimic or antagonize natural hormones (Shammas, 2007).

Humans and animals shared similar hormone receptor systems, and effects observed in wildlife species raise concerns of potential human health effects (Brusick, 1994; Shammas, 2007). Thus, EDCs are one of the toxicant contaminants which have been extensively studied in this few decades to identify their effects in wildlife, especially in fishes as model systems (Al-Sabti and Metcalfe, 1995; Vos et al., 2000; Obiakor et al, 2012). EDCs are synthetic or naturally occurring chemicals that interfere with the balance of normal hormone function in animals, including humans. There are three groups of endocrine disruptors. They can mimic, block or trigger a hormone response (Jesperson, 2003). EDCs can mimic hormones which respond like normal hormones inside the body. The second groups are blocker chemicals which interfere with naturally occurring hormone functions by preventing the natural hormone to send its message. The last group is trigger chemicals. Endocrine disruptors which belong to this group caused alteration of the normal endocrine system, or alter the synthesis, metabolism and activity of hormones (Shammas, 2007). This is because trigger chemicals attach to protein receptors, then they trigger an abnormal response in the cell and led growth at the wrong time (Jesperson, 2003). Some effects attribute from the disruption of endocrine system balance have caused reduced fertility, hatchability and viability of offspring, as well as impaired hormone activity and altered sexual behavior (Larkin et al., 2003).

The characteristics of EDCs are explained as 1) chemicals that can mimic the sex steroid hormones estrogen and androgens by binding to hormone receptors or influencing cell signaling pathways, 2) block, prevent, and alter hormonal binding to hormone receptors or influence cell signaling pathways, 3) alter production and breakdown of natural hormones, and 4) modify levels and function of hormone receptors (Larkin et al., 2003;

Shammas, 2007). EDCs can be herbicides, inseticides, metals, alkylphenols, pesticides, and mixtures of chemicals. Among existing EDCs, insecticides and herbicides have been extensively used especially in agricultural area, where the chemicals are an important strategy for the increment of production and controlling plagues and diseases. The accumulation rate of such chemicals in aquatic environment depends on several causes. It can be on the kind of associated food chain, on availability and persistent of the contaminant in the water, or on the physical and chemical characteristics of the agrochemical (Larkin et al., 2003). A numbers of chemicals have been studied to see their potential to cause endocrine disruption, which include atrazine and endosulfan.

Atrazine (2-chloro-4-ethylamino-6-Isopropylamino-s-triazine) is an herbicide which belongs to triazine group. Triazine is the oldest herbicide and is widely used in the world where it commands around 30% of the pesticide market in the world (Tomita and Beyruth, 2002). Atrazine is extensively used in agricultural fields such as corn, sorghum, sugar cane, pineapples, and the usage also extended to landscape vegetation (Nwani et al., 2010). It is considered as moderately toxic to aquatic species but it is mobile in the environment and it has high stability in water. Besides, atrazine also is highly persistent in aquatic environments. When the water has high acidity and high dissolving organic matter, the chances of atrazine to be persistent in water is also increased. The presence of atrazine in water source is due to runoff from herbicide used on row crops (Shammas, 2007). Thus it appears as one of pesticides detected in streams, rivers, ponds, reservoirs, and ground water (Brusick, 1994; Nwani et al, 2010). The appearance of atrazine in aquatic environment can be directly due to careless application in the agricultural area or due to the proximity of such area to water places.

The residual of atrazine herbicide which leaches into the soil and into near water resources due to agricultural activities can give adverse effect to the stability of aquatic ecosystems, especially the potential to promote damage to the genetic material of fishes. The effects of atrazine is not only limited to fishes. This compound has shown to be genotoxic and mutagenic actions in plants (Mohammed and Ma, 1999), and a cause of cytotoxicity effect in snails (Mona et al, 2013). *In vitro* studies on the effect of atrazine to mammalian cells have shownchromosomal aberration, DNA damage and cytogenetic effectto human lymphocytes(Meisner et al., 1993; Ribas et al., 1995; Clements et al., 1997; Lioi et al., 1998). In the fish, atrazine has been reported to affect fish kidney morphology (Fisher-Scherl et al, 1991), swimming behavior (Saglio and Trijasse, 1998) and alter hormonal pathway in various taxa (Moore and Waring, 1998; Spanò et al, 2004; Thibaut and Porte, 2004).Another report related to the occurrence of endocrine disruption showed that atrazine caused alteration of hepatic metabolism, and induction of estrogenic effects and oxidative stress on juvenile rainbow trout *in vivo*, where the effects are linked (Thibaut and Porte, 2004).

Endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9,9a-hexahydro-6,9-methano-2,4,3-benzodioxathiepin-3-oxide),is another potential EDC which has been shown to cause harm towards aquatic inhabitants. Endosulfan is an organochlorine pesticide that is widely used in agriculture (Da Cuňa et al., 2011). The characteristics of organochlorine include being very persistent, non-biodegradable and capable of biomagnifications as they move up in the food chain thereby making the compounds belonging to this group among the most hazardous with respect to environmental pollution. Endosulfan is used in agriculture field to increase agricultural productivity. It is extensively used throughout the whole world as a

contact and stomach pesticides as well as acaricide on field cereal crops oilseed, coffee, vegetables, and fruit crops. Therefore their presence in water bodies could affect aquatic life. Endosulfan contamination is frequently found in the environment at considerable distances from the point of its original application. Furthermore, it also has been detected in the atmosphere, soils, sediments, surface and rain waters, and food stuffs(Jaffery et al, 1990).

Compared to atrazine, endosulfan is extremely toxic to fish and aquatic invertebrates. Endosulfan residues or its metabolites are associated with mortality of fish in continental aquatic system. The half-life of endosulfan is 35 to 150 days (Romeo and Quijano, 2000). During this period, endosulfan degrades into endosulfan sulfate. Endosulfan sulfate is a principle metabolites from the process and highly toxic to some aquatic species compared to the parent compound (Shimmel et al, 1977; Rao and Murty, 1982). For the release of endosulfan in water bodies, the recommended safe limit is 74 µg/L (US EPA, 2001). However this concentration is 15 times more than the required concentration to cause reproductive damage in the red-spotted newt (Park et al, 2001). Even at low concentration, endosulfan is capable of causing oxidative damage and immune-suppression as seen in *O. mossambicus* (Kumar et al, 2011).Endosulfan as an organochlorine has the possibility to cause several physiological impairments which include immune system (Banerjee and Hussain, 1987), excretory system (Singh and Pandey, 1989), and reproductive system (Sinha et al, 2001).

For the past years, studies related to the effects of EDCs in aquatic organisms have been done in Malaysia. Most studies relate the environmental pollution cause by pesticides, especially organochlorines and their effects to aquatic organisms (Abu Zeid et al., 2005; Hii et al, 2007). Organochlorines are considered to be the most hazardous with respect to environmental pollution but they are still widely used in most agricultural places in the world (Ilyas and Javed, 2013). Apart from being highly toxic, they also exhibit potential for endocrine disruption in aquatic organisms. In Malaysia, organochlorine pesticides such as endrin aldehyde, heptachlor epoxide, lindane, aldrin,dichlorodiphenyltrichloroethane (DDT), and endosulfan have been found in agricultural sites such as paddy field and vegetable farms (Zakaria et al., 2003; Abu Zeid et al., 2005). The presence of organochlorine pesticides in water and sediment samples taken from the sites showed that environmental contaminations are high at places involve with agricultural activities (Zakaria et al., 2003).

Endosulfan is one of the most studied organochlorines in the world, including Malaysia. Exposure of sublethal dose of endosulfan to African catfish (*Claria gariepinus*) showed highest accumulation in the livers followed by the intestines, the gill, brain and skeletal muscles (Abu Zeid et al., 2005). Reports of endosulfan in aquatic environment showed several effects such as hematological and behavioral changes in the Asian swamp eel (*Monopterus albus)*(Hii et al, 2007), increase fatalities of freshwater species (Liong et al., 1988), and reduce survival and reproduction performance of *Moina macrocopa* (Chuah et al, 2007). Apart from endosulfan, atrazine is also among the pesticide which is widely used in Malaysia, especially in plantations. The study of atrazine in Malaysia mostly involved the presence of this pesticide in water source and means of removing it (Ahmad et

al., 2008; Ali et al., 2012). Atrazine is considered as a low toxic herbicide, but the presence of atrazine in surface water and groundwater shows the impacts of water quality and poses environmental pollution, even though its use is in a limited amount of compound despite its use is within the permitted dosage (Plakas et al., 2006; Ahmad et al., 2008).

2.2 Genotoxic Effects of EDCs in the Aquatic Environment

Genotoxicity is a branch of toxicology studies where it deals with the study of deleterious effects of toxic agents in the environment on the structure and function of DNA. The term "genotoxicity" is used in general to describe alteration to the gross structure, or content of chromosomes (clastogenicity), or base pair sequence (mutagenicity) by exposure to toxic contaminants. The focus of genotoxicity studies is to determine direct DNA damage, due to concern of the effects of genotoxins on the health of an organism and the possible implications to future generations if the germline is affected (Mohanty et al., 2011). The effects of genotoxicity in living systems can be seen in three types of genetic lesions. The first type of genotoxic effects are single gene mutations, or point mutations, which alter the nucleotide sequence of DNA, and may involve either the base substitution or frameshift mutations. Secondly, the structural chromosomal mutations or genomic mutations, which involve changes in chromosomal structure such as breaking of chromosome, or translocation of an arm, commonly known as clastogenensis. The third effects are numerical changes in the genome, aneuploidy and/or polyploidy (Cajaraville et al., 2003; Mohanty et al., 2011).

It is important to know the extent of genotoxicity in aquatic environment. This is because many toxic and potentially toxic chemical substances, either from natural source or man-made, are released into the environment daily (Obiakor et al, 2012). In genotoxic pollution of freshwater, the genotoxicants are mostly introduced into the water bodies through industrial, agricultural, domestic, and urban activities (Zakaria et al., 2003; Naeem et al, 2011; Naz and Javed, 2012). Current awareness of the potential hazards of EDCs in the aquatic environment has developed interest to study their effects in aquatic animals, especially in fish (Nwani et al., 2010; Obiakor et al., 2012). Recent reports have demonstrated the toxicity and effects of EDCs to fish under laboratory and field conditions (Nwani et al., 2011; Bűcker and Da Conceição , 2012; Ilyas and Javed, 2013).

There are a lot of tests to study the genotoxic effect of EDCs in fish *in vivo* and *in vitro*. MN test is the most common methods to study genotoxicity in fish since this method is simple and cost-effective (Ventura et al, 2008). This technique was first described by Schmidt (1975). Most studiesevaluate the genotoxic damage to blood erythrocytes of the fish when using this test (Udroiu, 2006). It is considered as a sensitive tool to measure the action of mutagenic chemicals, especially of those that cause clastogenic changes in exposed organisms. In most studies, after exposure to different EDCs under field and laboratory conditions, erythrocytes of fishes present significant increase of MN formation (Hughes and Hebert, 1991; Minissi et al., 1996; Souza and Fontanetti, 2006; Ventura et al., 2008; Nwani et al., 2011; Sponchiado et al., 2011). As a complement to the MN test, occurrence of morphological alteration, or also known as NA in blood erythrocytes is performed together as a possible indicator to genotoxicity (Carrasco et al., 1990;Ayllon and Garcia-Vazquez, 2001; Baršienė et al., 2006;Ventura et al., 2008).

Genotoxicity in fish also can be determined by assessing genetic patterns directly using RAPD. RAPD is a modification of PCR developed by Williams et al (1990). This technique is a molecular genetic based method which allows evaluation of molecular level (DNA) variation in populations (Belfiore and Anderson, 2001) since the amplified segments of DNA are random (Atienzar and Jha, 2006). This method is popular for comparing the DNA of biological systems that have not had the attention of the scientific community, or in a system in which relatively few DNA sequences are compared. The method does not require cloning, sequencing or any other form of the molecular characterization of the genome of the species in question.

The principle of RAPD is that short synthetic oligonucleotides (10 bases long) of random sequence as primers, which bind selectively at several priming sites on the complementary sequence in the template genomic DNA. This mechanism promotes generation of several discrete DNA products if the priming sites are within an amplifiable distance of each other. The profile of amplified DNA primarily depends on nucleotide sequence homology between the template DNA and oligonucleotide primer at the end of each amplified product (Bardakci, 2001). If two template genomic DNA sequences are different, different banding patterns are produced in the PCR products (Atienzar and Jha, 2006). Polymorphism result from mutation or chromosomal rearrangement such as insertions/deletions either at or between the primer binding sites will be detected as presence and absence of bands in the RAPD profile (Bardakci, 2001).

Originally, RAPD has been used in genetic mapping, taxonomy and phylogeny (Welsh and McClelland, 1990; Williams et al., 1990; Caetano-Anolles et al., 1991), since the RAPD technique surveys numerous loci in the genome (Clark and Lanigan, 1993; Bardakci, 2001). RAPD methodlogy has also been applied for genotoxicity assessment. The first study measuring genotoxic effects using the RAPD assay was done by Savva et al (1994). The study used rats exposed to benzo [a] pyrene and revealed the appearance and disappearance of bands in comparison to control patterns in the RAPD profiles. These changes observed in the fingerprint of exposed animals were supposed to be produced due to the presence of DNA adducts, mutations or DNA breaks. RAPD has been a useful tool to detect genotoxic potential of some chemicals and metals, including EDCs (Mona et al., 2013, Sayed et al., 2013). More recently, this technique has been applied in genotoxicity assessment in fish (Becerril et al., 1999; Castaňo et al., 2003; Abumourad et al., 2012; Sayed et al., 2013; Salem et al., 2014).

RAPD has numerous advantages over conventional methods such as hybridisation-based protocols. First, there is no requirement to have prior knowledge about the genome under study and very little source material (about 10 ng per reaction) is required to perform the assay. Thus it is useful when screening of rare and valuable samples. Next, RAPD used a single random oligonucleotide primer, which means when employing different primers, banding profiles can be generated that differ in complexity. In genotoxicity studies, RAPD give several advantages such as it has the potential to detect a wide range of DNA damage (e.g. DNA adducts, DNA breakage) as well as mutation (point mutation and large rearrangement). In addition, this technology is cheap and does not require specialized and expensive equipment (Atienzar and Jha, 2006).

2.3 Vitellogenin as a Biomarker for Water Contamination

Genotoxicity of EDCs, mostly of estrogenic compounds in wildlife is linked with estrogenic-modulating effects of the chemicals (Choi et al., 2010). As it is important to know the extent of genotoxicity of EDCs towards the aquatic inhabitants, especially in fish, certain biomarkers are required to study it. Biomarkers are measurement of body fluids, cells or tissues that indicate in biochemical or cellular terms the presence of contamination or the magnitude of the host response (Livingstone at al., 2000). One of the important biomarkers to study genotoxicity is Vitellogenin (Vtg). Vtg is used as a biomarker to study disruption on endocrine system which involves the interfering of protein levels production. Vtg is important as a precursor to develop yolk protein and it can be used to determine estrogenic effect in blood plasma or liver of fish tissues in mature female fish. Vtg is generally synthesised in response to endogenous estrogens.

Various mechanisms of action have been proposed to indicate disruption of the endocrine system due to contaminants. It is likely that EDCs affect reproduction either by disrupting the synthesis, or degradation of exogenous hormones, or by directly activating steroid hormone receptor-mediated gene activation pathways (Larkin et al., 2003). Interaction of xenobiotics with hormone-binding proteins induced vitellogenesis, which is a kind of estrogen receptor (ER)-responsive process, and produce vitellogenin as an after result. This interaction has been a center of focus where numerous studies have been conducted to see its relevance (Arukwe and Goksøyr, 2003). For example, when 17β-estradiol (E_2) is released into the bloodstream and stored in developing oocytes, females

undergoing oogenesis normally have high levels of Vtg. In males and juvenile fish, Vtg gene is naturally quiescent, which means the gene is present but normally not activated. Exposure to E_2 induced Vtg gene in males and juvenile fish (Larkin et al, 2003).

The presence of Vtg in blood or Vtg transcript in the liver of male or juvenile fish may be taken to indicate past or current exposure to estrogen or estrogenic EDCs. Hence, induction of Vtg in males or juveniles is a well-known effect of xenobiotics contamination in fish, and thus Vtg has been extensively used as a biomarker both in the laboratory and field studies (Arukwe and Goksøyr, 2003). So it is possible to used juvenile fish to establish whether xenobiotics metabolism affects the endocrine control of maturation of fish. This is because developmental stages that involve important hormonal changes such as early development and puberty are prone to endocrine disruption. Endocrine parameters in immature fish are relatively constant and provide easy assessment for xenobiotic-induced alterations (Salaberria et al, 2009).

Generally Vtg is produced only in the liver, but the gene for Vtg is present in other tissues besides the liver such as the gonad. Most of EDCs act as xenoestrogens. When EDCs are introduced in the endocrine system, EDCs will have their own specific gene profiles because they may bind with low affinity to more than one steroid resulting in a complex gene activation pattern (Larkin et al., 2003). Several methodologies have been developed for determination of Vtg which includes immunotechniques like radioimmunoassay, enzyme-linked immunosorbent assays (ELISA), western blot and immunobiochemistry. These techniques are based on the use of specific antibodies. There

are also other techniques which involves powerful molecular tools such as various PCR, including quantitative real time PCR (qPCR), Northen blotting and protein expression studies by proteomic approaches (Denslow et al, 1999; Arukwe and Goksøyr, 2003; Marin and Matozzo, 2004).

Researches on vitellogenin as biomarker for xenotoxicant contamination in aquatic environment have been widely studied in fish. qPCR technique has been used to quantify zona radiata and Vtg mRNA levels in rainbow trout (*Oncorhynchus mykiss*) treated with 17β-estradiol (E_2) and α-zearelenol (Celius et al., 2000). Estrogenic compounds such as 4-nonylphenol, 4-(tert -octyl)phenol, BPA, and E_2 exposed to male killifish produced plasma Vtg in a dose dependent manner (Pait and Nelson, 2003). Exposure to benzo[a]pyrene and hexachlorobenzene resulted in the induction of Vtg in male Nile tilapia (Rodas-Ortíz et al., 2008). Currently, Vtg studies have been done in Malaysia. For examples, Om et al (2013) used the MALDI-TOF technique to identify Vtg in male giant grouper (*Epinephelus lanceolatus*) treated with E_2. Vtg has also been studied in Asian sea bass (*Lates calcarifer*)using ELISA (Fazielawanie et al., 2011).

It is likely that EDCs will have their own specific gene expression profiles since they may bind with low affinity to more than one steroid receptor. A complex gene activation pattern resulted by this mechanism (Larkin et al, 2003). In order to determine gene expression, a direct technique like qPCR has been widely accepted in most studies to quantify gene expression, such as Vtg gene. It is a fairly new technology that came in the early 1990s (Higuchi et al., 1993; Heid et al., 1996). This method is more accurate and

sensitive compared to Northern blotting to measure gene expression. The qPCR reaction is a used mRNA monitored in real time by fluorescence either by SYBR green dye that fluoresces when it is intercalated into DNA or by a fluorescent probe that is complementary in sequence to the cDNA of interest. The fluorescence level increases with each PCR cycle as the amplified product increases. By using special software, the amount of PCR product can be monitored in 'real time' to identify the log-linear phase of the reaction. During the log linear phase, the increasing fluorescence signal is directly proportional to the initial amount of target mRNA in the sample. Expression levels of a gene can then be determined relative to other genes, or can be quantified by using a standard curve (Larkin et al, 2003).

A number of advantages have been proposed regarding qPCRsuch as the small amount of total RNA required (100 ng), the elimination post-PCR processing and radio-isotope labeling, the simplicity of the assay and most important is the sensitivity of the assay compared to ribonuclease protection assays, Northern blot or slot-blot hybridization, and competitive reverse transcription PCR for measuring gene expression. This is due to a single copy of mRNA of interest is enough to generate gene expression profile of interest. In addition, it is possible to generate the measurement of several genes at once (multiplexing), thus reducing time and making it a high throughput assay. The assay is also able to measure differences in gene expression over 7-8 log values (Larkin et al., 2003).

2.4 *Oreochromis niloticus* as a Test system in Aquatic Toxicology Studies

Fishes can accumulate pesticides in concentrations much higher than those found in waters where they receive xenotoxicants due to bioaccumulation as chemicals or they are connected to the particulate materials in suspension. These particulate materials can be ingested by organisms present in the environment and when the organisms are linked to the food chain, it leads to the accumulation of the pollutants in fish. Therefore fish is suitable as bioindicators of environmental pollutions. Blood of fish is usually used as sample to study the genotoxicity in fish since it is a pathophysiological reflector of whole body (Ventura et al, 2008).

Species of fishes like *O.niloticus*are excellent test-system to study genotoxicity and toxicity in marine.*O. niloticus* is one of the species which have been used widely in many studies as bioindicator of genotoxicity and environmental monitoring (Alves-Costa et al, 2006; Bűcker and Da Conceição, 2012). This is because *O. niloticus* has been a good biological model for toxicological studies due to diverse characteristics, such as their high growth rate, efficiency in adapting to diverse diets, great resistance to diseases and to handling practices, easy reproduction in captivity and prolific rate, and good tolerance to a wide variety of environmental conditions (Fontainhas-Fernandes, 1998). The fish can be found in estuaries all around the world. Due to its red colour, sometimes it can be confused with red snapper. In Malaysia, the fish have been cross-bred among *Oreochromis sp.* to produce red hybrid tilapia (*O. niloticus*)(Musa et al, 2009).

Studies of the effects of EDCs to *O. niloticus* have been done in the past. It was revealed that this speciesis susceptible to genotoxic and xenoestrogenic effects by EDCs (Ventura et al., 2008; Huang et al., 2010). BPA and nonylphenol have been shown to affect the estrogen receptor expression and induce male reproductive abnormalities in *O. niloticus* (Huang et al., 2010). It was found that atrazine is both mutagenic and genotoxic to *O. niloticus* based on the presence of mn and NA in the erythrocytes of the fish after exposure to this chemical. Atrazine is also able to cause DNA fragmentation in organisms exposed to that chemical (Ventura et al., 2008). Based on the study to determine the accumulation of endosulfan in the tissue of *O. niloticus* and *Lates niloticus*, a total of endosulfan level ranging between 0.02 and 0.2 mg/kg of fresh weight was obtained. The toxicity of the diets has caused accumulation of endosulfan in the fishes (Henry and Kishimba, 2006).

CHAPTER 3

METHODOLOGY

3.1 Experimental Fish Specimen and Chemicals

Hundred freshwater *O. niloticus* were obtained from Pusat Pengembangan Akuakultur, Bukit Tinggi from September 2012 until February 2013. The age of the fish was around six to eight months. The specimens were18.45 ± 0.85 cm in length and 146.63 ± 42.45 g in weight, respectively. The fishes were kept in glass aquaria in a flow of dechlorinated and well aerated water. The water temperature was at 22 ± 2 °C.Reverse osmosis water was used during acclimatization to laboratory conditions for two weeks**.** The fishes were fed on normal diet with artificial fish pellets during acclimatization. The fecal matter and other waste materials were siphoned off twice a week to reduce ammonia content in water. The test pesticides used in the study were atrazine and endosulfan.

3.2 Exposure to Atrazine and Endosulfan

There are a total of 24 male *O. niloticus* selected among hundred of fishes obtained for this study. The experiment was carried out in triplicatewith a total of three fishes used in every treatment, including control test for atrazine and endosulfan.Atrazine (PESTANAL®, Sigma-Aldrich, Germany) was exposed to *O. niloticus* via the water. There are three

different concentrations of atrazine exposure being studied according to the LC_{50} (96 hours) from the previous study (Nwani et al, 2010). The concentrations applied were 4.75 mg/L, 2.50 mg/L and 0.50 mg/L of atrazine. Atrazine in powdered form was dissolved in 0.1% (w/v) phosphate buffer saline (PBS) (Salaberria et al, 2009). The experiment was carried out with exposure period of 96 hours.

Another test pesticide used for this experiment was endosulfan (PESTANAL®, Sigma-Aldrich, Germany). The pesticide was exposed in three concentrations where the LC_{50} based on several studies conducted previously (Kumar et al, 2011; Da Cuňa et al., 2013). A 1000 µg/L of endosulfan stock was prepared beforehand. *O. niloticus* were exposed to 7.00 µg/L, 3.60 µg/L and 1.50 µg/L of endosulfan for 96 hours. *O. niloticus*unexposed to atrazine and endosulfan was used as control test.

3.3 Fish Sampling

After 96 hours of exposure, the fishes were sacrificed to collect blood and livers for the assays. Blood samples were collected from the spinal section. Meanwhile, whole liver of the fish was collected and weighed, and followed by washingthe liver samples with 0.1% (w/v) PBS to remove the blood and unwanted tissues. The liver was kept in a 5 ml bottle containing 1 ml of RNA later to avoid RNA degradation and stored in -40°C for preservation.

3.4 Genotoxicity Assessment by MN Test and NA

Blood smears were prepared immediately after sampling. After fixing with absolute ethanol for 20 min, slides were air-dried overnight and then stained with 5% Giemsa solution for 20 min. The slides were then washed with distilled water. Three slides per exposure concentration and control were prepared. From each slides, 1000 cells were scored under 1000x magnification. A total of 3000 cells were scored to determine the frequencies of micronucleated cells.Nuclear abnormality shapes were scored according to the following categories: (1) blebbed nuclei, (2) lobed nuclei, (3) notched nuclei, and (4) binucleated cells. The result was expressed as mean value for all individual abnormality observed. The appearances of NA were observed as describe by Carrasco et al (1990). The appearance of blebbed nuclei showed a relatively small evagination of the nuclear envelope, which seems to contain euchromatin. Meanwhile, lobed nuclei are those presenting larger evaginations than the blebbed nuclei. A notched nucleus has an appreciable depth, but absence of nuclear material and cells bearing binucleated nuclei observed as those presenting two nuclei in a cell.

3.5 Genomic DNA and RNA Extractions

3.5.1 Homogenizing Sample and Phase Separation

Genomic DNA was isolated from fresh liver tissue according to TRIzol® reagent protocol provided by the manufacturer (Life Technologies, USA). Approximately 100 mg of liver tissue was cut into small pieces and homogenized by using a power homogenizer (Fisher Brand, USA). The homogenize sample was incubated for 5 minutes at room temperature to permit complete dissociation of the nucleoprotein complex. After that, 0.2 ml of chloroform per 1 mL of TRIzol® reagent used for homogenization was added into the tube and shaken vigorously for 15 seconds. The sample was incubated for another 3 minutes at room temperature before it was centrifuged at 12,000 x g for 15 minutes at 4°C. There were three layers of phases formed in the solution after centrifugation which are aqueous phase, interphase and organic phenol-chloroform phase. The aqueous phase was transferred out from the tube and placed in a new tube for RNA extraction.

3.5.2 Isolation of DNA from liver of *O. niloticus*

DNA was isolated from the interphase and organic phenol-chloroform phase layer. After removing the aqueous layer, the remaining solution was used to isolate DNA by first adding 0.3 mL of 100% ethanol per 1 mL of TRIzol® reagent used for the initial homogenization and mixed before incubating the sample at room temperature for 3

minutes. Then the tube containing the sample was centrifuged at 2,000 x g for 5 minutes at 4°C to pellet the DNA. The phenol-chloroform supernatant was removed from the tube and then the step proceeded to DNA wash.

DNA was washed twice with 1 mL of sodium citrate/ethanol solution (0.1 M sodium citrate in 10% (v/v) ethanol, pH 8.5) and the sample was incubated for 30 minutes at room temperature with periodically mixing. Then, the tube was centrifuged at 2,000 x g for 5 minutes at 4°C and the supernatant was discarded. These steps were repeated once. After that, 1 mL of 75% (v/v) ethanol was added into the tube and the sample was incubated at room temperature for 20 minutes. During the incubation period, the sample was periodically mixed. Later, the tube was centrifuged at 12,000 x g for 5 minutes at 4°C. The supernatant was removed and the DNA pellet was air-dried for 10 minutes.

The next procedure involved resuspension of DNA where the DNA pellet was dissolved in 100 µL of ultrapure distilled water (GIBCO, USA) and incubated at 55°C in a waterbath. Then the sample was centrifuged at 12,000 x g for 10 minutes. Finally, the supernatant containing DNA was transferred into a new tube.The concentration and purity of DNA was determined using Nanodrop at 260 nm and 260/280 absorption. The DNA was stored in -40°C until used for PCR amplification.

3.5.3 RNA Isolation Procedure

RNA isolation from liver was done using TRIzol® reagent protocol provided by the manufacturer (Life Technologies, US). By using aqueous phase layer which was kept during phase separation step, 500 µL of isopropyl alcohol was added and mixed gently. The tube was then centrifuged at 7,500 x g for 5 minutes at 4°C and supernatant was carefully removed from the tube.

The second step involved RNA with 75% (v/v) ethanol and the sample was centrifuged at 7,500 x g for 5 minutes at 4°C, supernatant was removed and RNA was air dried for 10 minutes. Next, the RNA pellet was dissolved in 150 µL of ultrapure distilled water (GIBCO, USA) and the sample was incubated at 57°C in the waterbath for 5 minutes.

RNA was purifiedusing RNA Purification kit (Fermentas, USA).The procedure was done as provided by the manufacturer's protocol (Fermentas, USA). A total of 10 µL of reaction mixture containing 1.0 µL of 10X reaction buffer and $MgCl_2$ (Fermentas, USA), 1.0 µL of DNase 1 (Fermentas, USA), 1.0 µL RNA and 7.0 µL of ultrapure distilled water (GIBCO, USA). The reaction mixture was incubated at 37°C for 15 minutes. Then 1 µL of 25 mM EDTA was added and incubated at 65°Cfor 10 minutes. The RNA concentration was determined by Nanodrop at 260 nm before proceeding to reverse transcription step.

3.6 RAPD Test and Analysis

DNA amplification was performed in a final volume of 25 μL. The reaction mixture contained 2.5 μL of 10x reaction buffer (1^{st}Base), 2.0 μL of 2 mM $MgCl_2$ (1^{st}Base), 0.5 μL of dNTP mix (dATP, dTTP, dCTP, dGTP) (1^{st}Base), 1.5 μL of 0.5 μM primer, 0.5 μL Taq polymerase (1^{st}Base), 17.0 μL of ultrapure water (GIBCO, USA) and 1.0 μL of the 20 ng/μL extracted DNA. Five primers were used for PCR analysis (Table 3.1). These primers were purchased from Shanghai Genecore Biotechnology Co., Ltd.

Table 3.1: RAPD primer sequences used in the RAPD method analysis.

Name	Sequence (5'->3')	Base	MW	GC%	Tm (°C)
OPA 8	GTGACGTAGG	10	3038	60.0	22.3
OPA 13	CAGCACCCAC	10	3069	60.0	22.3
OPB 8	GTCCACACGG	10	3020	60.0	22.3
OPA 12	TCGGCGATAG	10	3038	60.0	22.3
OPC 11	AAAGCTGCGG	10	3038	60.0	22.3

The pre-amplification PCR procedure was treatment at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 27.5°C for 45 seconds and extension at 72°C for 1 minute and 30 seconds. Then, the final extension proceeded at 72°C for 5 minutes. A 3 μL aliquot of the PCR product was analyzed on a 1% (w/v) agarose gel

in 1.0 x TBE buffer. The gel was pre-stained with ethidium bromide (EtBr) prior electrophoresis. Electrophoresis is performed at a constant voltage of 100 V for 35 minutes and visualized under UV light. Sizes of DNA bands produced were estimated by comparison with the standard markers GeneRuler^{TM}1 kb DNA ladder (ThermoScientific, USA) and GeneRuler^{TM}100 bp DNA ladder (ThermoScientific, USA) and by referring to previous studies (Atienzar et al., 2002; Mohanty et al., 2011; Abumourad et al., 2012; Aksakal et al., 2013).

In RAPD analysis, the present and absent of RAPD bands were observed to define all genetically damaged DNA samples when comparing against control DNA (Abumourad et al., 2012). The gain or loss of bands was scored 1 or 0 respectively (Atienzar and Jha, 2006). Genomictemplate stability (GTS) was calculated for each experimental group of fish with the chosen primer, as follows:

$$GTS\ (\%) = (1 - a/n) \times 100$$

where "a" is the number of RAPD polymorphic profiles detected in each sample treated and "n" is the number of total bands in the control. Polymorphisms observed in the RAPD profile include disappearance of a normal band and appearance of a new band in comparison with control profile. The average was then calculated for each experimental group exposed to different EDCs. The GTS value of control was set to 100% and treated samples were calculated according to control (Atienzar et al., 1999).

Distance based phylogeny was generated by combining data from all primers with the software package PHYLIP (Felsenstein, 1989), using the program pars (parsimony) and then a majority-rule consensus tree was generated by CONSENSE. Finally, dendogram was produced with the PHYLIP program DRAWGRAM and viewed by using TREEVIEW software.

3.7 Vitellogenin Expression Assessment by qPCR

Reverse transcription of RNA was done according to High-capacity cDNA Reverse Transcription Kit manual (Applied Biosystem, USA). A total of 20 µL reaction mixture was prepared where 2X Reverse Transcription (RT) Master Mix was prepared first. 2X RT Master Mix contains 2.0 µL of 10X RT Buffer, 0.8 µL of 25X dNTP mix, 2.0 µL of RT Random Primers, 1.0 µL of MultiScribe™ Reverse Transcriptase, 1.0 µL of RNase inhibitor, and 3.2 µL of nuclease-free water. A total of 10 µL of RNA sample was used for cDNA reverse transcription reaction. Reverse transcription of cDNA started with treatment at 42°C for 15 minutes, then the temperature was increased to 99°C for 5 minutes, before cooling at 5°C for 5 minutes.

After reverse transcription step, target cDNA was amplified by using a set of Vtg primers, Vtg aa (NHK Bioscience) and β-actin (NHK Bioscience) as reference gene (Table 3.2). The total volume of real-time PCR reaction mixture was 20 µL. The reaction mixture contains 10.0 µL of Ssofast EverGreen Supermix, 1.0 µL of forward primer (VtgaaF), 1.0

µL of reverse primer (VtgaaR), 7.0 µL of RNase/Dnase-free water, and 1.0 µL of 0.5 µM cDNA, The thermal-cycling parameters were as follows: 15 minutes at 95°C, then 40 cycles of 20 seconds at 95°C, 20 seconds at 60°C and 20 seconds at 72°C. Flourescence data were collected at the end of each cycle. Following the amplification reaction, a melting curve analysis was carried out between 60°C and 95°C, fluorescence data were collected each 0.1°C. The C(*t*) is selected to be in the linear phase of amplification.

Table 3.2: Primer used to analyze gene expression of Vtg in *O. niloticus.*

Primers	Sequence (5'->3')	Bases
Vtgaa F	GAATGTGAATGGGCTGGAAATAC	23
Vtgaa R	TTTGTTTGATCTGGATGTCAGCTT	24
β-actin F	CCTGACAGAGCGTGGCTACT	20
β-actin R	TCCTTGATGTCACGCACGAT	20

3.8 Statistical Analysis

All statistical analysis was done by using SPSS 14. The one-way analysis of variance (ANOVA), followed byTukeywas used to compare the mean differences in MN and NA frequency, GTSand induction of Vtgbetween exposure concentrations against the control test.

CHAPTER 4

RESULTS

4.1 Micronucleus Test and Nuclear Abnormalities

The results obtained by analyzing irregularities (MN and NA) in erythrocytes of *O. niloticus* (Figure 4.1B-F), after exposure to atrazine and endosulfan were shown at Table 4.1 and Table 4.2 respectively. The formation of MN as described by Moron et al. (2006) was shown as not connected to the main nucleus, has the same colour and intensity of the main nucleus, and sizes with less than one third of the main nucleus size (arrow at Figure 4.1B). The type of NA found were erythrocytes with alterations on nuclear morphology, classified according to Carrasco et al. (1990) as notched nuclei (arrow at Figure 4.1C), blebbed nuclei (arrow at Figure 4.1D), lobed nuclei (arrow at Figure 4.1E), and binucleated cells (arrow at Figure 4.1F). From the experiment, the control test also showed nuclear alterations similar to those found in both chemicals tests, but in lower frequency (Table 4.1 and Table 4.2)

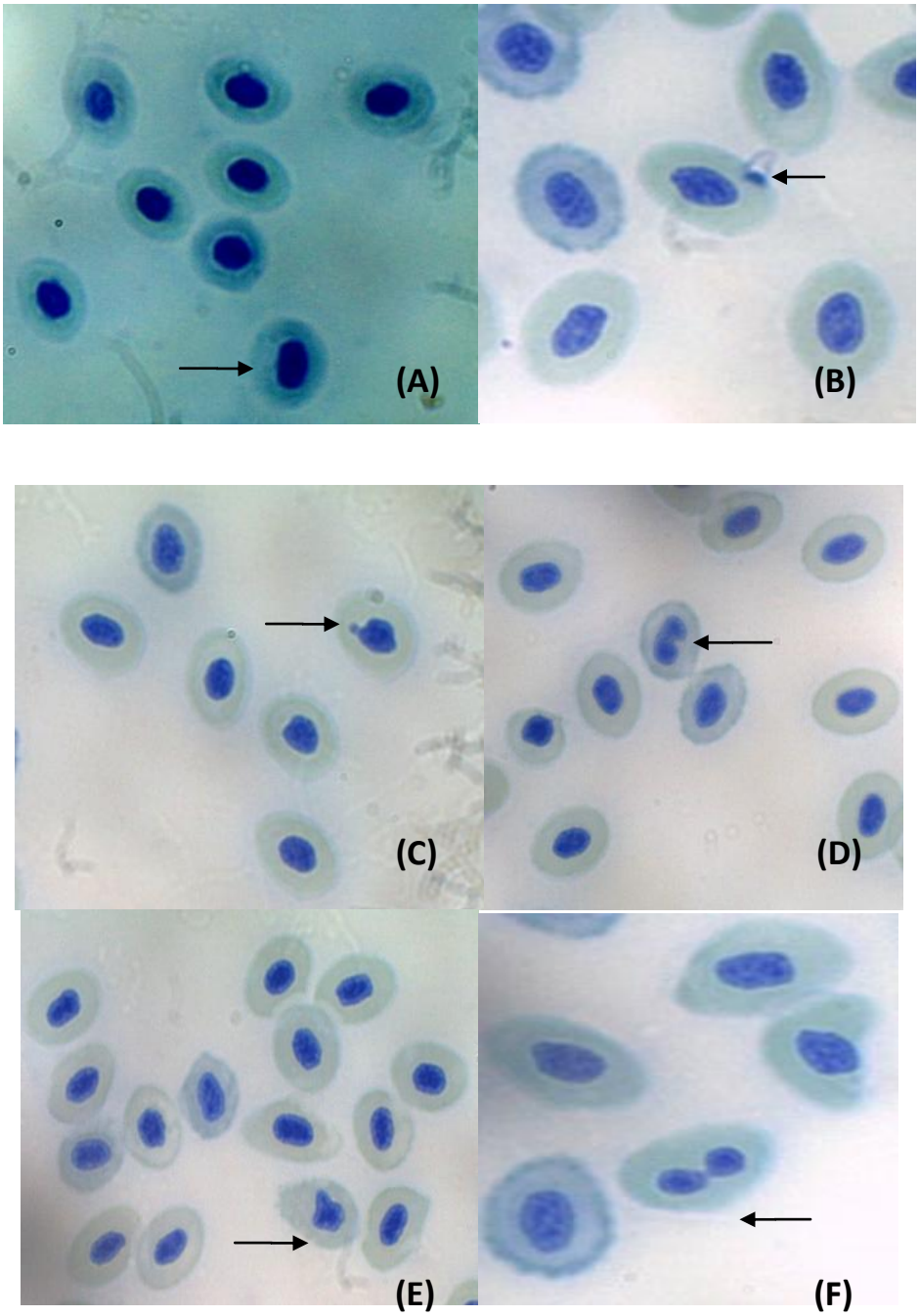

Figure 4.1: Genotoxic effect in erythrocytes of *O. niloticus*.

Normal erythrocytes were shown in (A). Genotoxic effect of erythrocytes were observed with (B) micronuclei, (C) notched nuclei, (D) blebbed nuclei, (E) lobed nuclei, and (F) binucleated cells, which were shown by arrows.

The result of MN and NA in erythrocytes of *O.niloticus* exposed to different concentration of atrazine and endosulfan were shownin Figure 4.1B-F. Normal erythrocyte cells were shown in Figure 4.1A. The normal cells have main nucleus which appeared round and without any MN presence. Erythrocyte cells containing the presence of MN was observed as shown in Figure 4.1B. The criteria of MN obtained in this experiment were in corcondance with description by Ventura et al. (2008). Based on Figure 4.1C-F,the kinds of NA most frequently observed in *O. niloticus* erythrocytes submitted to atrazine exposition for 96 hours were: cells with "blebbed" nuclei, cells with "lobed" nuclei, cells with "notched" nuclei, and binucleated cells, and the appearance of each NAwere observed as describe by Carrasco et al (1990).

Table 4.1:The frequency of MN and NA (average and standard deviation) in *O. niloticus* after exposure to atrazine.

Concentrations	Control	0.50mg/L	2.50mg/L	4.75 mg/L
MN	0.07 ± 0.02^{a}	0.14 ± 0.03^{b}	0.24 ± 0.02^{c}	0.36 ± 0.04^{d}
NA	0.09 ± 0.03^{a}	0.13 ± 0.05^{a}	0.23 ± 0.04^{b}	0.28 ± 0.07^{b}
Blebbed nuclei	0.02 ± 0.02^{a}	$0.03\pm 0.01^{a,b}$	$0.04\pm 0.03^{b,c}$	0.05 ± 0.02^{c}
Lobed nuclei	0.04 ± 0.01^{a}	0.04 ± 0.01^{a}	0.07 ± 0.02^{b}	0.10 ± 0.07^{c}
Notched nuclei	0.03 ± 0.01^{a}	0.05 ± 0.02^{a}	0.09 ± 0.02^{b}	0.10 ± 0.04^{b}
Binuclear cells	0.00 ± 0.00^{a}	0.02 ± 0.01^{b}	0.02 ± 0.02^{b}	0.02 ± 0.01^{b}

Different superscriptsindicate significant differences among treatments ($p<0.05$, One-way ANOVA, followed by Tukey's multiple comparison).Means by columnsfollowed by the different letter are significantly different at the $P< 0.05$ level as determined by Tukey's multiple comparison tests.

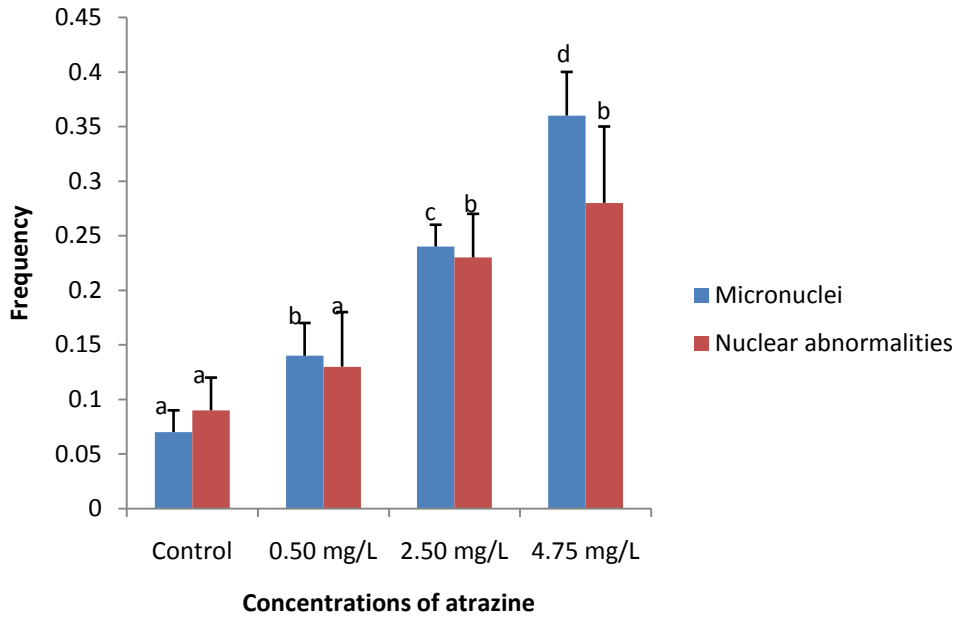

Figure 4.2:Frequency of MN and total NAin *O. niloticus*after atrazine exposure.

Control was untreated*O. niloticus*. Mean and standard deviation a, b, c, d: indicating a significant difference (*P<0.05*) between means recorded for different atrazine concentrations.

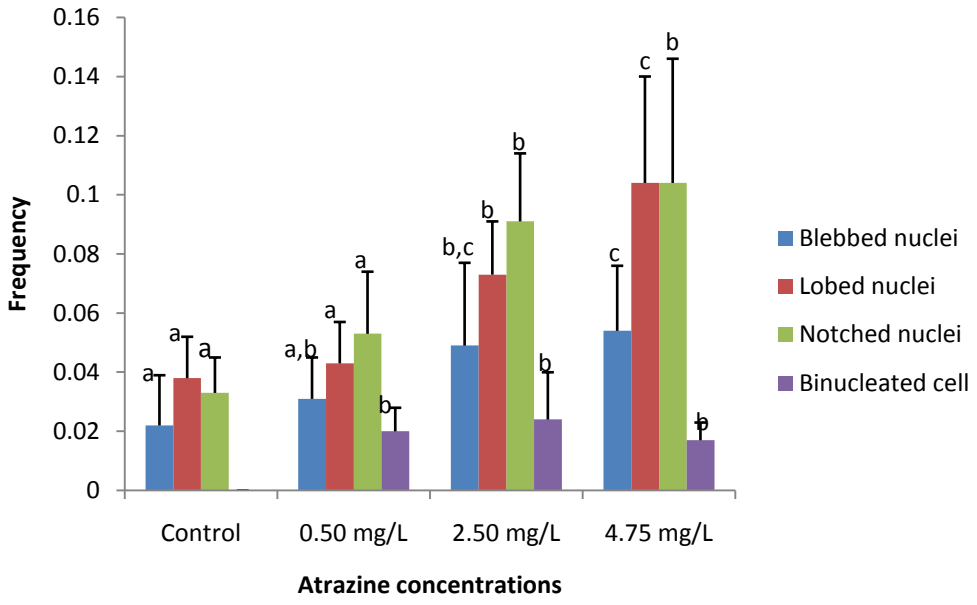

Figure 4.3:Frequency of NAin *O. niloticus*after atrazine exposure.

O. niloticus untreated with atrazine was used as control test. Mean and standard deviation a, b, c: indicating a significant difference ($P<0.05$) between means recorded for different atrazine concentrations.

Based on the results obtained for atrazine exposed to *O. niloticus* for 96 hours, there was a significant difference ($P<0.05$) for the frequencies of MN between control and treated group (0.50 mg/L, 2.50 mg/L and 4.75 mg/L atrazine). Meanwhile for NA, the frequencies showed significant differences ($P<0.05$) at concentration 2.50 mg/L and 4.75 mg/L atrazine when compared to the control test. In contrast, there was no significant difference showed at concentration 0.50 mg/L atrazine with control group (Table 4.1 and Figure 4.2). The highest frequencies of MN and NA among the three tested concentrations recorded were *O. niloticus* exposed to 4.75 mg/L (0.36 ± 0.04 and 0.28 ± 0.07 respectively). The data also showed that for 96 hours exposure at this concentration, the formation of micronuclei and NA were both significantly increased ($P<0.05$).

The 4.75 mg/L atrazine concentration showed the highest rates cells with blebbed nuclei, lobed nuclei and notched nuclei (0.05 ± 0.02, 0.10 ± 0.04 and 0.10 ± 0.04 respectively) and the rates were significant ($P<0.05$) when compared with the control test (Table 4.1). However, the data showed that the increase of cells with blebbed nuclei and notched nuclei at 4.75 mg/L atrazine was not significant ($P<0.05$) when compared to 2.50 mg/L atrazine. Meanwhile, binuclear cells were observed high at concentration 2.50 mg/L atrazine (0.02 ± 0.02) and the rate was significant ($P<0.05$) when compared to the control test, but not to the other test concentrations (Table 4.1 and Figure 4.3).

Table 4.2:The frequency of MN and NA (average and standard deviation) in *O. niloticus* after exposure to endosulfan.

Concentrations	Control	1.5 µg/L	3.6µg/L	7µg/L
MN	0.08 ± 0.02^{a}	0.10 ± 0.02^{a}	0.18 ± 0.02^{b}	0.23 ± 0.02^{c}
NA	0.04 ± 0.01^{a}	$0.10\pm 0.05^{a,b}$	0.18 ± 0.06^{b}	0.30 ± 0.12^{c}
Blebbed nuclei	0.01 ± 0.00^{a}	$0.02\pm 0.01^{a,b}$	$0.03 \pm 0.02^{b,c}$	0.04 ± 0.02^{c}
Lobed nuclei	0.02 ± 0.01^{a}	$0.05\pm 0.02^{a,b}$	$0.09\pm 0.06^{b,c}$	0.13 ± 0.06^{c}
Notched nuclei	0.02 ± 0.01^{a}	$0.04\pm 0.01^{a,b}$	0.05 ± 0.02^{b}	0.11 ± 0.04^{c}
Binuclear cells	0.00 ± 0.00^{a}	0.01 ± 0.01^{a}	0.02 ± 0.01^{a}	0.03 ± 0.02^{a}

Different superscripts indicating significant differences among treatments (*P<0.05*, One-way ANOVA, followed by Tukey's multiple comparison).Means by columnsfollowed by the different letter are significantly different at the $P< 0.05$ level as determined by Tukey's multiple comparison tests.

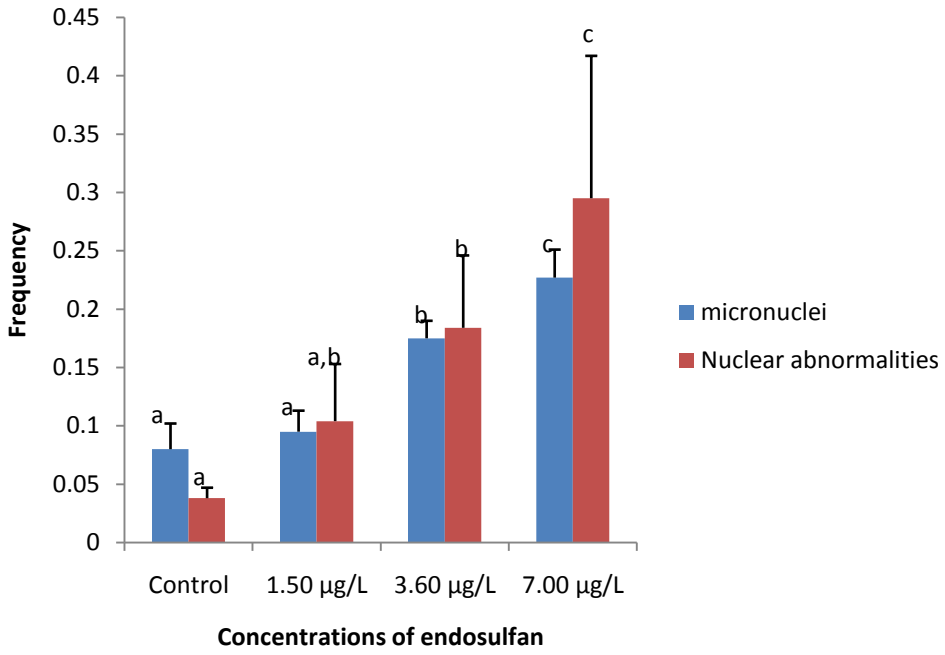

Figure 4.4:Frequency of MN and total NAin *O. niloticus*after exposure for 96 hours to endosulfan.

Untreated *O. niloticus*to endosulfan was used as control test. Mean and standard deviation a, b, c: indicating a significant difference ($P<0.05$) between means recorded for different endosulfan concentrations.

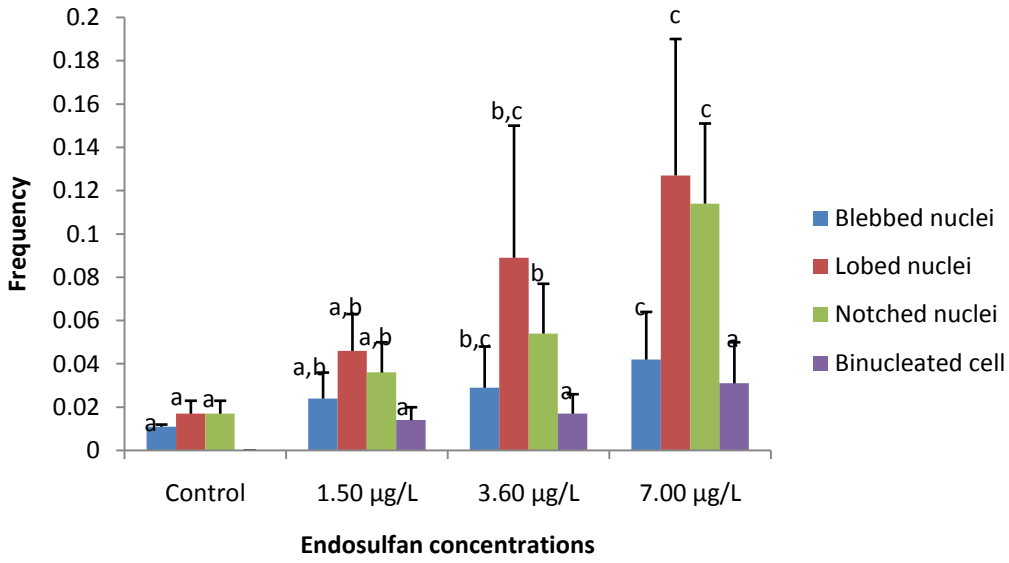

Figure 4.5:Frequency of NAin *O. niloticus*after exposure for 96 hoursto endosulfan.

Control was untreated*O. niloticus*to endosulfan. Mean and standard deviation a, b, c: indicating a significant difference ($P<0.05$) between means recorded for different endosulfan concentrations.

Results from Table 4.2 showed that there were significant differences (P<0.05) for the frequencies of MN and NA between control and treated group (3.60 µg/L and 7.00 µg/L endosulfan). Although the frequency of MN and NA were observed to increase between control and 1.50 µg/L endosulfan, it was not significantly different (*P<0.05*). The highest frequencies of MN and NA were shown at concentration 7.00 µg/L endosulfan (0.23± 0.02 and 0.30± 0.12 respectively) in erythrocytes of *O. niloticus* exposed for 96 hours, and the formation of nuclear alterations at this concentration also increased significantly (*P<0.05*) (Table 4.2 and Figure 4.4).

NA observed in erythrocytes of *O. niloticus* after exposure to endosulfan for 96 hours were the same as observed in *O. niloticus* exposed to atrazine (Figure 4.1C-F). From Table 4.2, the concentration 7.00 µg/L showed the highest rates of cells with notched nuclei (0.11± 0.04) and it increased significantly (*P<0.05*) when compared to the control test and the tested endosulfan concentrations exposed at 3.60 µg/L and 1.50µg/L. The frequencies of cells with blebbed and lobed nuclei were also high at concentration 7.00 µg/L endosulfan (0.04± 0.02 and 0.13± 0.06 respectively) and it was observed to increase significantly (*P<0.05*), when compared to the control test and 1.50 µg/L endosulfan but there was no significant difference when compared to 3.60 µg/L endosulfan. The cells bearing binucleated were also high at concentration 7.00 µg/L endosulfan (0.03± 0.02) but it was not significantly differentwhen compared to the control test and the other tested endosulfan concentrations (Table 4.2 Figure 4.5).

4.2 Genotoxicity Assessment by Using RAPD

A total of five RAPD primers were used on genomic DNA to all treated and untreated samples to generate RAPD profiles as shown from Figure 4.6 to Figure 4.10. Of the five RAPD primers used in this experiment, only OPA13 (5'-CAGCACCCAC-3') gave smear, but there was also visible band patterns observed (Figure 4.6). The other four RAPD primers (OPA8, OPB8, OPA12, and OPC11) produced reproducible and distinguishable banding profile between non-exposed and exposed samples. The banding patterns were varied among RAPD primers and gave a total of 31 bands (untreated control treatments) whose molecular weights ranged approximately from 1900 to 250 bp (Figure 4.6 - 4.10).

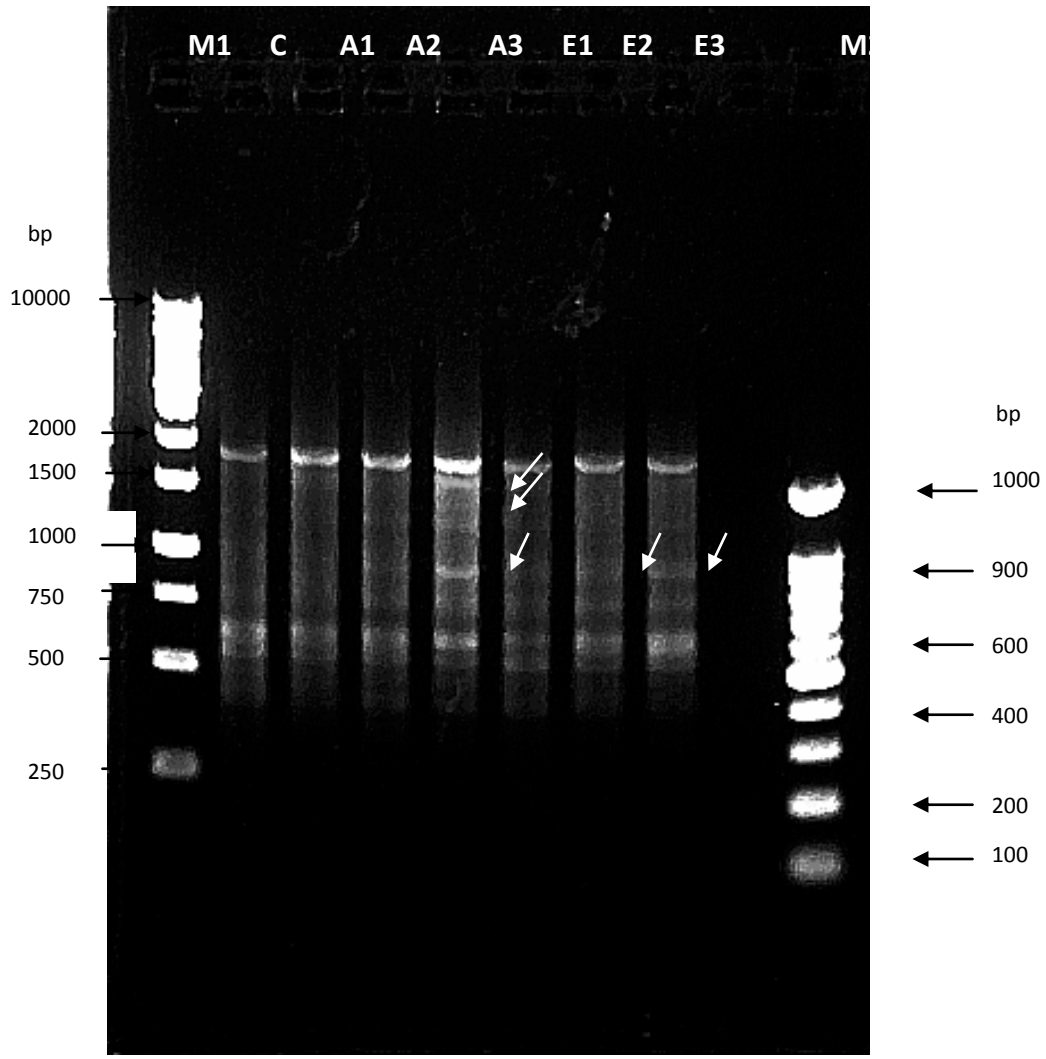

Figure 4.6: RAPD profiles generated by OPA13 (5'-CAGCACCCAC-3').

Figure 4.6 showed DNA polymorphic patterns in *O. niloticus* with DNA from control, (C), A1: 0.50 mg/L atrazine exposed fish; A2: 2.50 mg/Latrazine exposed fish; A3: 4.75 mg/L atrazine exposed fish; E1: 1.50 µg/L endosulfan exposed fish; E2: 3.60 µg/L endosulfan exposed fish and E3: 7µg/L endosulfan exposed fish. Lane M1 and M2 indicate molecular weight markers (1 kb ladder and 100 bp ladder respectively). Arrows indicate gain/loss differences for amplification products and the size for the amplified fragments in base pairs (bp).

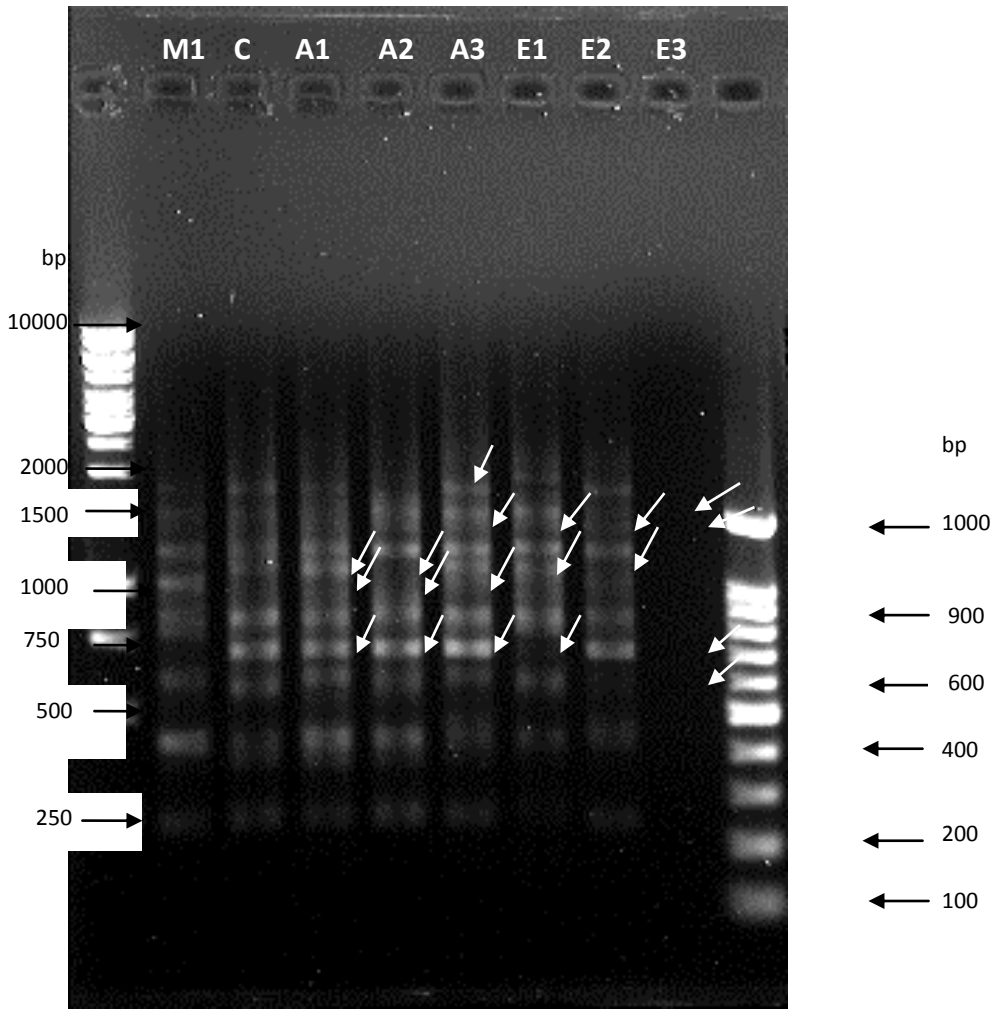

Figure 4.7: RAPD profiles generated by OPA8 (5'-GTGACGTAGG-3').

Figure 4.7 showed DNA polymorphic patterns in *O. niloticus* with DNA from control, (C), A1: 0.5 µg/L atrazine exposed fish; A2: 2.50 mg/L atrazine exposed fish; A3: 4.75 µg/L atrazine exposed fish; E1: 1.50 µg/L endosulfan exposed fish; E2: 3.60 µg/L endosulfan exposed fish and E3: 7µg/L endosulfan exposed fish. Lane M1 and M2 indicate molecular weight markers (1 kb ladder and 100 bp ladder respectively). Arrows indicate gain/loss differences for amplification products and the size for the amplified fragments in base pairs (bp).

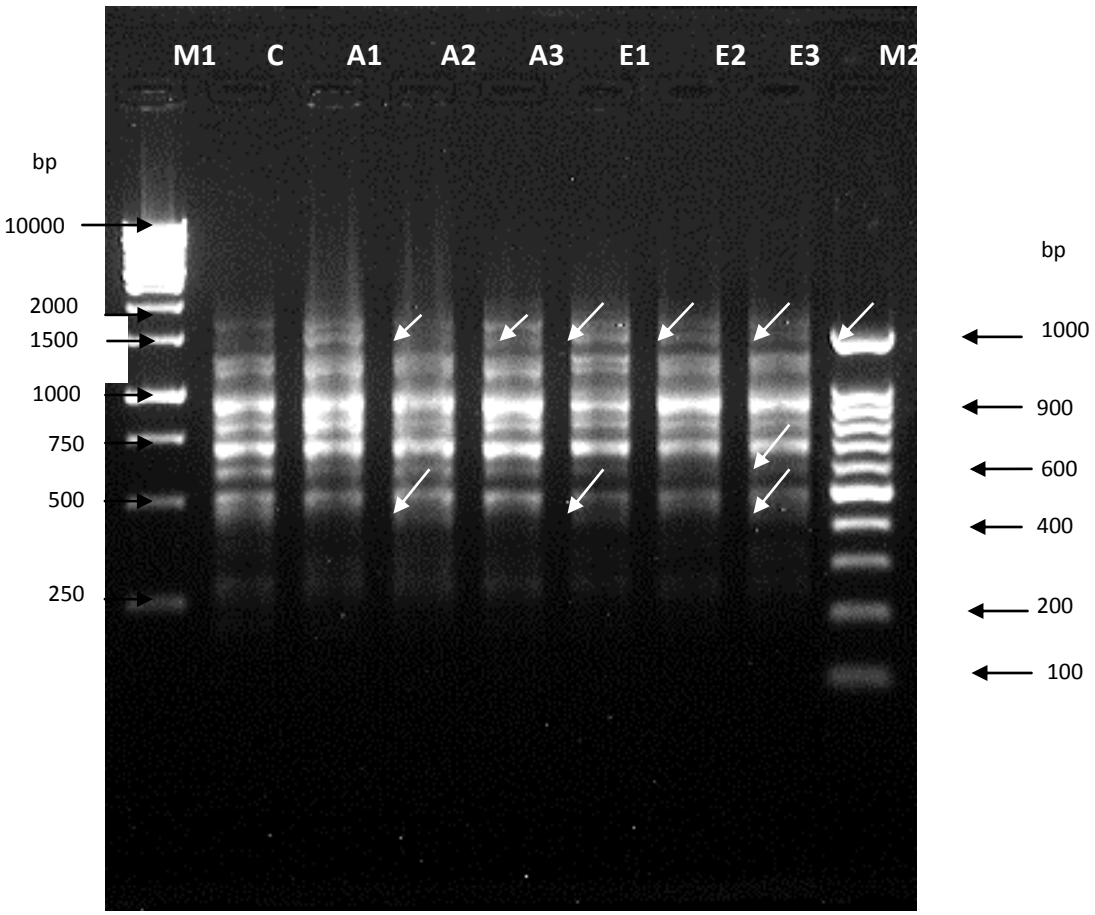

Figure 4.8: RAPD profiles generated by OPB8 (5'-GTCCACACGG-3').

Figure 4.8 DNA polymorphic patterns in *O. niloticus* with DNA from control, (C), A1: 0.5 µg/L atrazine exposed fish; A2: 2.50 mg/Latrazine exposed fish; A3: 4.75 µg/L atrazine exposed fish; E1: 1.50 µg/L endosulfan exposed fish; E2: 3.60 µg/L endosulfan exposed fish and E3: 7µg/L endosulfan exposed fish. Lane M1 and M2 indicate molecular weight markers (1 kb ladder and 100 bp ladder respectively). Arrows indicate gain/loss differences for amplification products and the size for the amplified fragments in base pairs (bp).

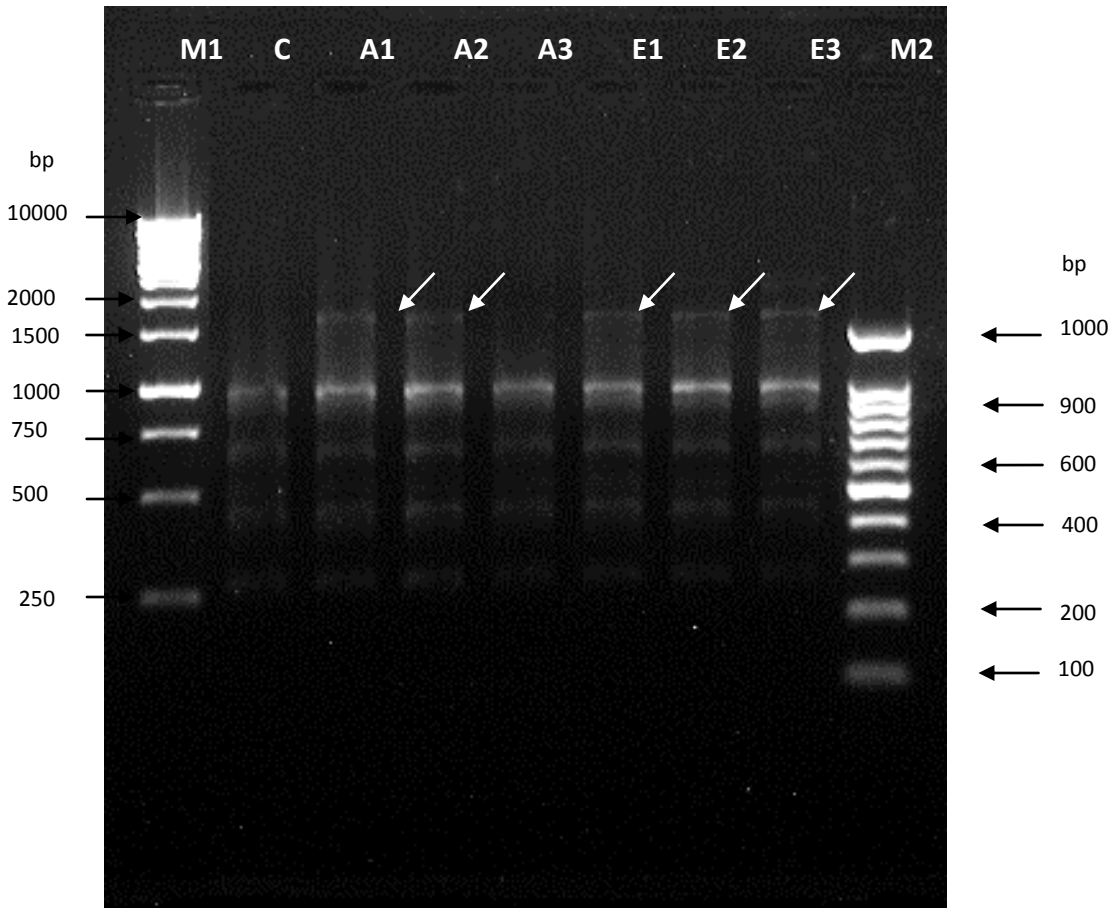

Figure 4.9: RAPD profiles generated by OPA12 (5'-TCGGCGATAG-3').

Figure 4.9 showed DNA polymorphic patterns in *O. niloticus* with DNA from control, (C), A1: 0.5 µg/L atrazine exposed fish; A2: 2.50 mg/Latrazine exposed fish; A3: 4.75 µg/L atrazine exposed fish; E1: 1.50 µg/L endosulfan exposed fish; E2: 3.60 µg/L endosulfan exposed fish and E3: 7µg/L endosulfan exposed fish. Lane M1 and M2 indicate molecular weight markers (1 kb ladder and 100 bp ladder respectively). Arrows indicate gain/loss differences for amplification products and the size for the amplified fragments in base pairs (bp).

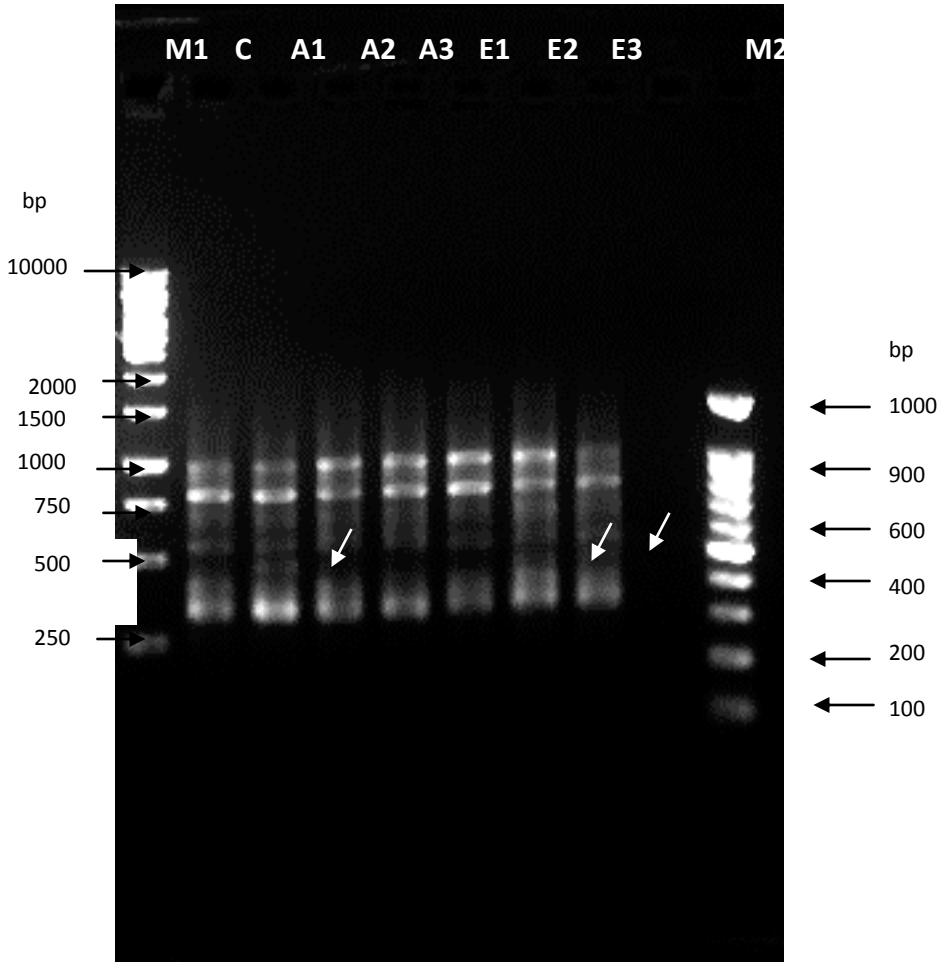

Figure 4.10: RAPD profiles generated by OPC11 (5'-AAAGCTGCGG-3').

Figure 4.10 showed DNA polymorphic patterns in *O. niloticus* with DNA from control, (C), A1: 0.5 µg/L atrazine exposed fish; A2: 2.5 µg/L atrazine exposed fish; A3: 4.75 mg/L atrazine exposed fish; E1: 1.50 µg/L endosulfan exposed fish; E2: 3.60 µg/L endosulfan exposed fish and E3: 7µg/L endosulfan exposed fish. Lane M1 and M2 indicate molecular weight markers (1 kb ladder and 100 bp ladder respectively). Arrows indicate gain/loss differences for amplification products and the size for the amplified fragments in base pairs (bp).

Table 4.3: The number of RAPD DNA fragmentsproduced in all treatments and their approximate molecular weights (bp).

Treatment		Atrazine (mg/L)						Endosulfan (µg/L)					
	Control	0.50		2.50		4.75		1.50		3.60		7.00	
Primer		a	b	a	b	a	b	a	b	a	b	a	b
OPA13	7	1000	1000, 800	1000	1100, 800	1900, 1000,	1400, 800	0	1400, 1100, 800, 700	0	1400, 1100, 800	1500, 600	1400, 800, 700
OPA8	5	0	0	0	0	0	1500, 1400, 900	0	0	0	900	0	900
OPB8	10	450	1500	0	1500	450	1500	0	1500	600. 450	1500	0	1500
OPA12	4	0	1900	0	1900	0	0	0	1900	0	1900	0	1900
OPC11	5	0	450	0	0	0	0	0	0	0	450	0	450
TOTAL	31	2	5	1	4	3	6	0	6	2	7	2	7
a + b		7		5		9		6		9		9	

(a) DNA band disappearance for all primers as compared to control test.

(b) DNA band appearance for all primers as compared to control test.

(a + b) Total of DNA band appearance and disappearance.

RAPD profiles showed differences between untreated male *O. niloticus* and treated samples with apparent changes (disappearance and/or appearance) in the number and size of the amplified DNA fragments for different primers (Table 4.3). The numbers of band disappearance and appearance were the highest in the sample exposed to 4.75 µg/L atrazine and 7.00 µg/L endosulfan. In both concentrations, there were nine polymorphic bands yielded. For endosulfan samples, the numbers of bands disappeared and appeared were increased with increasing concentration of the toxicant, although the numbers of polymorphic bands were same in 3.60 µg/L endosulfan and 7.00 µg/L endosulfan. As for atrazine samples, the bands disappearance and appearance were showed high in the lowest concentration examined (0.50 mg/L atrazine) where the numbers of polymorphic bands observed were seven, compared to 2.50 mg/L atrazine samples which yielded five polymorphic bands.

Table 4.4: Changes of GTS (%) all primers in *O. niloticus* exposed to atrazine and endosulfan.

Primer	Control	Atrazine concentration (mg/L)			Endosulfan concentration (µg/L)		
		0.50	2.50	4.75	1.50	3.60	7.00
OPA 13	100.00	57.14	57.14	42.86	42.86	57.14	28.57
OPA 8	100.00	100.00	100.00	40.00	100.00	80.00	80.00
OPB 8	100.00	80.00	100.00	80.00	90.00	70.00	90.00
OPA 12	100.00	75.00	75.00	100.00	75.00	75.00	75.00
OPC 11	100.00	80.00	100.00	100.00	100.00	80.00	80.00
Mean ± SD	100.00 ± 0.00[a]	78.43 ± 15.29[a]	86.43 ± 19.63[a]	72.57 ± 29.60[a]	81.57 ± 23.94[a]	72.43 ± 9.50[a]	70.71 ± 24.18[a]

Superscript indicating a significant difference (P<0.05) between means GTS (%) recorded for atrazine and endosulfan exposure in *O. niloticus*.Means by each column followed by the same letter are not significantly different at the P< 0.05 level as determined by Tukey's multiple comparison tests.

This statistical qualitative analysis allowed the correlation of genomic stability variations with exposure concentration to the chosen EDCs. The resulting mean and SD for GTS percentage (Table 4.4)showed that the genome stability was slightly reduced with increasing endosulfan concentration, where GTS value for *O. niloticus* exposed to 1.50 μg/L, 3.60 μg/L and 7.00 μg/L endosulfan were 81.57 ± 23.94, 72.43 ± 9.50 and 70.71 ± 24.18, respectively. On the other hands, GTS values for *O. niloticus* exposed to atrazine were shown with varied genomic stability. The result showed *that O. niloticus* treated with 0.50 mg/L, 3.50 mg/L and 4.75 mg/L atrazine gave GTS value of 78.43 ± 15.29, 86.43 ± 19.63 and 72.57 ± 29.60, respectively. For this result, GTS value for 0.50 mg/L atrazine was slightly lower as compared to GTS value for 2.50 mg/L atrazine.

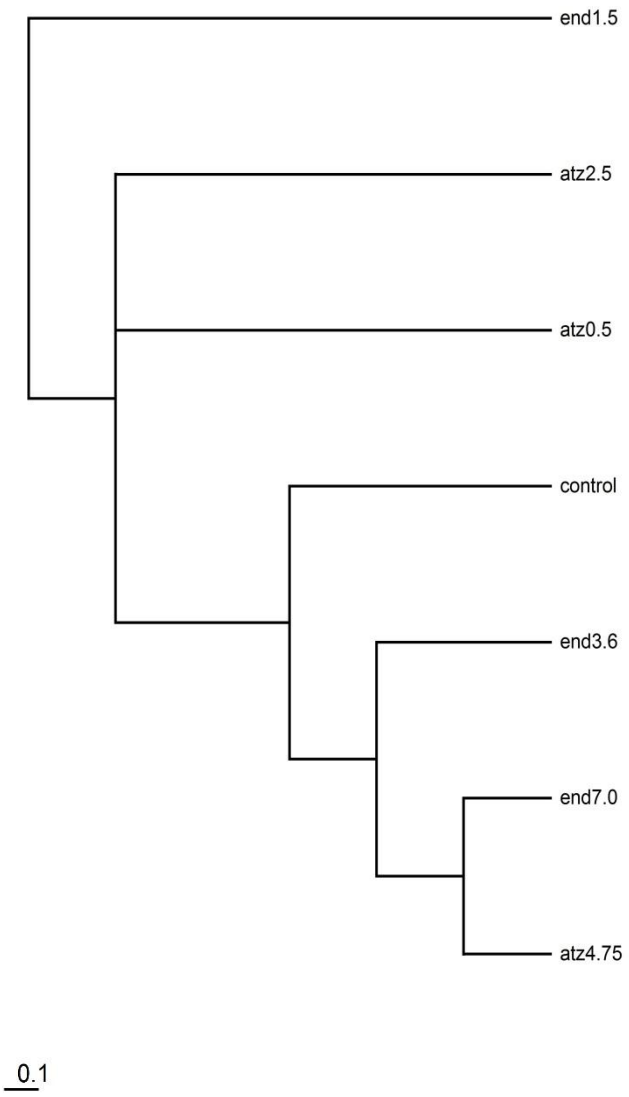

Figure 4.11: Dendogram representing the relationship among exposed concentrations of atrazine and endosulfan on male *O. niloticus* based on RAPD analysis.

The scale bar represents 0.1 substitutions per nucleotide site. Control: untreated male *O. niloticus*; atz0.5: male *O. niloticus* exposed to 0.5 µg/L atrazine; atz2.5: male *O. niloticus* exposed to 2.5 µg/L atrazine; atz4.75: male *O. niloticus* exposed to 4.75 mg/L atrazine; end1.5: male *O. niloticus* exposed to 1.50 µg/L endosulfan; end3.6: male *O. niloticus* exposed to 3.60 µg/L endosulfan; and end7.0: male *O. niloticus* exposed to 7.00 µg/L endosulfan.

Dendogram of Figure 4.11 showed a possible relationship between treated *O. niloticus* with atrazine and endosulfan and untreated *O. niloticus* based on the RAPD profiles obtained. Based on the dendogram, there were three branches and one cluster. Samples treated with endosulfan at concentration 1.50 µg/L which served as an outgroup. It showed that, at this concentration, the result was not significant as compared to the control test and the other concentrations tested. From the cluster, it showed that control samples have the closest relationship with samples exposed to endosulfan at concentration 3.6 µg/L, followed by 7.00 µg/L endosulfan and 4.75 mg/L atrazine (Figure 4.9). These relationships showed that the potential of genotoxicity effects caused by endosulfan and atrazine to *O. niloticus* was significant as compared to the control test. This data was correlated to the results obtained from genotoxic assessment by micronuclei assays (Table 4.1 and 4.2) and the significant increased ($P<0.05$) in male *O. niloticus* after exposure to these concentrations (Table 4.5).

4.3 Expression Level of Vtg Gene in Male *O. niloticus*

Amplification efficiencies of the reference gene and the target were 1.963 and 1.962 respectively. Meanwhile, the percentage of amplification efficiencies of the reference and the target were 96.3% and 96.2% respectively. Since the amplification efficiencies of the target and the reference gene were similar but the efficiency is not equal to 2, a modified form of $2^{-\Delta\Delta C_T}$method was used by replacing the 2 in the equation by 1.96. Therefore, the formula $1.96^{-\Delta\Delta C_T}$ was used to determine the relative expression of the target gene in different samples.

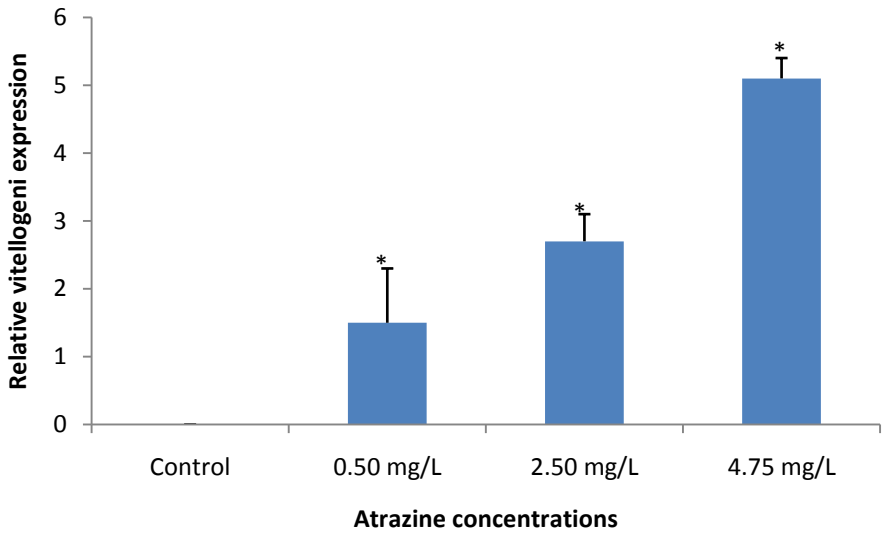

Figure 4.12: Normalized Vtg gene expression in male *O. niloticus* after atrazine exposure for 96 hours.

(*) indicating significant difference from control values ($P<0.05$, One-way ANOVA, followed by Tukey's multiple comparison).

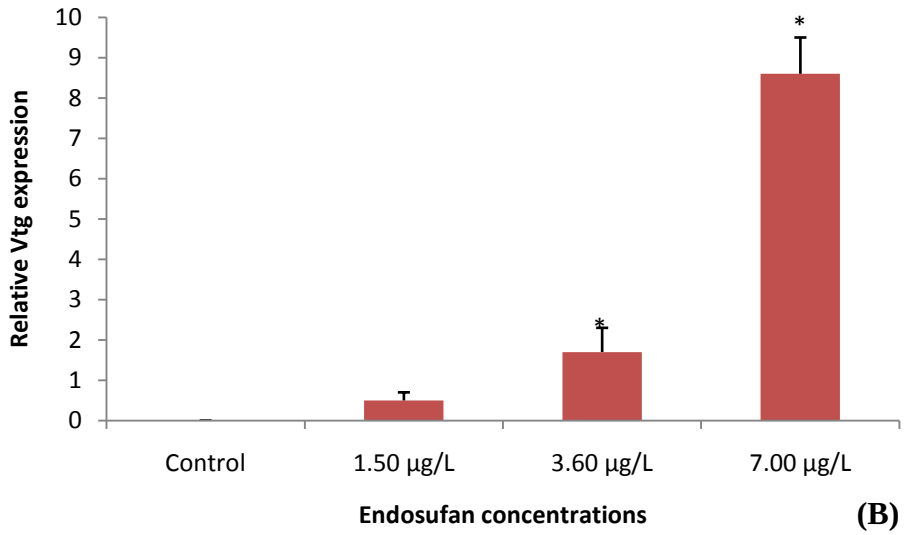

Figure 4.12: Normalized Vtg gene expression in male *O. niloticus* after endosulfan exposure for 96 hours.

(*) indicating significant difference from control values ($P<0.05$, One-way ANOVA, followed by Tukey's multiple comparison).

Table 4.5: Measurement of Vtg from liver *O. niloticus* exposed to atrazine and endosulfan for 96 hours.

Treatments	Control	Atrazine (mg/L)			Endosulfan (µg/L)		
		0.50	2.50	4.75	1.50	3.60	7.00
Vtg expression (mean ± SE)	0.00 ± 0.00^{a}	$1.50 \pm 0.80^{b,c}$	2.70 ± 0.40^{c}	5.10 ± 0.30^{d}	$0.50 \pm 0.20^{a,b}$	$1.70 \pm 0.60^{b,c}$	8.60 ± 0.90^{e}

Different superscipts indicating significant differences among treatments. Significant difference was analyzed by using One-way ANOVA, followed by Tukey's multiple comparison tests. Significant difference was ascertain at $P<0.05$.Means within each column followed by the different letters are significantly different at the $P<0.05$ level as determined by Tukey's multiple comparison tests.

The induction of Vtg in male *O. niloticus* following exposure to different concentrations of atrazine and endosulfan was investigated in this experiment. Samples exposed to 0.50 mg/L, 2.50mg/L and 4.75 mg/L atrazine were expressing Vtg at approximately 1.50, 2.70 and 5.10-fold, respectively (Table 4.5). It was found that Vtg production levels were significantly higher in all atrazine concentration tested (0.50 mg/L, 2.50 mg/L and 4.75 mg/L) as compared to untreated male *O. niloticus* ($P<0.05$) (Figure 4.12). Meanwhile, in samples exposed to endosulfan, the 3.60 µg/L and 7.00 µg/L endosulfan caused significant increases compared to the control group ($P<0.05$) (Figure 4.13). At concentration 3.60 µg/L and 7.00 µg/L endosulfan, the samples were expressing Vtg at approximately 1.70 and 8.60-fold respectively than the control group. In contrast, no statistically significant difference was observed in the 1.50 µg/L endosulfan treatment group (Table 4.5).

CHAPTER 5

DISCUSSION

The aims of this study were to assess genotoxicity effects and Vtg gene expression on *O. niloticus* after exposure to EDCs. Atrazine and endosulfan were chosen to assess their potential as endocrine disruptors and genotoxic effects to the target species. Previous studies regarding genotoxicity in fish to atrazine showed variability in response based on the concentration and time-dependent to the control test by verifying through different assays (Elia et al., 2002; Spanò et al., 2004; Nwani, et al., 2011). Endosulfan also is a concern since this chemical is harmful to fish species and other aquatic organisms. This chemical was demonstrated to have genotoxic effect to *Hyla pulchella* tadpoles when the samples were exposed *in vivo* at two sampling times, 48 ad 96 hours (Lajmanovich, et al., 2005). For this study, the concentration dependent relationship was evaluated to see the genotoxicity properties and estrogenic disruption which could occur to *O. niloticus* after exposure to atrazine and endosulfan for 96 hours.

5.1 Genotoxic Effect in *O. niloticus*

Atrazine and endosulfan were observed to have genotoxic effects to erythrocytes of *O. niloticus*. Atrazine and endosulfan concentrations tested showed a positive relation of dose reply of *O. niloticus*. There was an increase in the induction of MN and total NA in erythrocytes of *O. niloticus* with exposure to increasing concentrations of atrazine (Figure

4.2). This result is in agreement with previous studies that indicate that the higher atrazine concentration associate with higher percentile rate of cells bearing micronuclei and NA (Ventura, et al., 2008; Nwani, et al., 2011). At the 4.75 mg/L atrazine, there was a high incident of cells with micronuclei and NA, demonstrating the high mutagenicity of atrazine in that concentration for *O. niloticus*. It was in accordance with those previously reported by Nwani et al (2011), at the 4.74 mg/L atrazine, there was increased in DNA single strand breaks in the form of comet assay in blood and gill cells of *Channa punctatus*.

Significant increase for the formation of micronuclei at the 0.50 mg/L atrazine was observed, while the frequency of NA showed no significant difference compared to the control test at this concentration. However, findings from other researches showed that NA can significantly increase when exposed to *O. niloticus* at 0.05 mg/L concentration. Ventura et al. (2008) reported when atrazine was exposed to *O. niloticus* at 25 µg/L concentration, there was significant increase ($P<0.05$) to the frequency of MN and NA. Similar result was also shown when cadmium was treated to *O. niloticus* at 0.50 mg/L, where there was significant increase ($P<0.05$) of MN and NA frequencies as compared to control test (Őzkan et al., 2011). Therefore, it was justified as many chemical compounds may simultaneously induce the formation of both MN and other NA, or they may cause only one of these changes (Carrasco et al, 1990; Pacheco et al., 1998).

The induction of MN and NA were elevated significantly at 3.60 µg/L and 7.00 µg/L endosulfan concentrations. There was a high rate of micronuclei and total NA formation in *O. niloticus* at 7.00 µg/L endosulfan. These findings were corroborated to researches developed by some authors (Lajmanovich et al, 2005; Neuparth et al, 2006), where there was an increase in MN and NA in the erythrocytes of the test system studied in a concentration dependent manner following 96 hours exposure. It was also demonstrated that embryotoxicity and DNA strand breaks were observed in *Crassostea gigas* embryos at higher endosulfan concentration, and followed in a concentration dependant manner (Wessel et al., 2007). In contrast, at the lowest endosulfan concentration tested (1.50 µg/L), there was no significant difference for the frequency of MN and NA as compared to control test (Figure 4.3). Hence, endosulfan at this concentration slightly induced genotoxicity in *O. niloticus* as compared to the other treatment.

In this study, the total frequency of NA was directly proportional at the 2.50 mg/L and 4.75 mg/L atrazine concentrations, and at the 3.60 µg/L and 7.00 µg/L endosulfan concentrations. If each specific kind of nuclear alterations was considered, the 4.75 mg/L atrazine and the 7.00 µg/L endosulfan concentration showed the highest frequency of cells with blebbed nuclei, cell with lobed nuclei, and cells with notched nuclei. This result presents that at the 4.75 mg/L atrazine and 7.00 µg/L endosulfan, there was a high potential to induce the morphological changes of nuclear membrane (Ventura, et al., 2008). The induction of binucleated cells was observed high at 2.50 mg/L atrazine and 7.00 µg/L endosulfan. In this way, the action of atrazine and endosulfan may cause the failure of tubuline polymerization which could lead to the formation of binucleated cells in the erythrocytes of *O. niloticus* (Ventura et al, 2008).

MN test has been widely used in aquatic toxicology field to evaluate the genotoxicity of many compounds in polluted aquatic ecosystems (Ventura et al, 2008; Bűcker and Da Conceição, 2012). Since the results were based on the frequency of MN and NA, therefore these assays have been proposed as a monitoring system for potential genotoxity of an agent rather than by using chromosomal assay (Guha and Khuda-Bukhsh, 2002). Most methods developed for assessing MN and NAutilized blood as the sample. Blood smears could provide thousands of scorable erythrocytes. Hence, micronuclei assays in fish blood could provide endpoints in genotoxicity assessments which are simple, cost-effective and rapid (Baršienė, et. al, 2006).

The formation of MN could be originated from either acentric chromosome fragments or chromosome loss due to whole chromosome that lag behind at anaphase during nuclear division (Fenech, 2007). The lag at cell division may cause by lack of centromere, damage in centromere or defect in cytokinesis. (Baršienė, et al, 2006). According to the data (Table 4.1 and 4.2), the tested atrazine and endosulfan concentrations induced the formation of MN in *O. niloticus*. The induction may be related to chromosomal delays at anaphase, characterized by a bad functioning in the spindle, or the cause may due to the presence of acentric chromosome fragments (Al-Sabti and Meltcafe, 1995; Baršienė, et. al, 2006).

The occurrence of NA was taken into consideration when assessing MN analysis. The formation of NA may be associated to failure during cell division, cell death processes, and to genotoxicity and/or mutagenicity (Fenech, 2000). Notched cells which occur in erythrocytes of *O. niloticus* may probably be associated with aneuploids (Ghadially, 1982). The binucleated cells may have been originated from the failure to form mitotic fuse caused by aneugenic action of chemicals, and for this case, the cause were atrazine and endosulfan (Fernandes et al., 2007; Ventura et al., 2008). Although the mechanisms responsible for the formation of cells with blebbed nuclei and cells with lobed nuclei were poorly understood and have not fully been explained, these abnormalities are considered to be indicators of genotoxic damage (Őzkan, et al., 2011).

All tested atrazine concentrations and high concentrations of endosulfan induced mutagenic and genotoxic effects in *O. niloticus*, which agreed with the finding from previous studies (Clements et al., 1997, Ventura et al., 2008, Nwani et al, 2011). Thus, atrazine concentration at 0.50 mg/L, 2.50 mg/L and 4.75 mg/L,and endosulfan concentration at 3.60 µg/Land 7.00 µg/L induced micronuclei and NA. The increase in number of MN and NA could be potentially induced due to clastogenic effects of atrazine and endosulfan in the test system (Al-Sabti and Metcalfe, 1995; Ventura et al., 2008).

5.2 Changes in RAPD Band Pattern in *O. niloticus*

Subsequent RAPD analysis was investigated to determine the potential of atrazine and endosulfan genotoxicity in *O. niloticus*. Different and distinctive band pattern were observed from *O. niloticus*. Primers used in *O .niloticus* exposed to atrazine and endosulfan also yielded RAPD pattern differing from the control fish. This indicated that there was polymorphic region created in the *O. niloticus* genome when exposed to these chemicals (Abumourad et al., 2012). At highest concentration of atrazine and endosulfan studied (4.75 mg/L and 7.00 µg/L respectively), RAPD profiles showed increase in the disappearance of bands and the appearance of new bands, as well as a closer genetic distance relationship to the control test. This is relevant as high concentration of environmental pollutants can have deleterious effects as well as acute toxicity damaging cells, tissues and organs in living organisms (Conte et al., 1998). Moreover, the genotoxicity of atrazine is in accordance with findings found from the previous studies (Abbas and Ali, 2007; Mona et al., 2013), where atrazine caused appearance of new diagnostic bands due to high atrazine concentration. In addition, RAPD analysis data was correlated with micronuclei analysis in this study, thus confirmed that *O. niloticus* exposed to high concentration of atrazine and endosulfan yielded high genotoxicity to this species.

The gain or loss of different bands in the treated samples can be explained as a result of changes in primer binding sites, structural rearrangements in DNA caused by different types of DNA damages under the effect of atrazine and endosulfan exposures (Abumourad et al, 2012; Mona et al., 2013). These chemicals could cause these effects in

DNA, probably due to an increase of free radical activity or free radical life span in organisms following exposure to chemicals and could deteriorate antioxidant defensive system by Reactive Oxygen Species, as proposed by several authors (Lai and Singh, 2004; Lee et al., 2004; Guier et al., 2006, Abumourad et al., 2012).

The disappearance of some bands from normal *O. niloticus* may be correlated with the level of DNA damage after exposure to atrazine and endosulfan, as observed when *O. niloticus* was treated with ammonia (Abumourad et al., 2012). It can be explained that atrazine and endosulfan may interact with genomic DNA at specific sites, which lead to hot spot DNA damage and potentially to hot spot mutations, hence resulting in the disappearance of bands in the RAPD profiles (Vogelstein and Kinzler, 1992; Atienzar, et al., 2002; Sayed et al., 2013). The appearance of new bands may also relate to the level of DNA damage and the efficiency of DNA repair and replication (Atienzar et al., 1999). Appearing bands may be seen due to variations in the DNA sequences caused by mutations, large deletions and homologous recombinations (Atienzar et al., 1999), or may be due to different DNA structural changes such as breaks, transpositions, and deletions, which allow accessibility of primers to bind on some new oligonucleotide priming sites (Arillo et al., 1981; Abumourad et al., 2012, Cansaran-Duman et al., 2012).

Changes in the RAPD patterns are expressed as decreases in GTS, a qualitative measure reflecting the change in the number of RAPD profiles generated by the concentration of chemicals (Rocco et al., 2013). In this study, GTS reflects the genotoxicity for the concentration of atrazine and endosulfan used in relation to profiles obtained from

the control specimens. In this experiment, assessment of GTS showed that changes in the RAPD pattern in *O. niloticus* treated with endosulfan were decreased when applied to higher concentrations. This data was supported by research on genotoxity of zebrafish when exposed to the drug concentrations used (Rocco et al., 2013). Changes in RAPD patternsinduced by genotoxins could be regarded as modifications in GTSand such genotoxic effects can be directly compared to the alterationsin some parameters including DNA mutation or chromosomal rearrangement such as insertions/deletions either at or between the primer binding sites which observed as presence and dissapearance of DNA bands in the RAPD profile (Bardakci, 2001).

RAPD profile for atrazine treated *O. niloticus* showed variation in the mean GTS percentage at concentration 0.50 mg/L and 2.50 mg/l. It was possible that at 2.50 mg/L atrazine tested, this chemical may induced high level of DNA damage as compared to 0.50 mg/L atrazine, but the GTS value does not necessarily decreased since GTS may be related to different kinds of DNA damage, such as DNA adducts, mutations and rearrangement, thus it would be difficult to anticipate a dose-dependant relationship (Rocco, 2014). In Table 4.4, mean GTS percentage was not significant. This can be caused by lack of replicate for RAPD amplification for each treated samples. In order to give more significant mean GTS percentage value, genomic DNAs for all samples for all sample shoud be at least amplified twice before qualitative and quantitave analysis were done (Abumourad et al., 2012).

5.3 Vtg Gene Expression in Male *O. niloticus*

Vtg gene expression was also investigated in this study, as induction of Vtg in males and immature female fishes has been widely known to be a very useful biomarker to detect disruption of estrogen signaling by EDCs (Scholz et al., 2004; Reinen, et al., 2010). The induction of Vtg gene expression in male *O. niloticus* by atrazine and endosulfan showed that these chemicals were capable of interfering with the normal function of endocrine system of male *O. niloticus*. At 7.00 µg/L endosulfan, the Vtg gene was expressed at the highest followed by 4.75 mg/L atrazine, as compared to the control and the other concentrations tested. Thus, the endocrine system of male *O. niloticus* was highly disrupted by estrogenic effect of endosulfan as compared to atrazine.

The finding that atrazine induced Vtg in male *O. niloticus*is in contrast to the finding from studies on male carp hepatocytes *in vitro* (Sandersen et al., 2001) and in adult goldfish (Spanò et al., 2004). Following the result obtained in this study, atrazine which inducedVtg in male *O. niloticus* was supported by previous researches, where atrazine increased the circulating concentration of Vtg when administered orally to European quail (De la Casa-Resino et al., 2012), and this chemical was shownto produceVtg in male *Xenopus laevis* (Miyahara et al, 2003) and *Bufo marinus* (McCoy et al, 2002). It was suggested that *O. niloticus* is sensitive to atrazine estrogenic effect following concentration dependent manner, hence there is potential for this chemical toinduce feminization in male fish.

Based on the result, endosulfan was potentially harmful in aquatic environments as this chemical was shown to be toxic to *O. niloticus*. It has been demonstrated that endosulfan could severely affects the reproductive function and the synthesis of Vtg in the liver of other aquatic life, as shown in the zebrafish (Han et al., 2011, Wing et al., 2013), *Cichlasoma dimerus* (Da Cuňa et al, 2013), muddy loach (Min et al, 2010), and rainbow trout (Bisson and Hontela, 2002). In contrast, exposure to endosulfan at lower concentration failed to induce measurable levels of Vtg in male *O. niloticus*. This result supports a report from a study on sheepshead minnow (*Cyprinodon variegatus*), where the tested endosulfan concentrations applied were below 1.50 µg/L (Hemmer et al., 2001). The induction of vitellogenin was increased in the concentration-dependent manner for atrazine and endosulfan, which suggested that both chemicals were potential to disrupt the endocrine system and may cause feminization in male fish.

CHAPTER 6

CONCLUSION

6.1 Conclusion

Genotoxic effects of atrazine and endosulfan has been shown to have affected *O. niloticus* as the test subject for this experiment. Upon exposure to different concentrations of atrazine and endosulfan, the frequency of MN and NA in erythrocytes of *O. niloticus* were increased in a concentration dependent manner at 96 hours exposure. The response of concentrations tested for both chemicals against MN formation was summarized as below;

Control < end 1.50 µg/L < atz 0.50 mg/L< end 3.60 µg/L < atz 2.50 mg/L< end 7.00 µg/L < atz 4.75 mg/L

and the summary of NA formation when the concentration of atrazine and endosulfan was increased was shown below;

Control < end 1.50 µg/L < atz 0.50 mg/L< end 3.60 µg/L < atz 2.50 mg/L< atz 4.75 mg/L < end 7.0 µg/L

where, atrazine and endosulfan at highest concentration being tested (4.75 mg/L and 7.00 µg/L respectively) were showed to have the highest incident of MN and NA in *O. niloticus* blood cells. Therefore it was indicated that the genotoxicity in *O. niloticus* was the highest at these concentrations.

A subsequent RAPD assay also showed that atrazine and endosulfan induced genotoxic effect in *O. niloticus*. The five primers tested produced unique polymorphic band patterns and generated RAPD profile variations that displayed the disappearance of bands and appearance of new bands of amplified DNA in the atrazine and endosulfan-treated genomic DNA. At 4.75 mg/L atrazine, 3.60 µg/L endosulfan and 7.00 µg/L endosulfan, the number of bands disappearing and appearing were the highest at these concentrations. It was further verified through the dendogram created based on the RAPD profiles. Thus, it was showed the chance that RAPD band to disappear and new bands appeared was increased when *O. niloticus* was exposed with high concentration of genotoxicants.

Atrazine and endosulfan were also shown to induce Vtg in male *O.niloticus* with a concentration-dependent manner after being exposed to 96 hours. The summary of this finding was shown as below;

Control < end 1.50 µg/L < atz 0.50 mg/L< end 3.60 µg/L < atz 2.50 mg/L< atz 4.75 mg/L < end 7.0 µg/L

where, the expression of Vtg in male *O. niloticus* was the highest at 4.75 mg/L atrazine and 7.00 µg/L endosulfan as compared to the other concentration tested. Therefore, when the concentration of atrazine and endosulfan was increased, it was possible that the level of Vtg in male *O. niloticus* was also increasingly induced.

In summary, the finding obtained in this study suggested atrazine and endosulfan have the potential to be endocrine disruptor chemicals. Considering the genotoxic effect and Vtg expression results of atrazine and endosulfan, the action to induce mechanisms that lead to genotoxic properties may be possible due to atrazine and endosulfan clastogenic effect and estrogenic effect. Thus, exposure of EDCs to aquatic environment might posses threat to the aquatic livings especially, since these chemicals can gives adverse effect by interfering the stability of aquatic ecosystems due to their potentiality in promoting damage in the genetic material of fishes and also interrupt their reproductive system.

6.2 Recommendations and Future Works

Although MN test could provide rapid analysis, a more sensitive method is suggested to evaluate the genotoxicity of atrazine and endosulfan in *O. niloticus* in order to affirm the results. Comet assay has been usually applied along MN test to study the genotoxicity of EDCs in fish. This method is considered more sensitive than MN test (Bűcker and Da Conceição, 2012). Therefore, further studies will include comet assay to study the genotoxicity effect of atrazine and endosuflan in *O. niloticus*.

RAPD test has proven to be useful when studying the genotoxic effect of atrazine and endosulfan as seen in the present study. However, there was limited information to support the finding about genotoxicity in endosulfan exposed test system by using RAPD, while the present study found that this technique was applicable to determine genotoxicity

in endosulfan treated *O. niloticus* at high concentration. In order to affirm this finding and also to provide more knowledge, further studies with other species, should be developed to see the potential of genotoxic effect of endosulfan by RAPD assay.

The result presented here indicates that male *O. niloticus* respond to atrazine and endosulfan by producing Vtg. Therefore, it was suggested that both chemicals has been shown to potentially disrupt endocrine signaling and exhibit estrogenic effect in fish, which later may lead to fish feminization. Furthermore, Vtg was proven to be a useful biomarker or indicator of EDCs exposure in aquatic environment. However, a number of reports on the effect of atrazine causing production of Vtg especially in male aquatic vertebrates have been published, yet there is inconsistency in the effect reported, and between studies in different laboratories, as obtained in this current study.Hence, new tests are suggested to study the effect of atrazine on Vtg induction in males by applying other parameters and techniques, and also by using other test systems which can be used as supporting data to study the genotoxicity of EDCs, especially in fish.

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APPENDIX A

Statistical analysis for MN test and NA.

i) Atrazine samples

Oneway

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
MN	Between Groups	3474.333	3	1158.111	145.776	.000
	Within Groups	254.222	32	7.944		
	Total	3728.556	35			
NA	Between Groups	1797.861	3	599.287	28.804	.000
	Within Groups	665.778	32	20.806		
	Total	2463.639	35			
Blebbed	Between Groups	68.444	3	22.815	6.006	.002
	Within Groups	121.556	32	3.799		
	Total	190.000	35			
Lobed	Between Groups	200.306	3	66.769	16.780	.000
	Within Groups	127.333	32	3.979		
	Total	327.639	35			
Notched	Between Groups	234.889	3	78.296	13.110	.000
	Within Groups	191.111	32	5.972		
	Total	426.000	35			
Binuclear	Between Groups	14.333	3	4.778	4.556	.009
	Within Groups	33.556	32	1.049		
	Total	47.889	35			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Atrazine concentrations	(J) Atrazine concentrations	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
MN	control	atz 4.75	-26.000(*)	1.329	.000	-29.60	-22.40
		atz 2.5	-15.000(*)	1.329	.000	-18.60	-11.40
		atz 0.5	-5.889(*)	1.329	.001	-9.49	-2.29
	atz 4.75	control	26.000(*)	1.329	.000	22.40	29.60
		atz 2.5	11.000(*)	1.329	.000	7.40	14.60
		atz 0.5	20.111(*)	1.329	.000	16.51	23.71
	atz 2.5	control	15.000(*)	1.329	.000	11.40	18.60
		atz 4.75	-11.000(*)	1.329	.000	-14.60	-7.40
		atz 0.5	9.111(*)	1.329	.000	5.51	12.71
	atz 0.5	control	5.889(*)	1.329	.001	2.29	9.49
		atz 4.75	-20.111(*)	1.329	.000	-23.71	-16.51
		atz 2.5	-9.111(*)	1.329	.000	-12.71	-5.51
NA	control	atz 4.75	-17.889(*)	2.150	.000	-23.71	-12.06
		atz 2.5	-13.889(*)	2.150	.000	-19.71	-8.06
		atz 0.5	-5.000	2.150	.113	-10.83	.83
	atz 4.75	control	17.889(*)	2.150	.000	12.06	23.71
		atz 2.5	4.000	2.150	.265	-1.83	9.83
		atz 0.5	12.889(*)	2.150	.000	7.06	18.71
	atz 2.5	control	13.889(*)	2.150	.000	8.06	19.71
		atz 4.75	-4.000	2.150	.265	-9.83	1.83
		atz 0.5	8.889(*)	2.150	.001	3.06	14.71
	atz 0.5	control	5.000	2.150	.113	-.83	10.83
		atz 4.75	-12.889(*)	2.150	.000	-18.71	-7.06
		atz 2.5	-8.889(*)	2.150	.001	-14.71	-3.06
Blebbed	control	atz 4.75	-3.333(*)	.919	.005	-5.82	-.84
		atz 2.5	-2.889(*)	.919	.018	-5.38	-.40
		atz 0.5	-.889	.919	.769	-3.38	1.60
	atz 4.75	control	3.333(*)	.919	.005	.84	5.82
		atz 2.5	.444	.919	.962	-2.04	2.93
		atz 0.5	2.444	.919	.056	-.04	4.93
	atz 2.5	control	2.889(*)	.919	.018	.40	5.38
		atz 4.75	-.444	.919	.962	-2.93	2.04
		atz 0.5	2.000	.919	.151	-.49	4.49
	atz 0.5	control	.889	.919	.769	-1.60	3.38
		atz 4.75	-2.444	.919	.056	-4.93	.04
		atz 2.5	-2.000	.919	.151	-4.49	.49

Table multiple comparisons, continued.

Lobed	control	atz 4.75	-5.889(*)	.940	.000	-8.44	-3.34
		atz 2.5	-3.111(*)	.940	.012	-5.66	-.56
		atz 0.5	-.444	.940	.965	-2.99	2.10
	atz 4.75	control	5.889(*)	.940	.000	3.34	8.44
		atz 2.5	2.778(*)	.940	.028	.23	5.33
		atz 0.5	5.444(*)	.940	.000	2.90	7.99
	atz 2.5	control	3.111(*)	.940	.012	.56	5.66
		atz 4.75	-2.778(*)	.940	.028	-5.33	-.23
		atz 0.5	2.667(*)	.940	.037	.12	5.21
	atz 0.5	control	.444	.940	.965	-2.10	2.99
		atz 4.75	-5.444(*)	.940	.000	-7.99	-2.90
		atz 2.5	-2.667(*)	.940	.037	-5.21	-.12
Notched	control	atz 4.75	-6.333(*)	1.152	.000	-9.45	-3.21
		atz 2.5	-5.222(*)	1.152	.000	-8.34	-2.10
		atz 0.5	-1.778	1.152	.425	-4.90	1.34
	atz 4.75	control	6.333(*)	1.152	.000	3.21	9.45
		atz 2.5	1.111	1.152	.770	-2.01	4.23
		atz 0.5	4.556(*)	1.152	.002	1.43	7.68
	atz 2.5	control	5.222(*)	1.152	.000	2.10	8.34
		atz 4.75	-1.111	1.152	.770	-4.23	2.01
		atz 0.5	3.444(*)	1.152	.026	.32	6.57
	atz 0.5	control	1.778	1.152	.425	-1.34	4.90
		atz 4.75	-4.556(*)	1.152	.002	-7.68	-1.43
		atz 2.5	-3.444(*)	1.152	.026	-6.57	-.32
Binuclear	control	atz 4.75	-1.333(*)	.483	.044	-2.64	-.03
		atz 2.5	-1.667(*)	.483	.008	-2.97	-.36
		atz 0.5	-1.222	.483	.074	-2.53	.09
	atz 4.75	control	1.333(*)	.483	.044	.03	2.64
		atz 2.5	-.333	.483	.900	-1.64	.97
		atz 0.5	.111	.483	.996	-1.20	1.42
	atz 2.5	control	1.667(*)	.483	.008	.36	2.97
		atz 4.75	.333	.483	.900	-.97	1.64
		atz 0.5	.444	.483	.794	-.86	1.75
	atz 0.5	control	1.222	.483	.074	-.09	2.53
		atz 4.75	-.111	.483	.996	-1.42	1.20
		atz 2.5	-.444	.483	.794	-1.75	.86

* The mean difference is significant at the .05 level.

Homogeneous Subsets

MN

Tukey B

Atrazine concentrations	N	Subset for alpha = .05			
		1	2	3	4
control	9	6.67			
atz 0.5	9		12.56		
atz 2.5	9			21.67	
atz 4.75	9				32.67

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000

NA

Tukey B

Atrazine concentrations	N	Subset for alpha = .05	
		1	2
control	9	7.00	
atz 0.5	9	12.00	
atz 2.5	9		20.89
atz 4.75	9		24.89

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Blebbed

Tukey B

Atrazine concentrations	N	Subset for alpha = .05		
		1	2	3
control	9	1.56		
atz 0.5	9	2.44	2.44	
atz 2.5	9		4.44	4.44
atz 4.75	9			4.89

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Lobed

Tukey B

Atrazine concentrations	N	Subset for alpha = .05		
		1	2	3
control	9	3.44		
atz 0.5	9	3.89		
atz 2.5	9		6.56	
atz 4.75	9			9.33

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Notched

Tukey B

Atrazine concentrations	N	Subset for alpha = .05	
		1	2
control	9	3.00	
atz 0.5	9	4.78	
atz 2.5	9		8.22
atz 4.75	9		9.33

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Binuclear

Tukey B

Atrazine concentrations	N	Subset for alpha = .05	
		1	2
control	9	.00	
atz 0.5	9		1.22
atz 4.75	9		1.33
atz 2.5	9		1.67

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

ii) Endosulfan samples

Oneway

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
micronuclei	Between Groups	1046.444	3	348.815	105.746	.000
	Within Groups	105.556	32	3.299		
	Total	1152.000	35			
NA	Between Groups	2667.111	3	889.037	20.692	.000
	Within Groups	1374.889	32	42.965		
	Total	4042.000	35			
Blebbed nuclei	Between Groups	51.222	3	17.074	7.612	.001
	Within Groups	71.778	32	2.243		
	Total	123.000	35			
Lobed nuclei	Between Groups	529.111	3	176.370	10.830	.000
	Within Groups	521.111	32	16.285		
	Total	1050.222	35			
Notched nuclei	Between Groups	380.750	3	126.917	29.383	.000
	Within Groups	138.222	32	4.319		
	Total	518.972	35			
Binuclear cells	Between Groups	7.861	3	2.620	2.220	.105
	Within Groups	37.778	32	1.181		
	Total	45.639	35			

Post Hoc Tests

Multiple Comparisons

Tukey HSD

Dependent Variable	(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
micronuclei	7 microgram/L	3.6 microgram/L	4.667(*)	.856	.000	2.35	6.99
		1.5 microgram/L	11.889(*)	.856	.000	9.57	14.21
		control 2	13.222(*)	.856	.000	10.90	15.54
	3.6 microgram/L	7 microgram/L	-4.667(*)	.856	.000	-6.99	-2.35
		1.5 microgram/L	7.222(*)	.856	.000	4.90	9.54
		control 2	8.556(*)	.856	.000	6.24	10.88
	1.5 microgram/L	7 microgram/L	-11.889(*)	.856	.000	-14.21	-9.57
		3.6 microgram/L	-7.222(*)	.856	.000	-9.54	-4.90
		control 2	1.333	.856	.417	-.99	3.65
	control 2	7 microgram/L	-13.222(*)	.856	.000	-15.54	-10.90
		3.6 microgram/L	-8.556(*)	.856	.000	-10.88	-6.24
		1.5 microgram/L	-1.333	.856	.417	-3.65	.99
NA	7 microgram/L	3.6 microgram/L	10.000(*)	3.090	.014	1.63	18.37
		1.5 microgram/L	17.111(*)	3.090	.000	8.74	25.48
		control 2	23.111(*)	3.090	.000	14.74	31.48
	3.6 microgram/L	7 microgram/L	-10.000(*)	3.090	.014	-18.37	-1.63
		1.5 microgram/L	7.111	3.090	.119	-1.26	15.48
		control 2	13.111(*)	3.090	.001	4.74	21.48
	1.5 microgram/L	7 microgram/L	-17.111(*)	3.090	.000	-25.48	-8.74
		3.6 microgram/L	-7.111	3.090	.119	-15.48	1.26
		control 2	6.000	3.090	.231	-2.37	14.37
	control 2	7 microgram/L	-23.111(*)	3.090	.000	-31.48	-14.74
		3.6 microgram/L	-13.111(*)	3.090	.001	-21.48	-4.74
		1.5 microgram/L	-6.000	3.090	.231	-14.37	2.37
Blebbed nuclei	7 microgram/L	3.6 microgram/L	1.111	.706	.407	-.80	3.02
		1.5 microgram/L	2.111(*)	.706	.026	.20	4.02
		control 2	3.222(*)	.706	.000	1.31	5.14
	3.6 microgram/L	7 microgram/L	-1.111	.706	.407	-3.02	.80
		1.5 microgram/L	1.000	.706	.499	-.91	2.91
		control 2	2.111(*)	.706	.026	.20	4.02
	1.5 microgram/L	7 microgram/L	-2.111(*)	.706	.026	-4.02	-.20
		3.6 microgram/L	-1.000	.706	.499	-2.91	.91
		control 2	1.111	.706	.407	-.80	3.02
	control 2	7 microgram/L	-3.222(*)	.706	.000	-5.14	-1.31
		3.6 microgram/L	-2.111(*)	.706	.026	-4.02	-.20
		1.5 microgram/L	-1.111	.706	.407	-3.02	.80

Table multiple comparisons, continued.

Lobed nuclei	7 microgram/L	3.6 microgram/L	3.444	1.902	.287	-1.71	8.60
		1.5 microgram/L	7.333(*)	1.902	.003	2.18	12.49
		control 2	10.111(*)	1.902	.000	4.96	15.27
	3.6 microgram/L	7 microgram/L	-3.444	1.902	.287	-8.60	1.71
		1.5 microgram/L	3.889	1.902	.193	-1.27	9.04
		control 2	6.667(*)	1.902	.007	1.51	11.82
	1.5 microgram/L	7 microgram/L	-7.333(*)	1.902	.003	-12.49	-2.18
		3.6 microgram/L	-3.889	1.902	.193	-9.04	1.27
		control 2	2.778	1.902	.473	-2.38	7.93
	control 2	7 microgram/L	-10.111(*)	1.902	.000	-15.27	-4.96
		3.6 microgram/L	-6.667(*)	1.902	.007	-11.82	-1.51
		1.5 microgram/L	-2.778	1.902	.473	-7.93	2.38
Notched nuclei	7 microgram/L	3.6 microgram/L	5.333(*)	.980	.000	2.68	7.99
		1.5 microgram/L	7.000(*)	.980	.000	4.35	9.65
		control 2	8.667(*)	.980	.000	6.01	11.32
	3.6 microgram/L	7 microgram/L	-5.333(*)	.980	.000	-7.99	-2.68
		1.5 microgram/L	1.667	.980	.340	-.99	4.32
		control 2	3.333(*)	.980	.009	.68	5.99
	1.5 microgram/L	7 microgram/L	-7.000(*)	.980	.000	-9.65	-4.35
		3.6 microgram/L	-1.667	.980	.340	-4.32	.99
		control 2	1.667	.980	.340	-.99	4.32
	control 2	7 microgram/L	-8.667(*)	.980	.000	-11.32	-6.01
		3.6 microgram/L	-3.333(*)	.980	.009	-5.99	-.68
		1.5 microgram/L	-1.667	.980	.340	-4.32	.99
Binuclear cells	7 microgram/L	3.6 microgram/L	.222	.512	.972	-1.17	1.61
		1.5 microgram/L	.667	.512	.569	-.72	2.05
		control 2	1.222	.512	.100	-.17	2.61
	3.6 microgram/L	7 microgram/L	-.222	.512	.972	-1.61	1.17
		1.5 microgram/L	.444	.512	.821	-.94	1.83
		control 2	1.000	.512	.227	-.39	2.39
	1.5 microgram/L	7 microgram/L	-.667	.512	.569	-2.05	.72
		3.6 microgram/L	-.444	.512	.821	-1.83	.94
		control 2	.556	.512	.701	-.83	1.94
	control 2	7 microgram/L	-1.222	.512	.100	-2.61	.17
		3.6 microgram/L	-1.000	.512	.227	-2.39	.39
		1.5 microgram/L	-.556	.512	.701	-1.94	.83

* The mean difference is significant at the .05 level.

Homogeneous Subsets

Micronuclei

Tukey HSD

Concentration	N	Subset for alpha = .05		
		1	2	3
control 2	9	7.22		
1.5 microgram/L	9	8.56		
3.6 microgram/L	9		15.78	
7 microgram/L	9			20.44
Sig.		.417	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

NA

Tukey HSD

Concentration	N	Subset for alpha = .05		
		1	2	3
control 2	9	3.44		
1.5 microgram/L	9	9.44	9.44	
3.6 microgram/L	9		16.56	
7 microgram/L	9			26.56
Sig.		.231	.119	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Blebbed nuclei

Tukey HSD

Concentration	N	Subset for alpha = .05		
		1	2	3
control 2	9	.56		
1.5 microgram/L	9	1.67	1.67	
3.6 microgram/L	9		2.67	2.67
7 microgram/L	9			3.78
Sig.		.407	.499	.407

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Lobed nuclei

Tukey HSD

Concentration	N	Subset for alpha = .05		
		1	2	3
control 2	9	1.33		
1.5 microgram/L	9	4.11	4.11	
3.6 microgram/L	9		8.00	8.00
7 microgram/L	9			11.44
Sig.		.473	.193	.287

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Notched nuclei

Tukey HSD

Concentration	N	Subset for alpha = .05		
		1	2	3
control 2	9	1.56		
1.5 microgram/L	9	3.22	3.22	
3.6 microgram/L	9		4.89	
7 microgram/L	9			10.22
Sig.		.340	.340	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

Binuclear cells

Tukey HSD

Concentration	N	Subset for alpha = .05
		1
control 2	9	.00
1.5 microgram/L	9	.56
3.6 microgram/L	9	1.00
7 microgram/L	9	1.22
Sig.		.100

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 9.000.

APPENDIX B

RAPD band pattern scoring.

Table I: Loss/gain matrix of the bands on the gel picture for atrazine samples in Figure 4.4-4.8.

Primer	Sample (Atz)	Marker (bp)																			
		1900	1600	1500	1400	1200	1100	1000	950	900	800	750	700	600	550	500	450	400	350	270	250
OPA8	Control	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	1
	0.5	1	0	1	0	1	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1
	2.5	1	0	1	0	1	1	0	0	0	1	0	0	1	0	0	0	1	0	0	1
	4.75	0	0	1	1	1	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1
OPA13	Control	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
	0.5	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
	2.5	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
	4.75	1	0	1	1	1	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0
OPB8	Control	0	1	0	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0
	0.5	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0
	2.5	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0
	4.75	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0
OPA12	Control	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
	0.5	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
	2.5	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
	4.75	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
OPC11	Control	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0
	0.5	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	1	1	0	0
	2.5	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0
	4.75	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0

Table I, continued.

Primer	Sample (Atz)	TOTAL	Gain difference (a)	Loss difference (b)	a + b
OPA8	Control	7	0	0	0
	0.5	8	2	1	3
	2.5	8	2	1	3
	4.75	7	2	2	4
OPA13	Control	5	0	0	0
	0.5	5	0	0	0
	2.5	5	0	0	0
	4.75	8	3	0	3
OPB8	Control	10	0	0	0
	0.5	10	1	1	2
	2.5	11	1	0	1
	4.75	10	1	1	2
OPA12	Control	4	0	0	0
	0.5	5	1	0	1
	2.5	5	1	0	1
	4.75	4	0	0	0
OPC11	Control	5	0	0	0
	0.5	6	1	0	1
	2.5	5	0	0	0
	4.75	5	0	0	0

Table II: Loss/gain matrix of the bands on the gel picture for endosulfan samples in Figure 4.4-4.8.

Primer	Sample (End)	Marker (bp)																			
		1900	1600	1500	1400	1200	1100	1000	950	900	800	750	700	600	550	500	450	400	350	270	250
OPA8	Control	1	0	1	0	1	0	1	0	0	0	0	0	1	0	0	0	1	0	0	1
	1.5	1	0	1	1	1	1	1	0	0	1	0	1	1	0	0	0	1	0	0	1
	3.6	1	0	1	1	1	1	1	0	0	1	0	0	1	0	0	0	1	0	0	1
	7	1	0	0	1	1	0	1	0	0	1	0	1	0	0	0	0	1	0	0	1
OPA13	Control	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
	1.5	1	0	0	0	1	0	0	0	0	0	1	0	1	1	0	0	0	0	0	0
	3.6	1	0	0	0	1	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0
	7	1	0	0	0	1	0	0	0	1	0	1	0	1	1	0	0	0	0	0	0
OPB8	Control	0	1	0	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0
	1.5	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0
	3.6	0	1	1	1	1	0	0	1	0	1	0	1	0	0	1	0	0	0	1	0
	7	0	1	1	1	1	0	0	1	0	1	0	1	1	0	1	1	0	0	1	0
OPA12	Control	0	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
	1.5	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
	3.6	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
	7	1	0	0	0	0	0	0	1	0	0	0	1	0	1	0	0	0	0	1	0
OPC11	Control	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0
	1.5	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	0	1	1	0	0
	3.6	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	1	1	0	0
	7	0	0	0	0	0	0	1	0	0	1	0	0	0	1	0	1	1	1	0	0

Table II, continued.

Primer	Sample (End)	TOTAL	Gain difference (a)	Loss difference (b)	a + b
OPA8	Control	7	0	0	0
	1.5	10	4	0	4
	3.6	10	3	0	3
	7	8	3	2	5
OPA13	Control	5	0	0	0
	1.5	5	0	0	0
	3.6	6	1	0	1
	7	6	1	0	1
OPB8	Control	10	0	0	0
	1.5	11	1	0	1
	3.6	9	1	2	3
	7	11	1	0	1
OPA12	Control	4	0	0	0
	1.5	5	1	0	1
	3.6	5	1	0	1
	7	5	1	0	1
OPC11	Control	5	0	0	0
	1.5	5	0	0	0
	3.6	6	1	0	1
	7	6	1	0	1

APPENDIX C

Statistical Analysis for GTS (%)

Oneway

ANOVA

Genomic template stability

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	3221.731	6	536.955	1.370	.261
Within Groups	10971.534	28	391.841		
Total	14193.266	34			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Genomic template stability
Tukey HSD

(I) Concentration	(J) Concentration	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
Control	0.50 mg/L atz	21.57200	12.51943	.607	-18.1413	61.2853
	1.50 mg/L atz	13.57200	12.51943	.928	-26.1413	53.2853
	4.75 mg/L atz	27.42800	12.51943	.332	-12.2853	67.1413
	1.50 microgram/L	18.42800	12.51943	.758	-21.2853	58.1413
	3.60 microgram/L	27.57200	12.51943	.326	-12.1413	67.2853
	7.00 microgram/L	29.28600	12.51943	.261	-10.4273	68.9993
0.50 mg/L atz	Control	-21.57200	12.51943	.607	-61.2853	18.1413
	1.50 mg/L atz	-8.00000	12.51943	.995	-47.7133	31.7133
	4.75 mg/L atz	5.85600	12.51943	.999	-33.8573	45.5693
	1.50 microgram/L	-3.14400	12.51943	1.000	-42.8573	36.5693
	3.60 microgram/L	6.00000	12.51943	.999	-33.7133	45.7133
	7.00 microgram/L	7.71400	12.51943	.996	-31.9993	47.4273

Table multiple comparison continued,

1.50 mg/L atz	Control	-13.57200	12.51943	.928	-53.2853	26.1413
	0.50 mg/L atz	8.00000	12.51943	.995	-31.7133	47.7133
	4.75 mg/L atz	13.85600	12.51943	.921	-25.8573	53.5693
	1.50 microgram/L	4.85600	12.51943	1.000	-34.8573	44.5693
	3.60 microgram/L	14.00000	12.51943	.917	-25.7133	53.7133
	7.00 microgram/L	15.71400	12.51943	.866	-23.9993	55.4273
4.75 mg/L atz	Control	-27.42800	12.51943	.332	-67.1413	12.2853
	0.50 mg/L atz	-5.85600	12.51943	.999	-45.5693	33.8573
	1.50 mg/L atz	-13.85600	12.51943	.921	-53.5693	25.8573
	1.50 microgram/L	-9.00000	12.51943	.990	-48.7133	30.7133
	3.60 microgram/L	.14400	12.51943	1.000	-39.5693	39.8573
	7.00 microgram/L	1.85800	12.51943	1.000	-37.8553	41.5713
1.50 microgram/L	Control	-18.42800	12.51943	.758	-58.1413	21.2853
	0.50 mg/L atz	3.14400	12.51943	1.000	-36.5693	42.8573
	1.50 mg/L atz	-4.85600	12.51943	1.000	-44.5693	34.8573
	4.75 mg/L atz	9.00000	12.51943	.990	-30.7133	48.7133
	3.60 microgram/L	9.14400	12.51943	.989	-30.5693	48.8573
	7.00 microgram/L	10.85800	12.51943	.975	-28.8553	50.5713
3.60 microgram/L	Control	-27.57200	12.51943	.326	-67.2853	12.1413
	0.50 mg/L atz	-6.00000	12.51943	.999	-45.7133	33.7133
	1.50 mg/L atz	-14.00000	12.51943	.917	-53.7133	25.7133
	4.75 mg/L atz	-.14400	12.51943	1.000	-39.8573	39.5693
	1.50 microgram/L	-9.14400	12.51943	.989	-48.8573	30.5693
	7.00 microgram/L	1.71400	12.51943	1.000	-37.9993	41.4273
7.00 microgram/L	Control	-29.28600	12.51943	.261	-68.9993	10.4273
	0.50 mg/L atz	-7.71400	12.51943	.996	-47.4273	31.9993
	1.50 mg/L atz	-15.71400	12.51943	.866	-55.4273	23.9993
	4.75 mg/L atz	-1.85800	12.51943	1.000	-41.5713	37.8553
	1.50 microgram/L	-10.85800	12.51943	.975	-50.5713	28.8553
	3.60 microgram/L	-1.71400	12.51943	1.000	-41.4273	37.9993

Homogenous Subsets

Genomic template stability

Tukey HSD

Concentration	N	Subset for alpha = .05 1
7.00 microgram/L	5	70.7140
3.60 microgram/L	5	72.4280
4.75 mg/L atz	5	72.5720
0.50 mg/L atz	5	78.4280
1.50 microgram/L	5	81.5720
1.50 mg/L atz	5	86.4280
Control	5	100.0000
Sig.		.261

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 5.000.

APPENDIX D

Melt curve analysis of reaction (qPCR).

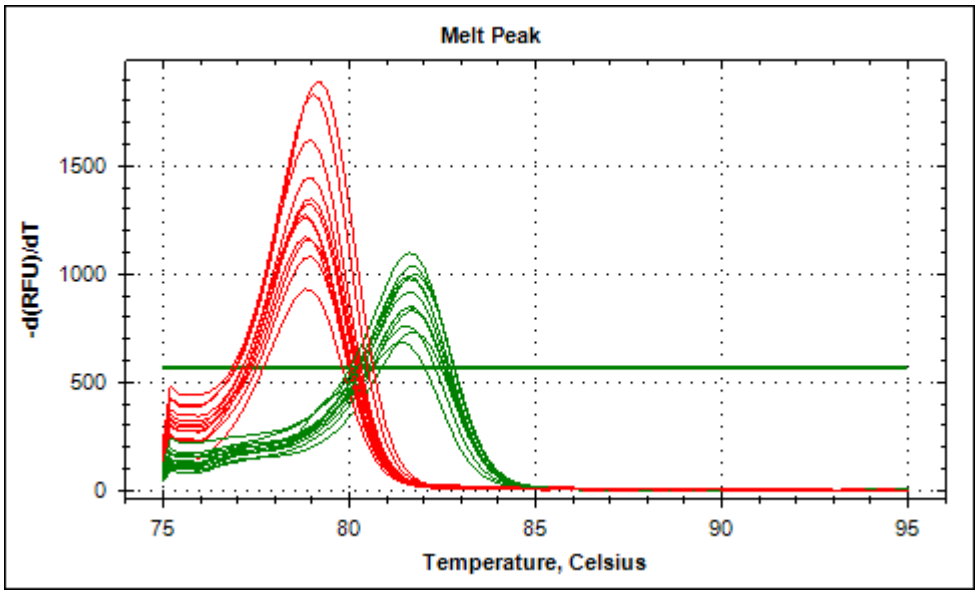

Figure I: Melt peak of atrazine sample (red) and β-actin (green).

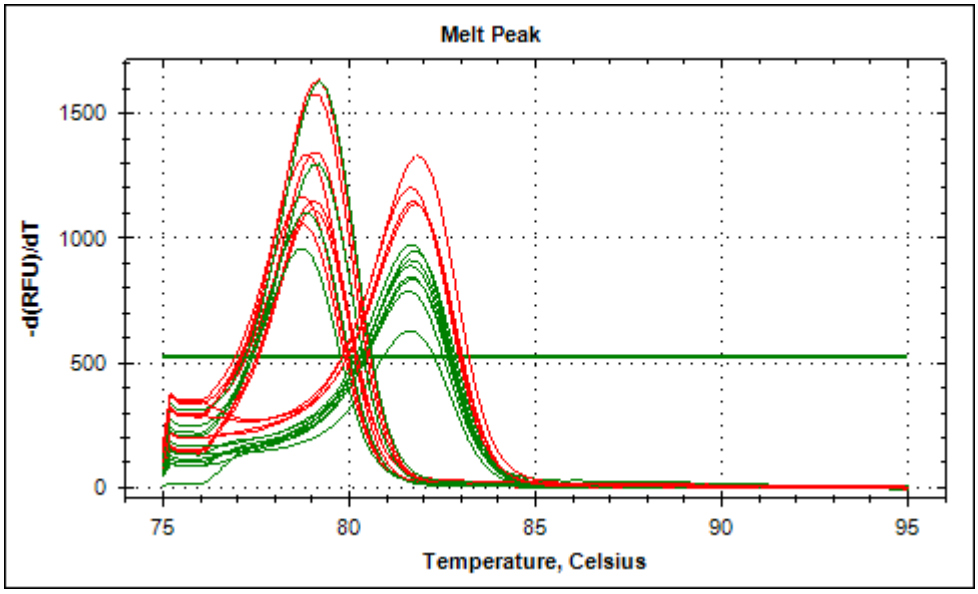

Figure II: Melt peak of endosulfan sample (red) and β-actin (green).

APPENDIX E

Standard curve from serial dilutions to determine amplification efficiency.

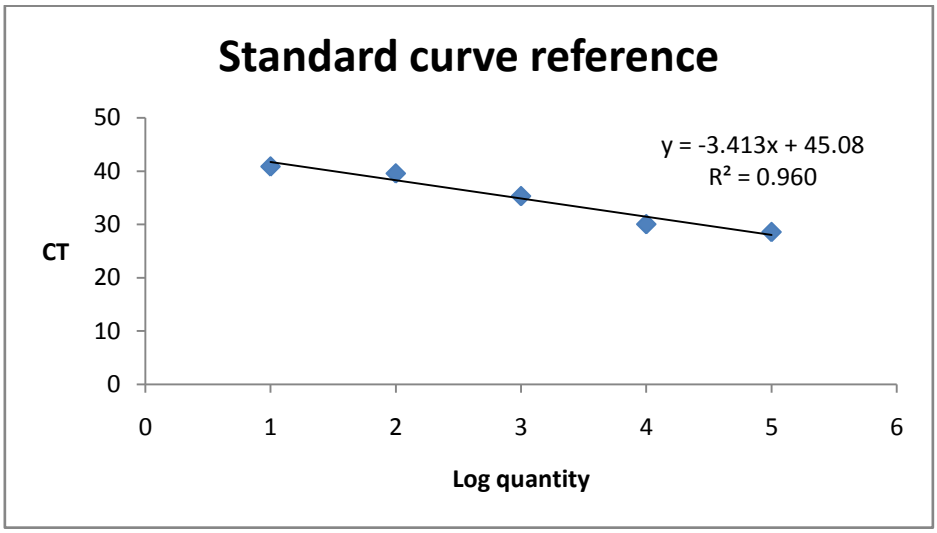

Figure I: Standard curve of β-actin (reference gene).

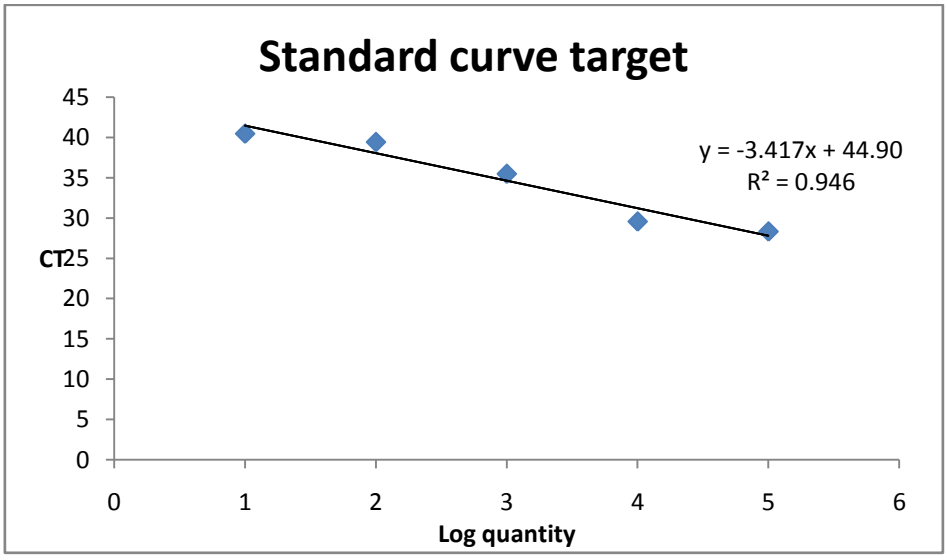

Figure II: Standard curve of samples.

APPENDIX F

Statistical analysis for vitellogenin gene expression.

Oneway

ANOVA

Relative gene expression

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	165.864	6	27.644	96.506	.000
Within Groups	4.010	14	.286		
Total	169.875	20			

Post Hoc Tests

Multiple Comparisons

Dependent Variable: Relative gene expression
Tukey HSD

(I) Concentratons	(J) Concentratons	Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
CONTROL	1.5 microgram/L	-.54667	.43700	.863	-2.0388	.9455
	3.6 microgram/L	-1.70667(*)	.43700	.021	-3.1988	-.2145
	7 microgram/L	-8.64333(*)	.43700	.000	-10.1355	-7.1512
	atz 0.5	-1.50667(*)	.43700	.047	-2.9988	-.0145
	atz 2.5	-2.65000(*)	.43700	.000	-4.1422	-1.1578
	atz 4.75	-5.12000(*)	.43700	.000	-6.6122	-3.6278
1.5 microgram/L	CONTROL	.54667	.43700	.863	-.9455	2.0388
	3.6 microgram/L	-1.16000	.43700	.182	-2.6522	.3322
	7 microgram/L	-8.09667(*)	.43700	.000	-9.5888	-6.6045
	atz 0.5	-.96000	.43700	.355	-2.4522	.5322
	atz 2.5	-2.10333(*)	.43700	.004	-3.5955	-.6112
	atz 4.75	-4.57333(*)	.43700	.000	-6.0655	-3.0812
3.6 microgram/L	CONTROL	1.70667(*)	.43700	.021	.2145	3.1988
	1.5 microgram/L	1.16000	.43700	.182	-.3322	2.6522
	7 microgram/L	-6.93667(*)	.43700	.000	-8.4288	-5.4445
	atz 0.5	.20000	.43700	.999	-1.2922	1.6922
	atz 2.5	-.94333	.43700	.373	-2.4355	.5488
	atz 4.75	-3.41333(*)	.43700	.000	-4.9055	-1.9212

Table multiple comparisons, continued.

7 microgram/L	CONTROL	8.64333(*)	.43700	.000	7.1512	10.1355
	1.5 microgram/L	8.09667(*)	.43700	.000	6.6045	9.5888
	3.6 microgram/L	6.93667(*)	.43700	.000	5.4445	8.4288
	atz 0.5	7.13667(*)	.43700	.000	5.6445	8.6288
	atz 2.5	5.99333(*)	.43700	.000	4.5012	7.4855
	atz 4.75	3.52333(*)	.43700	.000	2.0312	5.0155
atz 0.5	CONTROL	1.50667(*)	.43700	.047	.0145	2.9988
	1.5 microgram/L	.96000	.43700	.355	-.5322	2.4522
	3.6 microgram/L	-.20000	.43700	.999	-1.6922	1.2922
	7 microgram/L	-7.13667(*)	.43700	.000	-8.6288	-5.6445
	atz 2.5	-1.14333	.43700	.193	-2.6355	.3488
	atz 4.75	-3.61333(*)	.43700	.000	-5.1055	-2.1212
atz 2.5	CONTROL	2.65000(*)	.43700	.000	1.1578	4.1422
	1.5 microgram/L	2.10333(*)	.43700	.004	.6112	3.5955
	3.6 microgram/L	.94333	.43700	.373	-.5488	2.4355
	7 microgram/L	-5.99333(*)	.43700	.000	-7.4855	-4.5012
	atz 0.5	1.14333	.43700	.193	-.3488	2.6355
	atz 4.75	-2.47000(*)	.43700	.001	-3.9622	-.9778
atz 4.75	CONTROL	5.12000(*)	.43700	.000	3.6278	6.6122
	1.5 microgram/L	4.57333(*)	.43700	.000	3.0812	6.0655
	3.6 microgram/L	3.41333(*)	.43700	.000	1.9212	4.9055
	7 microgram/L	-3.52333(*)	.43700	.000	-5.0155	-2.0312
	atz 0.5	3.61333(*)	.43700	.000	2.1212	5.1055
	atz 2.5	2.47000(*)	.43700	.001	.9778	3.9622

* The mean difference is significant at the .05 level.

Homogeneous Subsets

Relative gene expression

Tukey HSD

		Subset for alpha = .05				
Concentratons	N	1	2	3	4	5
CONTROL	3	.0000				
1.5 microgram/L	3	.5467	.5467			
atz 0.5	3		1.5067	1.5067		
3.6 microgram/L	3		1.7067	1.7067		
atz 2.5	3			2.6500		
atz 4.75	3				5.1200	
7 microgram/L	3					8.6433
Sig.		.863	.182	.193	1.000	1.000

Means for groups in homogeneous subsets are displayed.
a Uses Harmonic Mean Sample Size = 3.000.