GENOTOXIC AND HEMATOLOGICAL EFFECTS OF ENDOCRINE DISRUPTIVE CHEMICALS, BISPHENOL A AND NONYLPHENOL, ON NILE TILAPIA (*Oreochromis niloticus*)

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GENOTOXIC AND HEMATOLOGICAL EFFECTS OF ENDOCRINE DISRUPTIVE CHEMICALS, BISPHENOL A AND NONYLPHENOL, ON NILE TILAPIA (*Oreochromis niloticus*)

ABSTRACT

This study was performed to evaluate the genotoxicity and hematology effects of single and binary exposure of bisphenol A and nonylphenol towards *Oreochromis niloticus*. *O. niloticus* was singly exposed to bisphenol A at concentration of 2 mg/l and nonylphenol at concentration of 0.04 mg/l in 16 L glass tank. *O. niloticus* was also synergistically exposed to the both concentration of 2 mg/l of bisphenol A and 0.04 mg/l of nonylphenol. Four fishes were randomly used for four exposures after two weeks acclimatization. All of them were exposed to the compound for 96 hours without water change prior observation. Fishes were anaesthetized and sacrificed for blood for the analysis of micronucleus, comet assay and hematology. The observation showed significant difference in the micronuclei frequency and nuclear abnormalities frequency in micronucleus test when they were singly exposed to 0.04 mg/l of nonylphenol. The obvious strand breakage of supercoiled duplex DNA had been determined in the comet assay analysis in single exposure of 0.04 mg/l of nonylphenol compared to others. Result from complete blood count also showed nonylphenol exposure at concentration 0.04 mg/l caused significant difference in some blood parameters when compared to the control. Based on this study, it was proved that single exposure of nonylphenol contributed stronger effect to *O. niloticus* compared to single exposure of bisphenol A and binary exposure of both chemicals.

Keywords: Micronucleus test, Hematology, Bisphenol A, Nonylphenol, *Oreochromis niloticus*.

GENOTOKSIK DAN KESAN-KESAN HEMATOLOGI KIMIA PEMUSNAH ENDOKRIN, BISPHENOL A DAN NONYLPHENOL, KE ATAS TILAPIA NIL (*Oreochromis niloticus*)

ABSTRAK

Kajian ini telah dijalankan untuk mengkaji genotoksik dan kesan hematologi terhadap pendedahan tunggal dan pendedahan binari bisphenol A dan nonylphenol terhadap *Oreochromis niloticus. O. niloticus* didedahkan secara tunggal kepada bisphenol A pada kepekatan 2 mg/l dan nonylphenol pada kepekatan 0.04 mg/l di dalam akuarium kaca 16 L. *O. niloticus* juga telah didedahkan secara binari bagi kedua-dua bahan kimia dengan kepekatan 2 mg/l bisphenol A dan 0.04 mg/l nonylphenol. Empat ekor ikan digunakan secara rawak bagi empat pendedahan berbeza selepas dua minggu penyesuaian. Kesemua ikan telah didedahkan kepada bahan kimia selama 96 jam tanpa tukaran air sebelum pemerhatian dijalankan. Ikan tersebut dipengsankan dan dikorbankan untuk darahnya bagi analisis mikronukleus, komet dan hematologi. Pemerhatian menunjukkan perbezaan signifikan terhadap frekuensi mikronuklei dan ketidaknormalan nuklear di dalam ujian mikronukleus apabila didedahkan secara tunggal terhadap 0.04 mg/l nonylphenol. Pecahan dwi-unsur DNA dapat dikenalpasti dengan jelas dalam analisis asset komet bagi pendedahan tunggal 0.04 mg/l nonylphenol berbanding yang lain. Keputusan dari pengiraan darah sempurna juga menunjukkan pendedahan tunggal nonylphenol pada kepekatan 0.04 mg/l menyebabkan perbezaan signifikan terhadap beberapa parameter darah apabila dibandingkan dengan kawalan. Ini membuktikan bahawa pendedahan tunggal nonylphenol memberikan kesan lebih kuat terhadap *O. niloticus* berbanding pendedahan tunggal bisphenol A dan pendedahan binari bagi bahan kimia tersebut.

Kata kunci: Ujian mikronukleus, Hematologi, Bisphenol A, Nonylphenol, *Oreochromis niloticus*.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF SYMBOLS AND ABBREVIATIONS

mg/ml	:	Milligram per millimeter
µg/kg dw	:	Mean concentration in sediment
g/kg dw	:	Mean concentration in sediment
mg/l	:	Milligram per liter
µg/l	:	Microgram per liter
N	:	Total number
V	:	Voltan
BPA	:	Bisphenol A
C.gariepinus	:	*Clarias gariepinus*
CBC	:	Complete Blood Count
Comp.	:	Component
DNA	:	Deoxyribonucleic Acid
EDCs	:	Endocrine Disruptor Chemicals
EDTA	:	Ethylenediaminetetraacetic Acid
EE2	:	17α-ethynyl estradiol
E2	:	17β-estradiol
GSI	:	Gonadosomatic Index
HCT	:	Hematocrit
HGB	:	Hemoglobin
LMP	:	Low Melting Point
Max	:	Maximum
MCH	:	Mean Corpuscular Hemoglobin
MCV	:	Mean Corpuscular Volume
MCHC	:	Mean Corpuscular Hemoglobin Concentration

Min	:	Minimum
NA	:	Nuclear Abnormalities
NPE	:	Nonylphenol ethoxylates
O. niloticus	:	*Oreochromis niloticus*
PBS	:	Phosphate Buffered Saline
RBC	:	Red Blood Cell
RNA	:	Ribonucleic Acid
SD	:	Standard Deviation
TDD	:	Total DNA Damage
WBC	:	White Blood Cell

LIST OF APPENDICES

CHAPTER 1: INTRODUCTION

1.1 Introduction

The antagonistic effects of xenobiotics compounds of endocrine disrupting chemicals (EDCs) namely nonylphenol (NP) and bisphenol A (BPA) to the aquatic environments have increased the global awareness (Vethaak et al., 2005; Auriol et al., 2006). Aquatic wildlife shows to be particularly risky by EDCs since rivers are submerged by types of EDCs comprising industrial by-product chemicals like NP and BPA also some pesticides number with distrustful activity of endocrine (Ternes et al., 2002; Nakada et al., 2004; Rutishauser et al., 2004). In fact, EDCs are able to mimic steroid features *in vivo* and induce desolate consequences on changes of hormonal and reproduction both in wildlife species and mankind mainly for males (Arukwe & Goksoyr, 2003; Brukcer-Davis et al., 2001). Many types of male fish species that was exposed to EDC's such as BPA, *p*-NP and *octyl*-NP have indicated increasing level of vitellogenin, gonadosomatic index reduction and intersex gonads formation under laboratory conditions or through contaminated watercourses (Wester et al., 2004; Mills & Chichester, 2005; Yamaguchi et al., 2005).

As purpose of industrial, commercial, household and agricultural benefits, raw materials or additives in manufacturing products are mainly produced from BPA and NP (Soares et al., 2008; Vandenberg et al., 2009). Due to extensive use of products consists of BPA and NP, different environmental compartments have been contaminated with large amounts of BPA and NP surplus (Staples et al., 1998; Vethaak et al., 2005; Zhang et al., 2011; Lee et al., 2013). Theoretically, range of acceptable amounts observed in effluent is between 0.15 µg/L to 37.3 µg/L for NP (Céspedes et al., 2005) and 0.01 µg/L to 44.7 µg/L for BPA (Lee et al., 2013). In aquatic animals, the multiple biological processes such as morphological, growth and sexual differentiation have been recorded

being affected from the use of BPA (Lam et al., 2011; Pastva et al., 2001). Apart from disruption of reproductive and endocrine system, BPA can also interfere the teleost's immune system (Milla et al., 2011). Concentrations of NP in soils, sediment and water could be reduced by action of microorganisms under aerobic or anaerobic conditions through NP biodegradation process (Mao et al., 2012). Wide range of organisms including fishes (Naderi et al., 2014; Shirdel & Kalbassi, 2014), mammals (Gan et al., 2015), crustaceans (Park & Choi, 2009) and algae (Zaytseva et al., 2015) are pointed out to be both acutely and chronically toxic to NP. Elevation of concentration of hepatic and plasma vitellogenin, significant increasing response to stimulus of aromatase, receptors of oestrogen and expression of vitellogenin gene could also led from NP exposure (Wu et al., 2014).

The genotoxicity of BPA and NP can be evaluated by using micronucleus test, comet assay and hematology analysis. All of these tests are crucial to indicate the toxicity of BPA and NP towards *O. niloticus*. The purpose of micronucleus test is to detect the chromosomal destruction, comet assay which perform under alkaline solution to measure DNA single and double strand breakage and hematology analysis is to determine changes of fish physiology regarding to different stress conditions such as pollutant exposure, hypoxia, metals and disease (Blaxhall, 1972: Dutie & Tort, 1985) based on haematological parameters that will be determined in this study as fish health indicators (Hesser, 1960).

Fish is important protein source for human body. However, plankton abundance and water quality in fish ponds are decreased due to pesticides sub-lethal toxicity (Sweilum, 2006). With respect to this matter, a lot of studies have been done using fish as models to study aquatic toxicology because of their responsiveness to toxicity by chemical contaminants which are similar to higher vertebrate (Ventura et al., 2008). Fish from

contaminated effluent could be used as important and sensitive indicator to determine contamination in the aquatic microbiota (Aravindakshan et al., 2004; Berntssen et al., 2010). Fish which act as pollutant effects bio-indicator are able to determine a very sensitive change in their ambience and play effective roles to evaluate the presence of potential risk associated with hazardous of new chemicals (Lakra & Nagpure, 2009).

1.2 Objectives of research

The main target of this research is to determine genotoxic and hematological effect of two endocrine disruptive chemicals namely bisphenol A and nonylphenol to the tilapia (*O. niloticus*) species. To achieve this target, several measurable objectives of the study are stated as below:

i. To determine the genotoxic effects of Nile tilapia (*O. niloticus*) on exposure to single and binary mixtures of BPA and NP using micronucleus test and comet assay.

ii. To determine the hematological effect of Nile tilapia (*O. niloticus*) on exposure to single and binary mixtures of BPA and NP using complete blood count.

CHAPTER 2: LITERATURE REVIEW

2.1 Genotoxic effects on aquatic system

A definite amount of the chemicals absorbing in aquatic environments potentially can induce DNA damage or disturb division cell processes (Depledge, 1998; Livingstone et al., 2000). A society health issue and aquatic ecosystem threat are unacceptable consequences urge to be accepted due to escalating number of industrial, agricultural and commercial chemicals disposal activity into the habitat of aquatic species (Mayon et al., 2006). Developing countries take serious concern on sustainable management and aquatic resources and fisheries in the matter of environmental pollution issue associated with human, wildlife and biota health (Robles-Molina et al., 2014). The aquatic ecosystem is the last point of industrial chemicals, pharmaceuticals and personal care products, pesticides and surfactants residues accumulated that finally cause to contamination of these ecosystems (Kolpin et al., 2013). Because of this factor, there are possibilities for production of complex biological effects on aquatic organisms including hormonal, physiology and reproductive effects due to environmental chemicals or their mixtures threats (Brian et al., 2005; Beresford et al., 2011). Nevertheless, the knowledge on the complex chemical mixtures that may influence on biological systems still not well understood or quantified mechanistically (Balaguer et al., 1996; Carvalho et al., 2014).

Thus, this raises concern to assess genotoxicity of polluted waters (Kligerman, 1982). The relative questions about the possible consequences of the exposure regarding to health status of current and future population of aquatic species being increases as various type of genotoxic chemicals are exposed to these aquatic species (Kligerman, 1982: Da Silva Souza & Fontanetti, 2006). In fact, there are evidences prove few number of pesticides are harmful to the aquatic ecosystem that make accidental spillage, untreated effluents discharge and spray drift as the major route of entrance into freshwater

ecosystem (Varó et al., 2008; Gaworecki et al., 2009). The adverse effect of genotoxic chemicals is they can induce male fish feminization and abnormality of male genitalia of alligators species in lakes and rivers (Howell et al., 1980; Anderson et al., 1988; Guillette et al., 1994; Prudom et al.,1994; Harries et al., 1996, 1997) aside from lots of studies reported the EDCs effects on reproduction in several invertebrate and vertebrate species (Degen & Bolt, 2000; Witorsch, 2002).

Due to the problems stated, recent researches in various animal models have been established to perform *in vivo* and *in vitro* assays in the study of genotoxic activity of different endocrine active chemicals (Tayama et al., 2008; Petridis et al., 2009; Quinn-Hosey et al., 2012). However, notable differences have been reported which lead to opposite conclusions even the use of same compound of NP and BPA is performed as differences in the screening test, use of organisms and the condition of exposure assayed might be their main factors (Grisolia et al., 2004; Lee & Choi, 2006; Park & Choi, 2007; Rivero et al., 2008; Tiwari et al., 2012). More researches shall be performed to assess the genotoxic possibility and DNA damage effects in different species apart from establish the potential correlation with other physiological effects. This is due to the issue of the fragmentary, limitation genotoxicity data for EDCs and restriction on only to a few species (Martínez-Paz et al., 2013).

2.2 Hematological effects of freshwater fish species

Hematological parameters are commonly used as fish and target organs of toxicity biomarker to indicate the level of xenobiotics exposure such as pesticides and metals (Singh & Srivastava, 2010; Ramesh et al., 2014b). Besides that, hematological parameters can also indicate water balance, nutritional status and overall fish health condition (Chang & Hur, 1999; Denson et al., 2003). Due to this reason, hematological parameters have been used in several species of fish to detect their health status. These parameters are able to determine the changes of physiology correspondingly to stress condition for instance exposure to pollutants, hypoxia, transportation, anaesthetic and adaptation (Akinrotimi et al., 2009). Interestingly, hematological parameters have been stated as decisive method (Katalay & Pariak, 2004; Akinrotini et al., 2012) to present the nutritional status and overall fish health condition (Akinrotini et al., 2012). Indeed, these useful parameters can be a good biomarker to monitor the toxicity of aquatic ecosystems apart from evaluating the environmental health because of its accuracy to indicate the pollutants (Fazio et al., 2012, 2013a,b,c).

2.3 Endocrine Disrupting Chemicals

EDCs capable to cause negative effects on reproduction of several invertebrate and vertebrate species in aquatic environment (Degen & Bolt, 2000; Witorsch, 2002). Several exogenous compounds namely phytoestrogens (Pelissero et al., 1991), synthetic oestrogens such as 17α-ethynyl oestradiol (EE2) that often used in birth control pills (Fent et al., 2006) and several other synthetic substances such as nonylphenol, insecticides, phthalates and hydroxylated-metabolites of polychlorinated biphenyls (Braathen et al., 2009) have been determined can create estrogenic effects in fish. Since rivers are submerged with various EDCs including industrial chemicals such as BPA, several pesticides with suspected endocrine activity or oestrogens excreted by humans for instance natural oestrogen, 17β-oestradiol (E2) or the synthetic oestrogen of the

contraceptive pill, 17α-ethynyl oestradiol (EE2), the chance for aquatic wildlife to survive seems to be at risk (Ternes et al., 2002; Nakada et al., 2004; Rutishauser et al., 2004).

In recent years, waste water treatment plants drainage and surface water contain numbers of degraded chemicals including pharmaceuticals and endocrine disrupting compounds (Jelic et. al., 2011; Joss et. al., 2005; Petrovic et. al., 2002b). EDCs are chemical compounds that belong to different chemical groups and separated by their properties to interrupt the exposed organisms' endocrine system through the action of mimicking or counteracting natural hormones and eventually lead to severe impacts even at very low concentrations (Céspedes et. al., 2005; Pascoe et. al., 2003). Elevating detail annotation indicate that few groups of environmental pollutants may produce estrogenic effects in comparable ways as natural hormones (Adeogun et. al., 2016; Bahamonde et. al., 2015; Wielogorska et. al., 2015). The main objective of systematic investigation is to study the properties of environmental contaminants, EDCs which able to mimic hormone's exposed organisms apart from determine the capability of these environmental contaminants to alter the reproduction and development of organisms involved (Adeogun et. al., 2016; Arukwe et. al., 1997; Bahamonde et. al., 2015; Wielogorska et. al., 2015). Because of the established knowledge about the production of significant number of these chemicals for specific purposes such as in agriculture, domestic and also industry as by-products or even degradation products of other compounds, these contaminants literally show a significant health threat of the society concerns. (Ahel et. al., 1993; Kvestak & Ahel, 1994). The aquatic wildlife will be in danger because of high admission of environmental pollutants into the aquatic ecosystem (Nohynek et al., 2013; Sheikh et al., 2016).

2.4 BPA

BPA is a well-known EDC and has hormone-mimicking chemical properties. BPA is made of polycarbonate plastic building block and widely utilized in daily life such as in reusable water bottles, polycarbonate baby bottles, inner coatings of metallic food can and polyvinyl chloride stretch films (Vandenberg et al., 2009). BPA is commonly found in aquatic system at concentration of μg/L to mg/L or mg/kg all over the world (Huang et al., 2012). A series of investigations have well-documented the adverse effects of BPA to the aquatic animals in spite of BPA has a relatively short half-life in aquatic environment with low bio-accumulation to aquatic organisms (Kang et al., 2007; Rogers et al., 2013). Classification of BPA is determined from slightly toxic to moderately toxic based on its toxicity effect to the adult fish. Consequently, the growth, sexual differentiation, morphology, behaviour and histological structure will significantly affected either to embryo of fish, fry or larvae in their early stages of life (Pastva et al., 2001; Lam et al., 2011; Kinch et al., 2015). Many studies performed related to BPA is about its physiological endocrine disrupting effects to the organisms. BPA could similar to oestrogen agonist that can create potential danger action toward reproduction system of fish for instance sex determination alteration, gonadal function alteration and stimulating a production of hepatic vitellogenin (Crain et al., 2007). Despite, receptor gene transcription assay recorded the estrogenic effects of BPA are still poor and existing only about 15,000-fold less active in its affinity compared to the estrogenic receptor α of an endogenous oestradiol (E2) based on steroid hormone made up of yeast-based (Gaido et al., 1997).

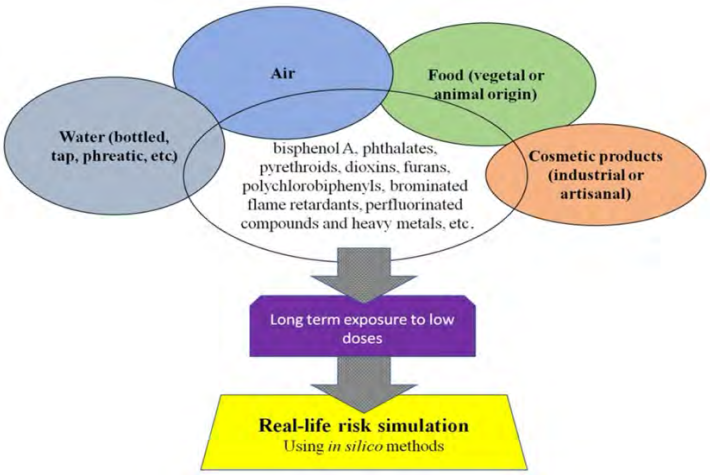

Figure 2.1: Low dose chronic exposure to chemical mixtures. An important issue in contemporary studies (Margina et. al. 2019).

Low dose effects of BPA have been looking forward as main objective for further studies due to performance of many studies and non-monotonic dose response curve of BPA with both a low and a high dose of BPA yield unclear low dose effects although high dose exposure is applied (Vandenberg, 2014). However, according to the result from genotoxicity and immune-toxicity studies performed by Rezg et. al. (2014) and Yang et. al. (2015), low dose of BPA exposure still can cause effect to the chromosomal disruption by elevating micronuclei frequency in fish erythrocytes and change the macrophage and lymphocyte functions such as improved bactericidal activity which is differed from the use of higher dose of BPA that react like inhibitory effects. Besides that, alteration of oxidative stress level and antioxidant parameters are other adverse effects of BPA exposure in fish embryos, larvae, adult fish and rat models (Wu et al., 2011; Xu et al., 2013). Some papers recorded BPA availability in aquatic ecosystem including surface water, rivers and estuaries (Klecka et al., 2009; De Kermoysan et al., 2013) and in fish sample (Liu et al., 2011; Mie`ge et al., 2012) as well although BPA half-life is only ½ to 6 days. As for instance, BPA has been successfully found in some rivers and lakes such as in Mondego River, Portugal, BPA is detected up to 880 ng/L (Ribeiro et al., 2009), Lake Superior, BPA is detected as much as 3.2 mg/mL (Environmental Health Division,

2014), River Elbe in Germany, BPA is detected as much as 0.105±0.204 μg/L (Heemken et al., 2001) and in Dutch surface waters, BPA is detected up to 0.33 μg/L (Belfroid et al., 2002; De Kermoysan et al., 2013). Furthermore, BPA level has been also detected in landfill leachate and pulp mill effluents as much as up to 17 mg/L (Flint et al., 2012), in river as much as 43 g/kg dw (Flint et al., 2012), in marine sediments as much as up to 191 g/kg dw (Koh et al., 2006) and in soils as much as 100 μg/kg dw (Canesi & Fabbri, 2015).

Literally, determination of the environmentally relevant concentration of BPA is complicated due to the different concentrations of BPA in many environmental matrixes (Canesi & Fabbri, 2015). Followed to BPA characteristics, BPA is biodegradable on surface water and also easily being degraded in sediments by the action of microorganisms (Klecka et al., 2001; West et al., 2001). However, aquatic organisms may be affected from the careless discharge of BPA from the various industrial processes (Mihaich et al., 2009). Even worse, fish are the most susceptible species to EDCs compare to other aquatic organisms (Eggen et al., 2003; Qin et al., 2013). Therefore, the use of ecosystem biomarkers are extremely necessary to monitor the quality of environment and the organisms' health in aquatic ecosystem. Biomarkers such as hematological and biochemical parameters are the most well-known biomarkers which often utilized in many studies (Ramesh et al., 2014a,b).

2.5 NP

NP, a type of xenobiotic compound is widely use in the production of nonylphenol ethoxylates surfactants and manufacturing antioxidants and lubricating oil additives (USEPA, 1990). In fact, NP in environment is initiated from the effluents discharge of the sewage treatment works (Ahel et al., 1994; Fries & Puttmann, 2003; Langford et al., 2005; Petrovic et al., 2002a, b; Sabik et al., 2003). Studies performed by Soto et al. (1991) coincidently found NP capable to induce growth of breast tumour cell. NP has mimicking

properties to the natural hormone of 17β-oestradiol by competing for the site of receptor binding and the natural oestrogen (Lee & Lee, 1996; White et al., 1994). Induction of photolysis by sunlight cause the concentration of NP be lowered down on the surface layer of natural waters (Ahel et al., 1994) and its half-life is more than 60 years in sediments (Shang et al., 1999). Although microorganisms can take over to degrade the NP, but there are limitations in oxygen supply (Hesselsoe et al., 2001; Topp & Starratt, 2000) and bioavailability (Bosma et al., 1997; Kelsey et al., 1997) in to do so. Interestingly, the contaminated areas of NP consisted of organisms that could degrade NP easily as they are indigenous microflora that have been acclimatized for past many years (Ahmed et al., 2001; Saagua et al., 2002; Soares et al., 2003).

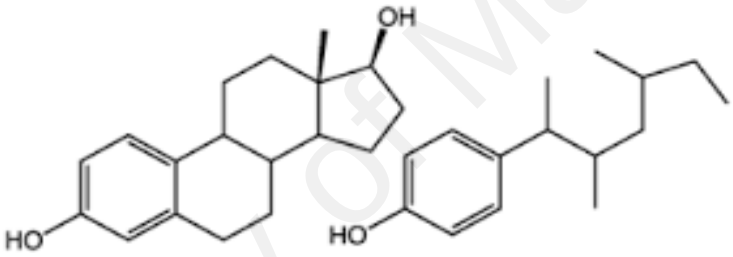

Figure 2.2: 17β-estradiol and 4-NP showing how 4-NP structure mimic the structure of estradiol hormone.

NP is mainly use in production of industrial and domestic cleaning agents, pesticides, paints, plastics and emulsifiers. It is originated from a microbial breakdown product of a non-ionic surfactant that is nonylphenol ethoxylate (NPE) (Vincent & Sneddon, 2009). Because of these products are extensively use, it causes NPE leftover is released into the aquatic environment via municipal, agricultural and industrial wastewaters and surface runoff (Mao et al., 2012). In order to reduce the NP concentration in water, sediment and soils, aerobic or anaerobic microorganisms are needed to degrade the NP (Mao et al., 2012). For long-chain alkylphenols-degrading purpose, the alkylphenol-degrading bacteria could be used as these bacteria will perform ortho-cleavage pathway and

multicomponent phenol hydroxylase to complete the degradation process (Nguyen et al., 2011).

Table 2.1: Physicochemical properties of 4-nonylphenol (Servos, 2008).

Molecular Formula	**$C_{15}H_{24}O$**
Molecular weight	220.34 g/mole
Appearance	Transparent or light-straw coloured, high viscosity liquid, slight phenolic order
Specific gravity	0.95 (20ºC)
Melting point	Approx. -8ºC
Vapour pressure	0.3Pa or lower (25ºC)
Aquatic solubility	6 mg/L (20ºC)
Log Kow	4.48

Recently, chemical pollutants of different sources continuously contaminate the aquatic ecosystems and NPE is one of the most dangerous pollutants which break up to simple single toxic product namely as 4-NP (Mekkawy et al., 2011; Rivero et al., 2008). Some studies related to hematotoxic, biochemical, disruption of hormone, histopathological, embryotoxic, and genotoxic effects of 4-NP on *C. gariepinus* are performed (Mekkawy et al., 2011; Sayed et al., 2011, 2012a,b, 2013) and the result of the studies indicate that fish which contaminated with 4-NP is the most expected cause of these consequences (Soares et al., 2008).The lack of high efficiency sewage treatments plants in developing countries such as Iran cause large amounts of untreated sewage are discharged into water resources (Tajrishy & Abrishamchi, 2005). According to Mortazavi et al. (2012, 2013), Anzali wetland consist of fish muscle, liver and sediments that contaminated with high value of 4-NP as much as up to 8.17, 10.43 and 29 μg/g dry

weight respectively. Therefore, the aquatic animals which living in southern part of the Caspian Sea especially the species of Caspian brown trout (*Salmo trutta caspius*) are totally in danger because the large amount of NP could threaten the key hormonal balance, development of reproduction system and histological structure of the aquatic wildlife. Nevertheless, just few studies are performed to evaluate nonylphenol effects on thyroid and growth hormone, homeostasis and non-gonadic histopathology in fishes (Robertson & McCormick, 2012; Naderi et al., 2014).

2.6 Nile tilapia (*O. niloticus*)

The special ability of fish to indicate the level of potential disruption effects of new chemical substances or even the possibility of environmental pollution make fish as the best experimental model to study on aquatic toxicology. Besides that, fish can also be classified as good test organisms in monitoring water quality activity especially related to small aquarium species as they can be kept in the laboratory apart from easily to be exposed to toxic substances that potentially harmful to the human health (Harshbarger & Clark, 1990; Al-Sabti & Metclafe, 1995). Furthermore, other characteristics present on fish to detect the effects of chemical substances are their gills and epithelium (Evans, 1987; Biagini et al., 2009) that become the main targets of several aquatic pollutants to dissolve (Kikuchi et al., 1978). Due to these reasons, fish have been chosen as good biomarkers to monitor water quality (Fontanetti et al., 2012). In addition, tilapia is well-known as ‘‘aquatic chicken’’ in the 1980s and the ‘‘poor man’s fish’’ (Smith & Pullin, 1984) has established important roles in aquaculture worldwide. In 21st century, tilapia become the most important species in aquatic ecosystem (Fitzsimmons, 2000). As for instance, Nile tilapia species (*O. niloticus*) is the top species that commonly farm in Egypt. Because of their vulnerability towards toxic are very high, tilapia is often choose as reliable model to involve in many mycotoxicosis studies (Kenawy et al., 2009).

Tilapia is one of the most important and widely reared fish species all over the world. Nile tilapia (*O. niloticus*) is the famous species among other tilapia because of its can grow rapidly, easy to reproduce, delicious taste and can be obtained at low price (He et al., 2013). Tilapia has unique characteristic which can tolerate to adverse environmental conditions during cultivation. Besides that, tilapia is superior species because it can survive even the dissolved oxygen is very low, euryhaline and efficient food conversion (Asad et al., 2010). In fact, at least 85 countries farm the tilapia and this makes tilapia as the highest species of farmed finfish in the world and become the second position in volume behind to carps and salmonids. In recent years, production of tilapia is drastically increase from about 1 million metric tons in 1998 to about 2.4 million tons in 2006 and almost 2 million tons of the total metric tons are contributed by a single species that is Nile tilapia (FAO, 2006).

Figure 2.3: Nile tilapia (*Oreochromis niloticus*) used as a model of the study.

CHAPTER 3: METHODOLOGY

3.1 Sample preparation

Twenty fishes of *O. niloticus* were obtained from Razham Agro Trading, Beranang, Selangor. The fishes were reared in brick-made tanks completely supplied with aerated water from the nearby river in Razham Agro Trading. Fishes were enough supplied with oxygen gas in transparent plastic while transported back to University Malaya. Those twenty fishes were early acclimatized upon reached in Ladang Mini ISB, University Malaya for two weeks. After two weeks later, four out of twenty fishes that were acclimatized in Ladang Mini ISB were randomly taken and acclimatized again in 16 litres glass tank for three days before used for exposure treatments. Reverse osmosis water was used and the fishes were fed with artificial fish pellets during acclimatization. The waste product of the fishes was siphoned off every two days to reduce ammonia content in the water.

Figure 3.1: Acclimatization of fishes for two weeks upon reached in Ladang Mini ISB, University Malaya from Razham Agro Trading.

Figure 3.2: Acclimatization of fishes in 16 L glass tank before chemical administration.

3.2 Preparation of BPA and NP

BPA (purity: >95%) and NP (purity: 99%) were purchased from Sigma-Aldrich which existed in powder and solution form respectively. BPA and NP were weighed, prior diluted in acetone solution (purity: 99.5%) because of insolubilities characteristic in water. NP was prior diluted in 1 L (1.28×10^4 M) stock solution and BPA was directly mixed to the water for the administration.

3.3 Genotoxicity studies

Three different analysis were used to evaluate the genotoxicity of BPA, NP and their binary exposure towards *O. niloticus*. The analysis involved were micronucleus test, comet assay and Complete Blood Count (CBC) through hematological analysis.

3.3.1 BPA and NP exposure

Total four fishes were used for the experiment whereby one fish was only used for one type of exposure which carried out simultaneously in triplicate studies for single and binary exposure of BPA and NP. Fishes were randomly chosen and acclimatized for three days in separated aquarium which completely supplied with oxygen and dechlorinated

water without feed. Both BPA and NP treatments were left for 96 hours exposure without water change. The dose used for single exposure was 2 mg/l for BPA ($1/6^{th}$ of 96h LC_{50} value of BPA) and 0.04 mg/l for NP ($1/5^{th}$ of 96h LC_{50} value of NP). Meanwhile, the dose for binary exposure was taken by added 2 mg/l BPA with 0.04 mg/l of NP. The concentration of LC_{50} for both EDCs were prior determined in the preliminary studies.

3.3.2 Fishes sacrification

Upon fishes sacrification, all fishes were prior anaesthetized by exposed them in a special container that mixed with clove oil and left for 10 minutes to assure the movement of the fishes were unidentifiable. Then, the blood of the fishes were suctioned out from the area of the fish spinal section using 3 ml of Terumo syringe. They were all collected in 1 ml of purple vacutainer tubes for hematological analysis together with 1 ml tube containing RNA later for micronucleus and comet assay analysis. EDTA was being used during blood collection to prevent any blood clotting which reduce accuracy in observation of the result.

3.3.3 Micronucleus test

The micronucleus test procedures had been reported by Schmidt (1975) and some minor modifications were applied as followed to Grisolia & Cordeiro (2000). Blood samples from *O. niloticus* were collected by punctured on caudal vein of *O.niloticus* with a heparinized syringe and quickly smeared onto the sterile glass slides. The samples were air dried and fixed in absolute methanol for 15 minutes and left for overnight. The samples were finally stained with 5% of Giemsa solution for 15 minutes and analysed under light microscope. There were about 500 erythrocytes for each *O.niloticus* specimens were examined. Micronucleus on the slides were identified and scored microscopically under 100x objective. The criteria of the test scoring were counted individually according to its specific type such as binucleated nuclei, blebbed nuclei, lobed nuclei and notched nuclei.

Whilst for statistical analysis, the data collected from both micronuclei and nuclear abnormalities tests were analysed by SPSS software. Control of untreated any chemical exposure was constantly used as reference in this study. Data obtained were represented by nonparametric test through Kruskal-Wallis.

3.3.4 Comet assay

In order to measure the relationship between DNA damage and genotoxic pollutants exposure to the aquatic organisms in environment, comet assay procedure could be a powerful tool to do so (Andrade et al., 2004). By referring to Hartman et al., (2004), alkaline comet assay procedures were performed. A syringe was used to collect the samples of fish blood from the caudal vein. 75 µL of 0.5% low melting agarose (LMP) concentration was used as a media to mix with a 10 µL aliquot of blood from each diluted sample. For the purpose of agarose solidification, the suspension was spread thoroughly on the slides that was prior coated with 1.0% of normal agarose. This was prepared in phosphate-buffered saline (PBS) and covered with a cover slip. Then, the second layer of LMP agarose with concentration of 0.7% was added and left dried. The cover slip was removed and the slides were immersed in the lysis solution for 20 minutes once the agarose was fully solidified. Afterwards, ice-cold PBS was used to wash the slides to clean such detergents and salt residue. Then, the slides were quickly left in electrophoresis buffer for 20 minutes with 0.8 V to unwind the DNA. Once the electrophoresis finished, the slides were neutralized in a neutralizing buffer for 10 minutes and fixed in the ethanol solution for 10 minutes later. As for staining process, the slides were left dried and stained with 50 µL of ethidium bromide and finally analysed under fluorescence microscopy for observation purpose.

Table 3.1: Cell analysed against comet assay score under alkaline solution.

Comet scoring	Cell analysed
0	Healthy
1	Mild DNA destruction
2	Medium DNA destruction
3	High DNA destruction
4	Highest DNA destruction

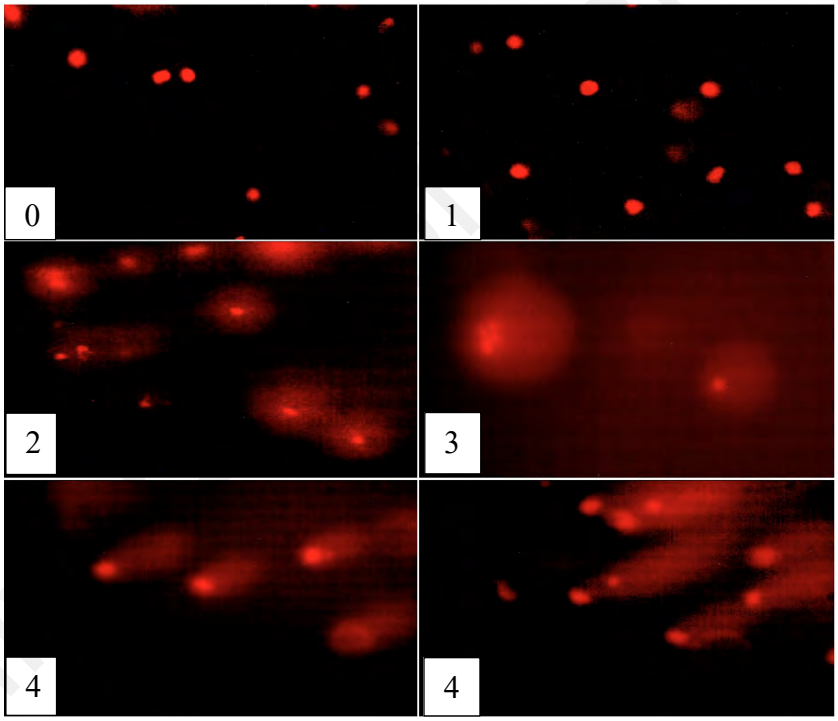

Figure 3.3: Example of destruction DNA level in comet assay from healthy to the highest destruction cells (Ambreen and Javed, 2018).

3.3.4.1 Comet scoring

One fish was only used for each treatment and three slides were used for each treatment for triplicate studies. These slides were analysed and 50 cells were counted based on

breakage of DNA from the nucleus. Each comet was scored as 0, 1, 2, 3 or 4 (from normal to highest destruction) regarding to its destructive level.

3.3.5 Hematology assay

The concept of CBC was blood samples were collected by venipuncture of the caudal vein, which lied just ventral to the spinal cord, either by ventral or lateral approach. The blood was then immediately transferred to a tube coated with heparin. Utilization of heparin tubes allowed separation of the plasma from blood cell without delay thus it would decrease the risk of blood clotting during scarification of fishes.

All four tubes from four fishes of different exposures respectively were filled at least 0.3 ml of blood for triplicate analysis. The results of the analysis were taken after three days submission. The results were analysed by using SPSS software.

CHAPTER 4: RESULTS

4.1. MN and NA detection using micronucleus test

4.1.1 MN evaluation

The existence of MN and NA determination in the blood of *O. niloticus* after 96 hours single exposure towards 2 mg/l for BPA ($1/6^{th}$ of 96h LC_{50} value of BPA), 0.04 mg/l for NP ($1/5^{th}$ of 96h LC_{50} value of NP) and binary exposure (mixture of 2 mg/l of BPA and 0.04 mg/l of NP) were tabulated in data analysis. Data was tabulated in mean±SD (Table 4.1) which represented triplicate samples for each exposure. Total of red blood cells in the Table 4.1 indicated the decisive constant amount of red blood cell present on the slides that should be computed for the data analysis. The frequencies (mean±SD) were obtained by calculating the average mean for triplicate slides per total of red blood cell multiply by hundred percent. MN evaluation showed that all groups of chemicals exposure had significantly increased when compared to the control.

Table 4.2 represented the descriptive analysis for concentration and exposure in mean±SD. Table 4.3 showed different exposure based on mean rank. Single exposure of NP recorded the highest mean rank which was 48.10 followed by single exposure of BPA which was 38.27 and binary exposure which was 27.63. Thus, it was proven that single exposure of NP give strongest effect to the *O. niloticus* compared to the others. The test revealed significance influence of type of exposure and concentration. The categorial data was further analysed for its possible influence on level of concentration using Kruskal-Wallis test. The test revealed insignificant influence type of exposure on the concentration, $H(3) = 4.3660$, $P = 0.00$. The P value is 0, thus it could be said the idea that the difference was due to random sampling was totally rejected otherwise it concluded that the populations have different distributions.

Different types of nucleus fragments such as micronuclei, binucleated cells, blebbed nuclei, lobed nuclei and notched nuclei were determined in the blood of the fish exposed to single exposure of BPA, NP and binary exposure of both chemicals after 96 hours exposure. The presence of MN and NA in blood cells were obviously monitored under 100x objective for the exposure treatment and 30x objective for the controlled treatment in the Figure 4.1. The drawn arrow in the pictures indicated the fragmented nuclei that occurred resulted from the effect of the exposure treatment.

4.2 DNA strand breakage detection through comet assay

Total cell with comet and total DNA damage (TDD) scores in the blood of *O. niloticus* of single and binary exposure of BPA and NP together with the control by comet assay were tabulated in Table 4.5 and Table 4.6 respectively. As shown in Table 4.5, total cell with comet of *O. niloticus* that exposed to single exposure of NP was the highest compared to the single exposure of BP and binary mixtures. The mean score indicated the degree of destruction in the analysis of the fishes blood showed single exposure of NP (155±1.2) was approximately tripled times of the mean score of single exposure of BPA (47.33±11.06) and almost 1/5 times of the mean score of the binary mixtures (124±16.6). In fact, single exposure of BPA however showed the lowest mean score of DNA destruction among the three exposures determined.

TDD score of the control groups was 90% blood without destructed DNA (Table 4.6). However, there was still 10% blood was destructed in the control group because of environmental factor which affect the condition of the blood itself which was unintentionally occurred. In general, most of blood fish was destructed in class 1 which was mild DNA destruction group when compared to the control groups (Figure 4.2). Table 4.6 was tabulated to show the percentage of different classes of DNA damage for different exposures found in blood of *O. niloticus* after 96 hours exposure. The analysis

of the comet assay was separated according to the classes of the destruction. In simple way, the higher the number of the class, the higher the destruction of the blood of *O. niloticus.* TDD for single exposure of NP was the highest followed by single exposure of BPA which was at the second highest.

Table 4.8 showed different exposure based on mean rank. Single exposure of NP recorded the highest mean rank which was 34.90 followed by single exposure of BPA which was just a bit lower than single NP which was 34.33 and binary exposure which was 32.27. Hence, the numbers stated was obviously prove that single exposure of NP give strongest effect to the *O. niloticus* compared to the single exposure of BPA and binary exposure. The categorial data was further analysed for its possible influence on level of concentration using Kruskal-Wallis test and indicated insignificant influence type of exposure on the concentration, $H(3) = 6.799$, $P = 0.079$. In fact the P value was larger than 0.5, thus it could be said the idea that the difference was due to random sampling was retained and the populations had same distributions.

The destruction of the DNA strand were visualized as in Figure 4.2. There were separated into four groups which represented level of destruction. Class 0 was control group that unexposed to any chemical, class 1 was mild DNA destruction which DNA strand only broken in small tail, class 2 was medium DNA destruction which DNA strand produced longer tail than class 1, class 3 was high DNA destruction which DNA shape almost totally broken and class 4 was the highest DNA destruction which DNA shape totally lost. All the shapes for each class were circled with white circle for the analysis.

4.3 Hematology analysis of *O. niloticus* blood

The hematological parameters of *O. niloticus* was tabulated as mean±SD (Table 4.10). Single exposure of NP indicated statistically increased in the level of red blood cell (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and platelet while drastically decreased in the level of mean corpuscular hemoglobin concentration (MCHC) and white blood cell (WBC) with respect to control. Table 4.12 was tabulated to show the exposure differences based on mean rank. Single exposure of NP recorded the highest mean rank which was 53.90 followed by single exposure of BPA which was 48.25 and binary exposure which was 43.38. Therefore, this observation suggest that single exposure of NP was undeniable give strongest effect to the *O. niloticus* compared to other both exposure.

The categorial data was further analysed for its possible influence on level of concentration using Kruskal-Wallis test and indicated insignificant influence type of exposure on the concentration, $H(3) = 1.715$, $P = 0.634$. Thus the P value was larger than 0.5 and the idea that the difference was due to random sampling was retained and the populations had same distributions. The current findings based on micronucleus test, comet assay and hematology indicated that the single exposure of NP was able to give huge drawback to the fishes especially for *O. niloticus* even only used in small amount.

4.4 Data analysis

4.4.1 MN and NA evaluation

Table 4.1: MN and NA frequencies of *O. niloticus* exposed to different exposures.

MN and NA	Total of red blood cell	Frequencies (mean±SD)			
		Control	BPA	NP	Binary
Micronuclei	1500	0.29±0.22	0.71±0.10	0.91±0.15	0.69±0.10
Binucleated cell	1500	0.18±0.04	1.29±0.17	1.60±0.13	1.00±0.13
Blebbed nuclei	1500	0.07±0	1.29±0.10	1.58±0.14	1.02±0.08
Lobed nuclei	1500	0.20±0.07	1.29±0.08	1.53±0.18	0.96±0.08
Notched nuclei	1500	0.18±0.04	1.13±0.18	1.51±0.10	1.16±0.04

Table 4.2: Descriptive statistic of MN and NA evaluation.

	N	Mean	S.D	Min	Max
Concentration	60	14.1000	7.69702	1.00	26.00
Exposure	60	2.5000	1.12747	1.00	4.00

Table 4.3: Kruskal-Wallis Test based on ranking exposure.

	Exposure	N	Mean Rank
Concentration	Control	15	8.00
	BPA	15	38.27
	NP	15	48.10
	Binary	15	27.63
	Total	60	

Table 4.4: Test Statistics on comet assay.

	Concentration
Chi-Square	43.660
df	3
Asymp. Sig.	.000

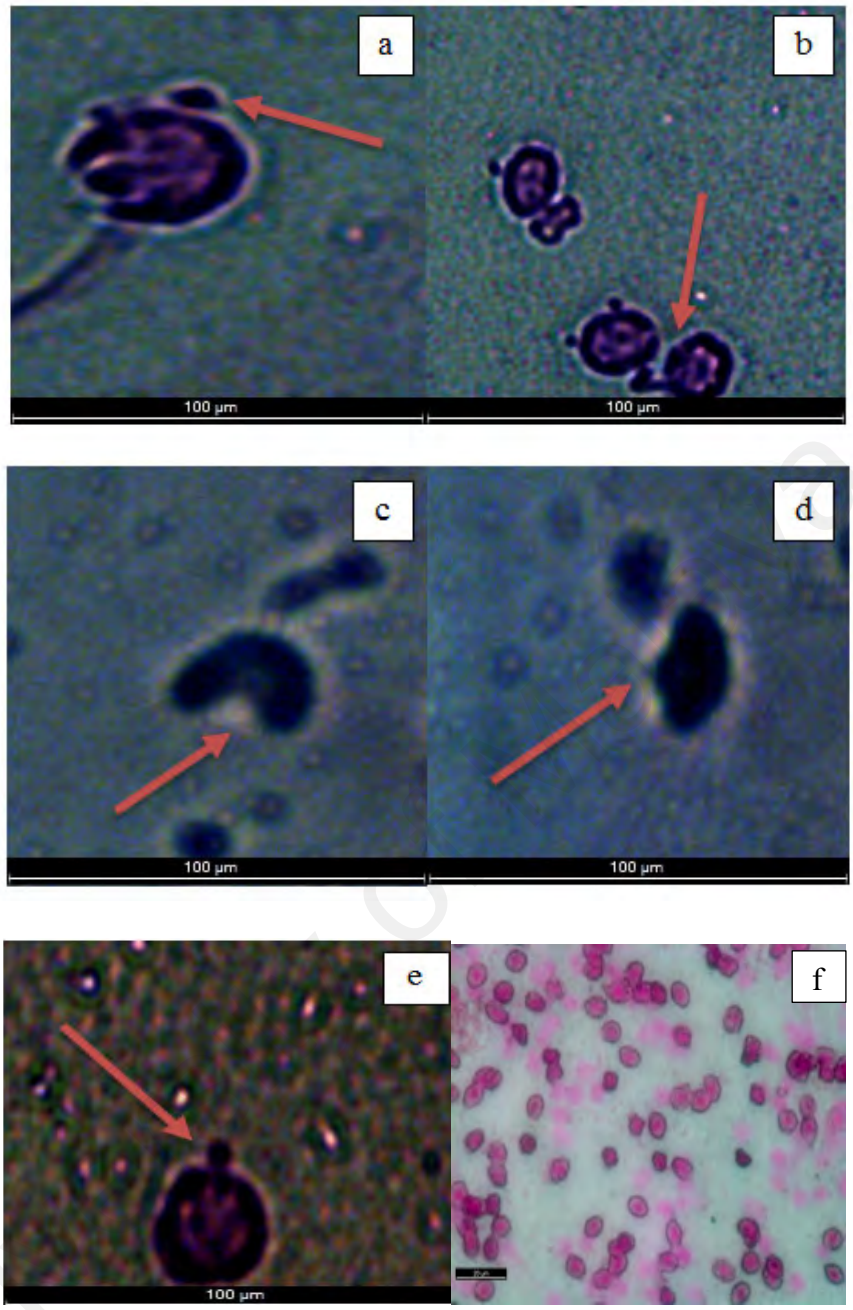

Figure 4.1: Presence of MN and NA in blood cells (a) micronuclei, (b) binucleated cells, (c) blebbed nuclei, (d) lobed nuclei, (e) notched nuclei and (f) control cells.

4.4.2 DNA strand breakage detection through comet assay

Table 4.5: DNA destruction analysis in *O. niloticus* blood exposed to BPA, NP and binary exposure.

Groups	Total cells analysed	Total cells with comet	Classes					Score
			0	1	2	3	4	
Control	50	5	45	5	0	0	0	5
	50	6	44	6	0	0	0	6
	50	4	46	4	0	0	0	4
Total	150	15	135±1.0	15±1.0	0	0	0	5±1.4
BPA	50	19	28	8	6	3	2	37
	50	21	26	8	5	4	4	46
	50	24	32	6	7	5	6	59
Total	150	64	86±3.1	22±1.2	18±1.0	12±1.0	12±2.0	47.33±11.1
NP	50	28	22	14	8	3	3	51
	50	29	21	13	11	4	1	51
	50	31	19	17	8	4	2	53
Total	150	88	62±1.5	44±2.1	27±1.7	11±0.7	6±1.0	155±1.2
Binary	50	20	35	7	8	4	6	59
	50	13	30	6	3	2	2	26
	50	19	33	8	5	3	3	39
Total	150	52	98±2.5	21±1.0	16±2.5	9±1.0	11±2.1	124±16.6

Table 4.6: Total DNA damage scores in blood of *O. niloticus* exposed to BPA, NP and binary exposure.

Exposure	Percentage of score (%) Classes					Total DNA Damage (TDD)	Total cell analysed
	0	1	2	3	4		
Control	90	10	0	0	0	10	150
BPA	57.33	14.67	12	8	8	42.67	150
NP	41.33	29.33	18	7.33	4	58.67	150
Binary	65.33	14	10.6	6	7.3	34.67	150

Table 4.7: Descriptive statistic of comet assay evaluation

	N	Mean	S.D	Min	Max
Concentration	60	10.0833	12.14362	.00	46.00
Exposure	60	2.5000	1.12747	1.00	4.00

Table 4.8: Kruskal-Wallis Test based on ranking exposure

	Exposure	N	Mean Rank
Concentration	Control	15	20.50
	BPA	15	34.33
	NP	15	34.90
	Binary	15	32.27
	Total	60	

Table 4.9: Test Statistics on comet assay

	Concentration
Chi-Square	6.799
df	3
Asymp. Sig.	.079

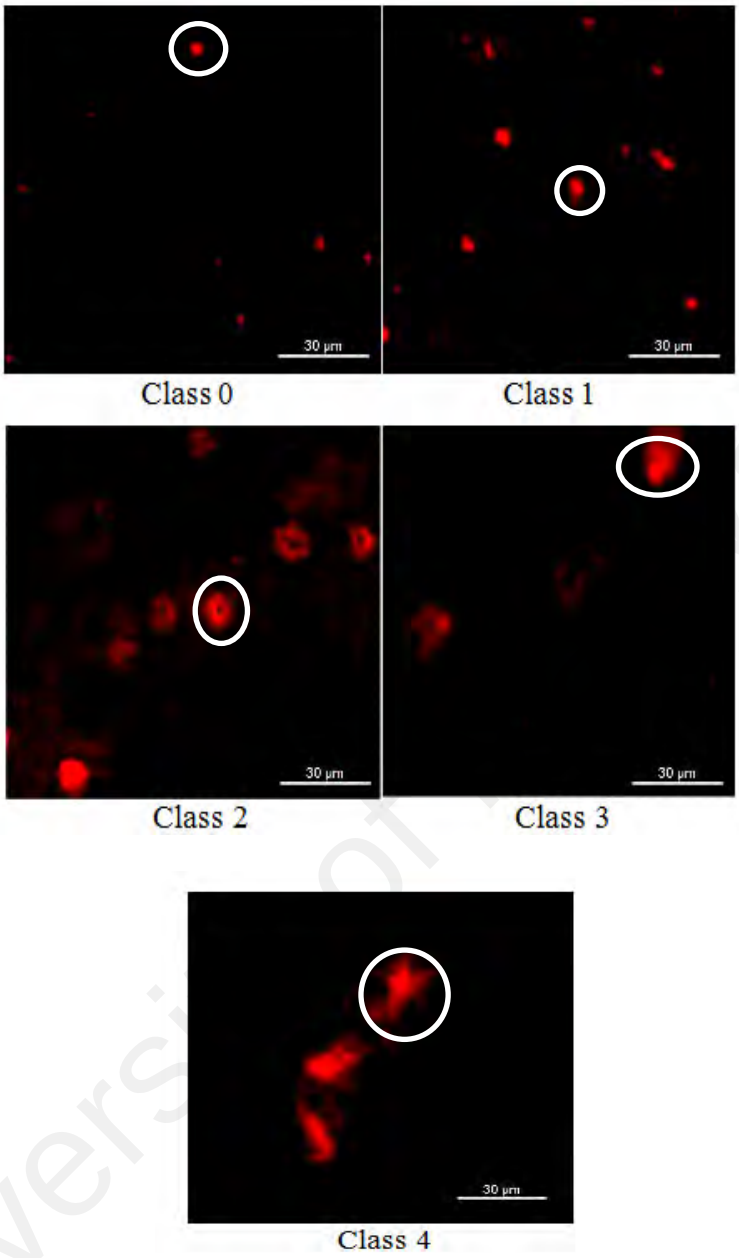

Figure 4.2: Visualization of comet assay from different analyzed plates according to the specific classes of destruction: class 0 (healthy), class 1 (mild DNA destruction), class 2 (medium DNA destruction), class 3 (high DNA destruction) and class 4 (highest DNA destruction).

4.4.3 Hematology analysis of *O. niloticus* blood

Table 4.10: Hematological changes (mean±SD) of *O. niloticus* exposed to chemicals

Parameters	Chemicals exposure			
	Control	BPA	NP	Binary
RBC (g/L)	1.20±0.33	1.18±0.50	1.77±0	0.83±0.07
HGB (L/L)	35.00±14.00	40.67±1.53	54.67±3.06	26.00±2.65
HCT (10^{12}/L)	0.17±0.05	0.20±0.01	0.26±0.05	0.13±0.00
MCV (fl)	138.00±6.08	141.00±6.93	150.67±17.04	157.33±11.59
MCH (pg)	28.70±5.72	29.20±1.65	31.07±1.81	31.20±0.76
MCHC (g/L)	208.00±37.47	207.33±2.08	207.67±26.54	199.33±19.09
Platelet (10^9/L)	9.67±2.52	6.67±2.52	53.33±8.62	6.00±1.00
WBC (10^9)/L)	147.97±1.93	125.37±13.60	42.53±3.03	31.13±0.70

Table 4.11: Descriptive statistic of hematology evaluation

	N	Mean	S.D	Min	Max
Concentration	96	66.0690	73.64141	.11	250.00
Exposure	96	2.5000	1.12390	1.00	4.00

Table 4.12: Kruskal-Wallis Test based on ranking exposure

	Exposure	N	Mean Rank
Concentration	Control	24	48.48
	BPA	24	48.25
	NP	24	53.90
	Binary	24	43.38
	Total	96	

Table 4.13: Test Statistics on hematology

	Concentration
Chi-Square	1.715
df	3
Asymp. Sig.	.634

CHAPTER 5: DISCUSSION

5.1 Genotoxic studies

The complimentary studies of physiological and hematological methods in genetic toxicology can explain the determination of genotoxic effects and ease the understanding of the data at the fundamental level. In fact, micronucleus test, comet assay and hematological analysis are performed in order to gain understanding in the genotoxic effects of BPA and NP either for single exposure or binary exposure as well towards *O. niloticus*. Furthermore, micronucleus test and comet assay have been extensively used in previous studies to determine DNA destruction and genotoxicity analysis. This can be seen from the function of each test itself which permits the determination of DNA destruction at the level of chromosomes in micronucleus test while comet assay method allows the measurement of direct DNA strand breakage capacity based on the level of destruction due to chemicals exposure. In addition, hematological analysis can also increase the reliability of the data by observing the specific parameters in CBC respect to control treatment to consider contamination of blood occurred during exposure of *O. niloticus* towards chemicals.

5.1.1 Micronuclei and nuclear abnormalities in blood of *Oreochromis niloticus*

Micronucleus test and comet assay are reliable methods to indicate level of chromosomes fragments and DNA strand breakage that happened due to toxicity of the chemicals. In fact, DNA is very susceptible to the contaminant's exposure and their effects for instance DNA breakage apart from act as early warning parameters based on data and criteria studied by Hartmann et al., (2004). Besides that, Lemos et al. (2005) stated that comet assay is a safe procedure to preserve both the organism and ecosystem.

The application of micronucleus test is important both *in vivo* and *in vitro* bioindicator because it is commonly used in the molecular epidemiology and cytogenetic damages in populations exposed to genotoxic agents (Bonassi et al., 2007: Terradas et al., 2010: Samanta & Dey, 2012). Even since 1959, Kirsch-Volders et al., (2003) reported that the micronucleus has been suggested as an indicator to determine cytogenetics damages. However, micronucleus test been widely used special only for genotoxicity studies (Norppa & Falck, 2003).

Although the micronucleus test has more benefits in relation to the chromosomal aberration test and comet assay, the necessity of more than one mutagenic test still have to be reinforced when related to the toxicology genetic studies (Araldi et al., 2014: Celik et al., 2014). Therefore, the complimentary of micronucleus test and comet assay can be classified as the best standard among mutagenic tests. This is because the combination of both micronucleus test and comet assay could produce more accurate outcomes, statistical number more realistic, simple procedure, versatile and just involve little time and investment.

Among all exposures, single exposure of NP at concentration of 0.04 mg/l has emerged the highest frequencies of micronuclei and nuclear abnormalities whilst the binary exposures of both chemicals indicates the lowest frequency of micronuclei and nuclear abnormalities. The single exposure of NP on *O. niloticus* resulted in significantly increase in micronuclei evaluation and nuclear abnormalities of blood cell compared to control (Table 4.1). This result was consistent with the data obtained by Kramer et. al. (1998) which utilized alkaline-labile phosphate (ALP) as indicator of vitellogenin presence for the tadpoles. NP exposure treatments triggered ALP became higher than BPA exposure treatments. In fact, BPA and NP were demonstrated to bind to amphibian oestrogen receptors (Lutz & Kloas, 1999) and induced feminization of male *Xenopus laevis* larvae

(Kloas et. al., 1999). Also, in primary cultured amphibian hepatocytes showed the single exposure of NP stimulated VTG-mRNA at a concentration of 10^{-8} M while singe exposure of BPA needed 10^{-7} M for a similar increase (Kloas et. al., 1999). Thus, it proved that single exposure of NP caused strongest effect on metabolism of *O. niloticus* compared to other exposure of BPA and binary accordance to the previous and current studies made.

5.1.2 BPA, NP and binary mixtures effects on hematological parameters

The hematologic parameters are used because they have abilities to show variability of chemicals impacts on the hematological indices such as RBCs, HGB, HCT, MCV, MCH, MCHC, Platelet and WBCs efficiently. These hematologic parameters eventually show not only reliable stress indicators but also indicate the different influences of their environment and the state of fish organism itself (Rowan, 2007). The effects of external stressors and toxic substances can be verified through hematological parameters because circulatory system and external environment had been combined altogether (Wendelaar Bonga, 1997). In order to detect pollutant toxicity, some factors such as hematology, growth rate, biochemical changes and oxygen utilization of fish have to be used (Wepener, 1997).

Single exposure of NP was severely affecting the hematological parameters of *O. niloticus*. These effects were similar to those monitored in fishes treated with the synthetic esterogen ethinylestradiol (EE2) (Schwaiger et al., 2000), ultraviolet radiation (Sayed et al., 2007; Mekkawy et al., 2010; Osman et al., 2010), pesticide (Adedeji et al., 2009) and heavy metals (Ololade & Oginni, 2010). As stated in Table 4.10 for hematological analysis of *O. niloticus* blood, single exposure of NP spiked the RBC, HGB, HCT, MCV, MCH and platelet became the highest values when compared to control treatment and other exposures. This finding was consistent with toxicology studies according to Atamanalp et.al. (2011) which stated that some factors might be believed affected the

increasing of RBC, HGB and HCT after pollutant and heavy metals exposure, including compensation of low amount of oxygen-carrying capacity of HGB and RBC thus increasing RBC count spontaneously. Moreover, increasing value of MCV perhaps caused by the existence of a large amount of larger or older RBC and could be also due to increasing amount of immature RBC (Carvalho & Fernandes, 2006).

Interestingly, MCHC and WBC were drastically dropped in single exposure of NP when compared to the control. The decrease change in MCHC was consistent with the studied made by Adeyemo (2005) which stated that MCHC was decreased significantly for the exposed groups when compared to control was a marker for changes in erythrocyte shape, size and hemoglobin content which also led to anaemia (Eastham & Slade, 1993). The presence of WBC were to fight any infection occurred, shield the body against foreign microorganisms and acted as immune response. In fact, there is a direct relationship between the count level of WBC and severity level of infection in animals (Douglas & Jane, 2010). The significant decrease in WBC count synchronized with increasing of NP concentration suggested that the catfish was literally exposed to high possibility of infection. Hence, this finding was also similar to the previous study made by Adedeji et. al, (2009) which recorded a significant decrease of WBC was occurred after acute exposure to diazinon. The significant changes in WBC count could be utilized as a marker for immunity decrease after exposed to the toxic substances (Adedeji et al., 2009).

5.2 Interaction chain between BPA and NP

BPA and NP have successfully attract public attention due to their capabilities as EDCs which often found in the freshwater. BPA is extensively used as a plastic monomer and plasticizer in the epoxy resins and polycarbonate plastic production. Even though BPA has a short half-life for bioaccumulation, it still actively cause destroying effects on the

aquatic ecosystem as an effect of large-scale production and wide application (Staples et. al., 1998). While NP existence in the environment produced from the nonylphenol ethoxylates degradation which one of the highly cost-effective surfactants usually used in commercial, household, industrial and institutional applications as well (Ying et. al., 2002). NP has high resistant to biodegradation and result in bioaccumulated by aquatic organisms (Soares et. al., 2008). In fact, both BPA and NP are classified as endogenous oestrogen mimics that capable to bind to the estrogenic receptors (Vivacqua et. al., 2003). Their antagonistic effects on endocrine system and being toxic to reproductive system have been well documented (Sohoni et. al., 2001; Ishibashi et. al., 2006).

Based on the whole findings obtained from this study, single exposure of NP indicated most significantly changes when compared to the control and other exposure (single exposure of BPA and binary exposure). This result however was inconsistent with previous study made by Staples et. al. (1998) and Soares et. al. (2008) that stated a significant antioxidant response inhibition was determined in the binary exposure compared to the single BPA exposure. Besides that, it was also known that the bioaccumulation rate of BPA in organisms became lower than NP (Staples et. al., 1998: Soares et. al., 2008) which might contributed the cause that NP gave in stronger inhibition of antioxidant response compared to BPA even at the same exposure concentrations. Furthermore, study made by Mustafa (2010) proved that mixture of BPA and NP administration has caused less additive consequences on puberty onset as well as testicular damage in treated rats rather than individual exposure of both chemicals BPA and NP when compared to the control. This was consistent with the data obtained from the whole findings which showed binary exposure cause the least additive effects in micronucleus test, comet assay and hematology analysis.

CHAPTER 6: CONCLUSION

This study is focused on the genotoxicity and hematological effect of 96 hours of single and binary exposures of BPA and NP towards freshwater fish species, *Oreochromis niloticus*. For the purpose of genotoxicity studies, micronucleus and comet assay were used to determine the different level of DNA destruction whereas hematological studies was purposely performed to assess the certain parameters of blood that influenced by the toxicity of the chemicals.

Generally, the results gained from the study showed that BPA and NP were capable to induce toxicity towards genetic of *Oreochromis niloticus*. However, the clearest observation could be seen was NP caused greater effects towards DNA destruction rather than BPA. It was proven when the dose used in this study for NP was only 0.04 mg/l yet gave higher significant destruction compared to the dose used for BPA which was 2 mg/l. Apart from that, single exposure of NP was eventually given stronger impact besides binary exposures of both chemicals.

For the micronucleus test, single exposure of NP emerged as the cell with highest frequency on micronuclei and nuclear abnormalities followed by single exposure of BPA at the second. Binary exposures indicated the lowest frequency of micronuclei and nuclear abnormalities although the values were not far away from the single exposure of BPA.

The second finding through alkaline comet assay indicated the genotoxicity of single exposure of NP on the erythrocytes of *O. niloticus* was the highest compared to single exposure of BPA and binary exposures of both chemicals. In fact, the mean score, level of destruction in the blood analysis found in single exposure of NP was tripled the value observed in single exposure of BPA and 1/5 times of the value in binary exposures.

The third finding through hematological analysis showed the specific parameters in blood influenced by the toxicity of the chemicals. Single exposure of NP indicated significantly different in most of the parameters observed respect to control and other exposure. However, there were some differences in changes of parameters involved when referred to previous studies due to the independent factors during the study.

Despite all the results obtained, the synergistic uses of physiological and hematological tools in genetic toxicology also have certain drawbacks that cannot be avoided. The validity and reliability of the results gained from different kinds assays are continuously be questioning. Considering to sensitiveness of cells towards xenobiotics is a complex issue to be recognized and resolved. There are many factors that could be highlighted to improve uncertainties for future study related to *O. niloticus* and EDCs exposure especially on BPA and NP. The purity of erythrocytes of *O. niloticus* from foreign substances during the collection step should be concerned. Besides, the factors of denaturation erythrocytes during hematological analysis also should be aware to decrease uncertainties in analysis.

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