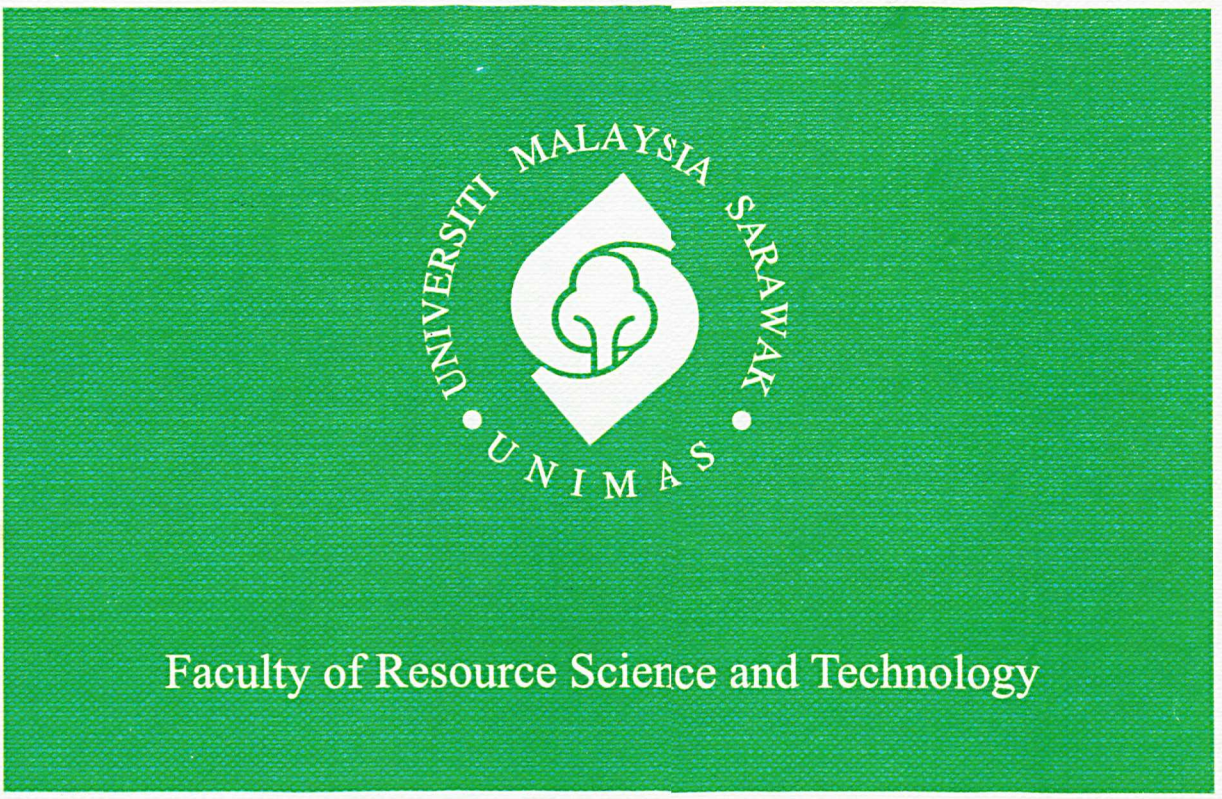

Toxicogenomic Screening on Dipropyl Phthalate Treated Embryos

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(49261)

Bachelor of Science with Honours
(Resource Biotechnology)
2017

Toxicogenomic Screening on Dipropyl Phthalate Treated Embryos

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A Thesis Submitted in Partial Fulfillment of the Requirement of the Degree of Bachelor of Science with Honors (Resource Biotechnology)

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2017

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Toxicogenomic Screening on Dipropyl Phthalate Treated Embryos

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ABSTRACT

Dipropyl phthalate (DPrP), belongs to the phthalate ester (PAEs) family and is widely used as addictive in the plastic products, could be released into the environment through commercial products and food, and it might cause impact to human health by several pathways. Several PAEs had been reported could act as estrogen and thyroid endocrine disruptor. The aim of this research is to test the toxic effect of DPrP by using Zebrafish (*Danio rerio*) embryo as the animal model. Zebrafish eggs were treated with nine different DPrP concentrations from 4 hour post-fertilization (hpf) until 96 hpf. DPrP had induced the lethal effect in the embryos, with LC_{50} was calculated as 5.16 mg/L. Phenotypic analysis using Zebrafish embryo has revealed that DPrP can cause uninflated swimbladder, pericardial oedema, yolk sac oedema, shorten body length, yolk opaque, microphthalmia in Zebrafish embryos, and the hatching ability had been affected too. Using toxicogenomic screening, gene expression of *ttr* that involved in thyroid hormone metabolism were examined by using semi-quantitative Reverse-Transcript PCR. Result shown that the band intensity of *ttr* gene was altered and the effect was dose-dependent. Overall, these results demonstrate that DPrP can cause morphology deformations in Zebrafish embryo and it might be associated with the effect of thyroid endocrine disruptor.

Keywords: Phthalate ester, Dipropyl phthalate, Toxicogenomic, *ttr*, Zebrafish

ABSTRAK

Dipropil flatat (DPrP) tergolong kepada famili ester ftalat (PAEs) banyak digunakan sebagai bahan tambahan dalam pembuatan plastik. Komponen-komponent dalam ftalat boleh dilepaskan ke alam sekitar melalui produk komersial dan makanan, dan akan menjejaskan kesihatan manusia dengan beberapa laluan. Beberapa PAEs telah dilaporkan boleh bertindak sebagai pengacau endokrin estrogen dan tiroid. Kajian ini bertujuan untuk menguji kesan toksik DPrP dengan menggunakan embrio Zebrafish (Danio rerio) sebagai model haiwan. Telur-telur Zebrafish telah terdedah kepada sembilan kepekatan DPrP yang berbeza memulai 4 jam persenyawaan (hpf) sehingga 96 hpf. DPrP telah mendorong kesan maut dalam embrio, dengan LC_{50} dikira sebagai 5.16 mg/L. Analisis fenotip menggunakan embrio Zebrafish telah mendedahkan bahawa DPrP menyebabkan embrio Zebrafish kehilangan gelembung renang, edema pericardium, edema kantong kuning telur, memendekkan panjang badan, kuning telur legap, micropthalmia, dan keupayaan penetasan telah diubah juga. Melalui toxicogenomic idenfikasi, ungkapan gen ttr yang terlibat dalam metabolisme hormone tiroid telah dikaji dengan mengunakan semi-kuantitatif Songsang-Transkrip PCR. Perubuhan ungkapan ttr dalam embrio Zebrafish yang terdedah kepada DPrP menunjukkan bahawa DPrP mengamalkan ketoksikan tiroid endokrin. Secara kesuluruhan, keputusan ini menunjukkan bahawa DPrP boleh menyebabkan perubahaan morfologi embrio Zebrafish dan mungkin dikaitkan dengan kesan pengacau tiroid endokrin.

Kata kunci: Ester ftalat, Dipropil flatat, Toxicogenomic, ttr, Zebrafish

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List of Abbreviations

DBP	Dibutyl phthalate
DEPC	Diethyl pyrocarbonate
dpf	Days post-fertilization
DPrP	Dipropyl phthalate
E3	Embryo medium
EDCs	Endocrine disruptive chemicals
FET	Fish Embryo Acute Toxicity Test
hpf	Hours post-fertilization
LC_{50}	Median lethal concentration 50%
NOEC	No observed effect concentration
PAEs	Phthalate ester
RT-PCR	Reverse Transcription Polymerase Chain Reaction
TAE Buffer	Tris Acetate EDTA Buffer
TH	Thyroid hormone
ttr	transthyretin

1.0 Introduction

Phthalates, or phthalic acid are a family of the artificial organic compound of ester that widely used as plasticizers to manufacture variety commercial products such as food packing, cosmetics, biomedical device, building materials and personal care products (Wan *et al.*, 2013). Phthalate ester (PAEs) was first introduced in the 1920's, different types of PAEs serve in different usage, chemical structure and also the toxicity profiles (Barlow *et al.*, 2007; Graham, 1973). In recent, the global manufacture of PAEs had grown from 1.8 to 4.3 million tons (Habert *et al.*, 2009).

As PAEs are not chemically bound to the polymer, these chemicals will easily emit from the product to environment, and PAEs have been detected in food, air, water source, soil and sediments (Magdouli *et al.*, 2013). Human may get exposed to these chemicals via ingestion, inhalation, intravenous injection tubing and solution, or via dermal contact (Barlow *et al.*, 2007). There are possible hazards that could be released by PAEs, with high levels of this chemicals will disrupt genital development, serve as endocrine disruptors, carcinogen, cause chronic organ effect, reproductive defects and immunology defects (Barlow *et al.*, 2007; Milla *et al.*, 2011). Some of the PAEs have been reported acts as estrogenic endocrine disruptor and thyroid endocrine disruptor, causing disrupting effects in estrogen and thyroid function, and been classified as priority pollutants by United States Environmental Protection Agency (Chen *et al.*, 2014; Kim *et al.*, 2005; Zhai *et al.*, 2014).

Dipropyl phthalate (DPrP) is the metabolite product of dibutyl phthalate (DBP), it is used in fuel cells production and olefins polymerization (Begum *et al.*, 2004; Gangolli, 1999). DPrP has been detected in Taiwan rivers (Yuan *et al.*, 2002). Many types of research have been done on several types of phthalate ester to understand the toxicity effect of the chemicals towards human health. However, the findings of the toxicity and endocrine disrupting activity of DPrP on vertebrate animals remained largely unknown. Hence, this

research is aimed to study the effect of DPrP towards vertebrate development and to evaluate the gene expression in the early vertebrate embryo by using Zebrafish embryo as the animal model.

To date, Zebrafish (*Danio renio*) is widely used as model organism in research. This is due to several benefits: Zebrafish is highly fecund, the development of the embryos is external, speedy and visually, and lower cost and simpler protocol are needed to maintain the Zebrafish (Clark & Ekker, 2015). By using fish embryo acute toxicity test (FET), any defect in the development of fish embryo can be observed under the microscope easily due to its transparency (Veldman & Lin, 2008). Most importantly, FET is the alternative approach to reduce the amount of using live animals in toxicity that is not congruent with animal welfare legislation (Lammer *et al.*, 2009). In addition, the genetic of Zebrafish is highly conserved with other vertebrates, including human, thus the findings in Zebrafish are counterparts to human (Veldman & Lin, 2008).

In this research, the lethal and sublethal effect of DPrP on Zebrafish embryo was evaluated by using different concentrations of DPrP. Toxicogenomic studies on *ttr* was carried out by using semi-quantitative Reverse-Transcript PCR. The result may be used to study the effect of dipropyl phthalate and provide data for human risk assessment.

1.1 Problem Statements

Phthalate ester that found in many products may release possible hazards, with exposed to high level of these chemicals will cause reproductive defects. It also reported that phthalate esters also act as endocrine disruptor that will affect the hormonal system. It has been suggested that children posing higher risks than adults, this is due to children are exposed to products that contain phthalates more frequent than adults. Many types of research on several types of phthalates have been done to understand the toxicity effect of the chemical towards human health. However, the findings of the toxicity of dipropyl phthalate (DPrP) on vertebrate development, especially early developmental stage of fish remained largely unknown. Hence, this research is aimed to study the effect of DPrP towards vertebrate development and to evaluate the *ttr* gene expression in the early vertebrate embryo by using Zebrafish embryo as the animal model.

1.2 Objectives

The objectives throughout the completion of this project are following:

i. To study the toxicity effects of dipropyl phthalate towards early embryo development of Zebrafish.

ii. To observe the effect of dipropyl phthalate on the gene expression of *ttr* in Zebrafish embryo at 96 hpf.

2.0 Literature Review

2.1 Background of Phthalate Ester

Phthalates, or phthalic acid diesters are artificial chemicals that use phthalic anhydride as basic raw materials, generally either naphthalene or o-oxylene and chemically mixed with variety alcohol (Graham, 1973). Phthalate ester (PAEs) was introduced as plasticizer in the 1920's to overcome the shortage of camphor (plasticizer in 1870) (Graham, 1973). PAEs used as plasticizer extensively due to their high flexibility, low volatility, colourless and odourless properties, able to extend the lifespan and providing shapes to some plastics products (Barlow *et al.*, 2007). In between 1970 and 2006, the global manufacture of phthalate had grown from 1.8 to 4.3 million tons (Habert *et al.*, 2009).

PAEs are considered as ubiquitous contaminants because they are not chemically bound to the polymer and will easily emit from the products to the environment (Wittassek *et al.*, 2011). PAEs have been detected in food, air, water source, soil and sediments (Magdouli *et al.*, 2013). Humans may get exposed to these chemicals via ingestion, inhalation, intravenous injection tubing and solution, or via dermal contact (Barlow *et al.*, 2007). Among the sources of exposure, food and consumer products are the main pathways human exposed to phthalate (Wittassek *et al.*, 2011). Data collected by NHANES (as cited in National Research Council (US) Committee (NRC), 2008) showed that children age 6-11 possess higher urinary phthalate metabolites concentration than adults and adolescent, this might due to present of PAEs in children's toys and milk. Besides, youngsters have higher respiratory rates than elders, hence higher chance in children exposed to phthalate via inhalation (NRC, 2008).

Guo *et al.* (2011) had collected human urine samples from seven different countries in Asia. From the analysis, 14 phthalate metabolites were detected from all the urine samples, with highest median concentration (the summation of 14 phthalates) was found in the

samples from Kuwait (1050 ng/mL), followed by India (389 ng/mL), China (234 ng/mL), Vietnam (133 ng/mL), Japan (120 ng/mL), Korea (117 ng/mL), and the lowest is in Malaysia (94.9 ng/mL).

2.1.1 Toxicity of Phthalate Ester

PAEs are classified as endocrine disruptor because of their complicated effects in the hormonal system. These endocrine disruptive chemicals (EDCs) can affect the hormonal system by intruding to receptor signalling such as estrogen receptors and androgen receptors, or by activating other signalling pathways and alter gene expression in cells (Swedenborg *et al.*, 2009). Chen *et al.* (2014) have reported that some phthalate such as dibutyl phthalate (DBP), diethyl phthalate (DEP), diisodecyl phthalate (DIDN) and mixture of several types of PAEs possess estrogenic endocrine disrupting activity. Besides, the potential of PAEs which can act as thyroid endocrine disruptor has also been investigated by Zhai *et al.* (2014).

The exposure to PAEs will cause several health defects, such as malformations in the reproductive system and abnormalities in development (Mariana *et al.*, 2016). For instance, in the studies of Rozati *et al.* (2002), the infertile men are having higher PAEs in their total semen with altered sperm morphology. Besides, adult female rodents exposed to diethylhexyl phthalate (DEHP) has prolonged estrous cycles and ovulation is absent (Mariana *et al.*, 2016). Farzanehfar *et al.* (2016) reported that dibutyl phthalate (DBP) causes neurobehavioral adverse effect in mice, where treated mice have reduced total distance movement, impaired memory function and being more anxiety. Exposed to phthalate ester also leads to peroxisome proliferation and alter liver function in beta-oxidation and fatty acid transport (Ganning *et al.*, 1984).

Due to those phthalate toxicity effects, dibutyl phthalate (DBP), butyl benzyl phthalate (BBP) and di(2-ethylhexyl) phthalate (DEPH) are permanently prohibited; while

di-n-octyl phthalate (DnOP), diisononyl phthalate (DINP) and diisodecyl phthalate (DIDP) are prohibited on an interim basis if the individual phthalate concentration is more than 0.1% in children toys under The Consumer Product Safety Improvement Act of 2008 (CPSIA) (United States Consumer Product Safety Commission, 2010). Europian Union (EU) also bans the use of dibutyl phthalate and diethyhexyl phthalate in comestic products (Hubinger, 2010). United States Environmental Protection Agency has classified DEPH, BBP, DBP, DnOP, diethyl phthalate (DEP) and dimenthyl phthalate (DMP) as priority pollutants (Kim *et al.*, 2005).

2.2 Dipropyl Phthalate

Dipropyl phthalate (DPrP), also known as dipropyl 1,2-benzenedicarboxylate, is involved in the fuel cells productions and acts as catalyst of olefins polymerization (Gangolli, 1999). DPrP is the metabolite product of dibutyl phthalate (DBP) through demethylation of DBP (Begum *et al.*, 2004). The chemical formula of DPrP is $C_{14}H_{18}O_4$, the molecular weight is 250.29 g/mol, while the density of DPrP at 20°C is 1.078 g/mL. Boiling point and flash point of DPrP are 317.5 °C and >110 °C respectively (Gangolli, 1999). In spite of being easily degraded under aerobic conditions, DPrP was detected in Taiwan rivers and was placed at second category of concentration levels (Yuan *et al.*, 2002). It has been reported that DPrP was detected in milk formulae, packaged food, vegetable oil, house dust and air (Biomonitoring California, 2015; Saillenfait *et al.*, 2010).

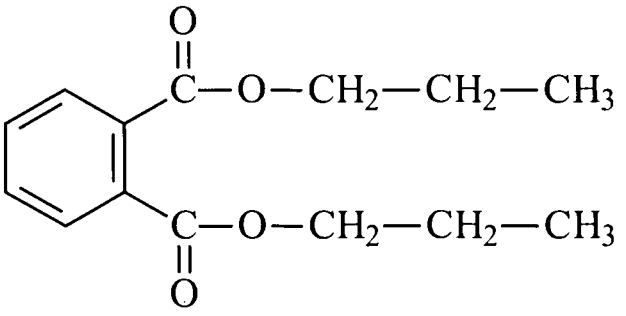

Figure 1. Molecular structure of dipropyl phthalate. Adapted from Yamasaki *et al.*, 2004.

2.2.1 Effects of Dipropyl Phthalate

Similar to the other PAEs, dipropyl phthalate (DPrP) had shown weak estrogenic effect in *in vitro* yeast two hybrid assays (Kawagoshi *et al.,* 2002). Reproductive and immune defects may relate to the phthalate estrogen disrupting activities (Chen *et al.*, 2014; Milla, 2011). Heindel *et al.* (1989) had reported that CD-1 mice were infertile or having lower fertility rate (live pups/litter) when exposed to high concentration of DPrP, and the toxicity effect is in female is more severe than in male. Another research done by Saillenfait *et al.* (2010) had shown that DPrP treated Sprague-Dawley rats had increased in liver weight, decrease in anogenital distance and delayed of ossification centers in the paws. However, several results showed neither estrogenic nor anti-estrogenic effects in both *in vivo* and *in vitro* assay (Akahori *et al.*, 2008; Okubo *et al.*, 2003; Takeuchi *et al.*, 2005). In spite of the decrease in the anogenital distance in rats which is believed to be due to the antiandrogenic effect of DPrP in mammals, no antiandeogenic effect was shown in *in vivo* Hershberger assay (Mathieu-Denoncourt, 2015; Yamasaki *et al.*, 2004). Different exposure conditions and concentrations might produce incompatible results (Zhai *et al.*, 2014). DPrP also causes effects in the immune response. DPrP treated *Neocaridina denticulate* had increased the expression of immune mediators (Sung *et al.*, 2011). Table 1 summarise the effects of DPrP.

Table 1. Effects of dipropyl phthalate on animal model from previous studies.

Study	Effect	Literature
Oral treatment on CD-1 Swiss mice	5.0% (roughly 8600mg/kg/d) cause infertile; 2.5% (4100mg/kg/d) fertility of mice is reduced; Effect in female is more severe than male Toxic dose low (TD_{LO}): 1260 mg/kg	Heindel *et al.*,1989
Oral treatment on pregnant Sprague-Dawley rats	1.0 g/kg/d or 1.5 g/kg/d cause induction of peroxisomal enzyme in dams; 1.5 g/kg/d cause body weight is decreased, liver weight is increased in dams 1.5 g/kg/d cause foetus body weight decreased; 1.0 g/kg/d and 1.5 g/kg/d cause foetus anogenital distance (in male) decreased, malpositioned testis; formation of cervical and thoracic rudimentary ribs; delayed ossification in paws; No teratogenicity effect; No observed adverse effect level (NOAEL): 0.5 g/kg/d	Saillenfait *et al.*, 2010
Green neon shrimp (*Neocaridina denticulate*)	Immune functions were affected; Increase in susceptibility when exposed to infection; Immune-related gene (AcP, ANAE and β-Glu) expressed varied depends on the concentration; Response occurred most obviously within 10 days after exposure to DPrP	Sung *et al.*, 2011
Protozoan (*Tetrahymena pyriformis*)	No observed effect concentration (NOEC): 10 mgL^{-1}; Lowest observed effect concentration (LOEC): 25 mgL^{-1}	Yoshizawa *et al.*, 1977
In vivo immature rat uterotropic assay	Neither estrogenic nor anti-estrogenic properties	Akahori *et al.*, 2008
In vitro test on human estrogen receptor α, receptor β, androgen receptor, and MCF-7 cell proliferation assay	Neither estrogenic nor anti-estrogenic properties	Akahori *et al.*, 2008; Okubo *et al.*, 2003; Takeuchi *et al.*, 2005
In vitro yeast two-hybrid assay	Weak estrogenic activity at 10^{-5} M	Kawagoshi *et al.*, 2002
In vivo Hershberger assay on rats	No androgen antagonistic affinity was shown	Yamasaki *et al.*, 2004

2.3 Toxicogenomic Screening

In molecular biology, toxicogenomic is one type of genomic technologies that use high-throughput techniques, predictive and translational informatics tools in toxicology studies (Khan *et al.*, 2014). Toxicogenomic acts as a holistic tool to omics which enclosing transcriptomics, epigenomics, global miRNA analysis, proteomics, metabolomics, bioinformatics and cheminformatics (Khan *et al.*, 2014).

Toxicogenomics is widely used in toxicology as this technique able to incorporate toxicant-specific alteration in the pattern of gene, protein and metabolite expression correspond with phenotypic responses, with the principle of almost identical gene expression profiles can be performed in the compounds that having similar mechanisms of toxicity and efficacy (Institute of Medicine (US), 2008). Compared to traditional approaches, the prediction of gene expression through toxicogenomics is more sensitive, and a number of samples can be measured in once at the same time (Institute of Medicine (US), 2008).

2.4 Zebrafish Embryo Model

The Zebrafish, *Danio renio*, was first described by Francis Hamilton in 1822, and introduced used as experimental model organism since the early 1980s to study the developmental biology of vertebrate (Clark & Ekker, 2015). Zebrafish is tropical freshwater teleost which belongs to the *Cyprinidae* family, native to the stream of India and South Asia (Eaton & Farley, 1974). The sex of Zebrafish can be distinguished by the colour and shape of the body, in which male Zebrafish has silver bands with reddish tints on slimmer body; while female has a swollen body (Eaton & Farley, 1974).

Zebrafish are preferably used as a model organism due to its large family size, highly fecund, development of embryos are external, speedy and visually, and the cost

and maintenance protocols of Zebrafish is much lower and easier (Clark & Ekker, 2015). According to Scholz *et al.* (2008), each female Zebrafish able to spawn approximate 100 eggs per day, and under laboratory condition, the fecundity able to reach several thousands of embryos daily for parallel experiment treatments.

In fish acute toxicity test, hypothesizing will leads to severe pain and distress in fish. Hence, instead of using free-feeding larvae, juvenile or adult fish for toxicity test, *in vitro* fish embryo toxicity test was introduced as an alternative approach to reduce the conflict of current animal rights legislation (Lammer *et al.*, 2009). Besides, to date, fish embryos and eleutheroembryos are not yet under the protection of various governmental definitions, hence ethical issues related to laboratory animals can be avoided (Lammer *et al.*, 2009).

The transparent form of embryo and chorion enable the researchers to observe the development of Zebrafish from a fertilized egg until larva fish stage under microscope (Veldman & Lin, 2008). Essentially, after 24 hpf (hours post-fertilization), the basic body plan of Zebrafish is exhibited, and at estimated 2-3 dpf (days post-fertilization), the embryo is hatched. At 5 dpf, the major organ systems of Zebrafish embryo are developed and begin of external feeding, at 3 months, Zebrafish is matured (Scholz *et al.*, 2008). The developmental disorders can be screened and act as an indicator for teratogenic effects, and it has been reported that acute toxicity in embryos and adult are correlated (Scholz *et al.*, 2008).

In addition, the genetic of Zebrafish is highly conserved with other vertebrates, including humans, thus the findings in Zebrafish are counterparts to human (Veldman & Lin, 2008). The full genome of Zebrafish was begun sequenced by Sanger Institute in 2001. It has been reported that Zebrafish have 26,260 protein-coding genes, the number is higher than other vertebrates because there is additional teleost-specific

genome duplication in Zebrafish (Howe *et al.*, 2013). Compared to human protein-coding genes, 71.4% of human gene possess at least one Zebrafish orthologue, with 47% is one-to-one relationship; while 69% of Zebrafish genes possess at least one human orthologue (Howe *et al.*, 2013).

2.5 Experiment Design

2.5.1 Fish Embryo Toxicity Test

In fish embryo acute toxicity test (FET), fertilized eggs should be promptly exposed to test chemical to ensure early onset of exposure, where fertilized eggs should be immersed into the test solutions at latest at the high blastula stage (stage 11), or before 4 hpf, hence chemical effect can be evaluated throughout the gastrulation stage (Lammer et al., 2009; McGrath, 2012; Organisation for Economic Co-operation and Development (OECD), 1992). Although the early larva stage of Zebrafish is started at 72 hpf, European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM) has suggested that the toxicity exposure time should be extended to 96 hpf as Zebrafish embryo at before 72 hpf is surrounded by chorion that will decrease the exposure of embryo to chemical, and this principle has been added into OECD guideline (EURL-ECVAM, 2014; OECD, 2013). First screening on embryos can be done at 4 hpf to select the healthy embryo prior to valid the developmental toxicity test (McGrath, 2012). According to OECD (2013), observation in FET should be done at every 24 hours, begins at 24 hpf, followed by 48 hpf, 72 hpf and 96 hpf. At 24 hpf (pharyngula period), embryo has developed to the phylotypic stage, classic vertebrate body plan can be observed (Kimmel *et al.*, 1995). Meanwhile, 48 hpf is the hatching period, and 72 hpf is the early larva period (Kimmel *et al.*, 1995). The lethal endpoints at every 24 hours are shown in Table 2 (OECD, 2013).

A parameter LC_{50} (Median Lethal Concentration) is commonly used in toxicity testing. LC_{50} acts as a quantitative measure in environment risk assessment where the concentration of a compound will cause lethal of 50% of organisms (Oliveira *et al.*, 2010).

Table 2. The lethal endpoint for fish embryo acute toxicity test.

Apical Observations	Exposure Time			
	24 hpf	48 hpf	72 hpf	96 hpf
Coagulated Embryos	•	•	•	•
Lack of somite formation	•	•	•	•
Non-detachment of the tail	•	•	•	•
Lack of heartbeat		•	•	•

2.5.2 Transthyretin (*ttr*)

In vertebrate, thyroid hormone such as tri-iodothyronine (T_3) and thyroxine (T_4) are essential to develop and maintain the function of the cardiovascular, nervous, immune and reproductive system (Wendi *et al.*, 2002). There are three different thyroid hormone-binding proteins (THBP) present in vertebrate: transthyretin (TTR), thyroxine-binding globulin (TBG) and albumin (ALB). THBP acts as transporter to transport TH in the bloodstream, with *ttr* is the major TH carrier protein in teleost (Morgado *et al.*, 2007). Yamauchi *et al.* (1999) had mentioned that *ttr* is the major THs carrier protein in teleost, where T_3 and T_4 will bind to *ttr* to form a stable tetrameric structure and THs are transported through bloodstream to the target tissue (Morgado *et al.*, 2006). According to Richardson *et al.* (2007), *ttr* is synthesized in the liver of fish, the binding affinity is higher for T_3 than T_4.

Margado *et al.* (2006) had stated when present of endocrine disruptor, the concentration of free TH in fish might be affected by the binding affinity of TH towards the *ttr*. In the studies done by Zhang *et al.* (2013), downregulation of *ttr* might due to the pollutants had interfered the binding of free THs to *ttr* and reduce the *ttr* protein translation.