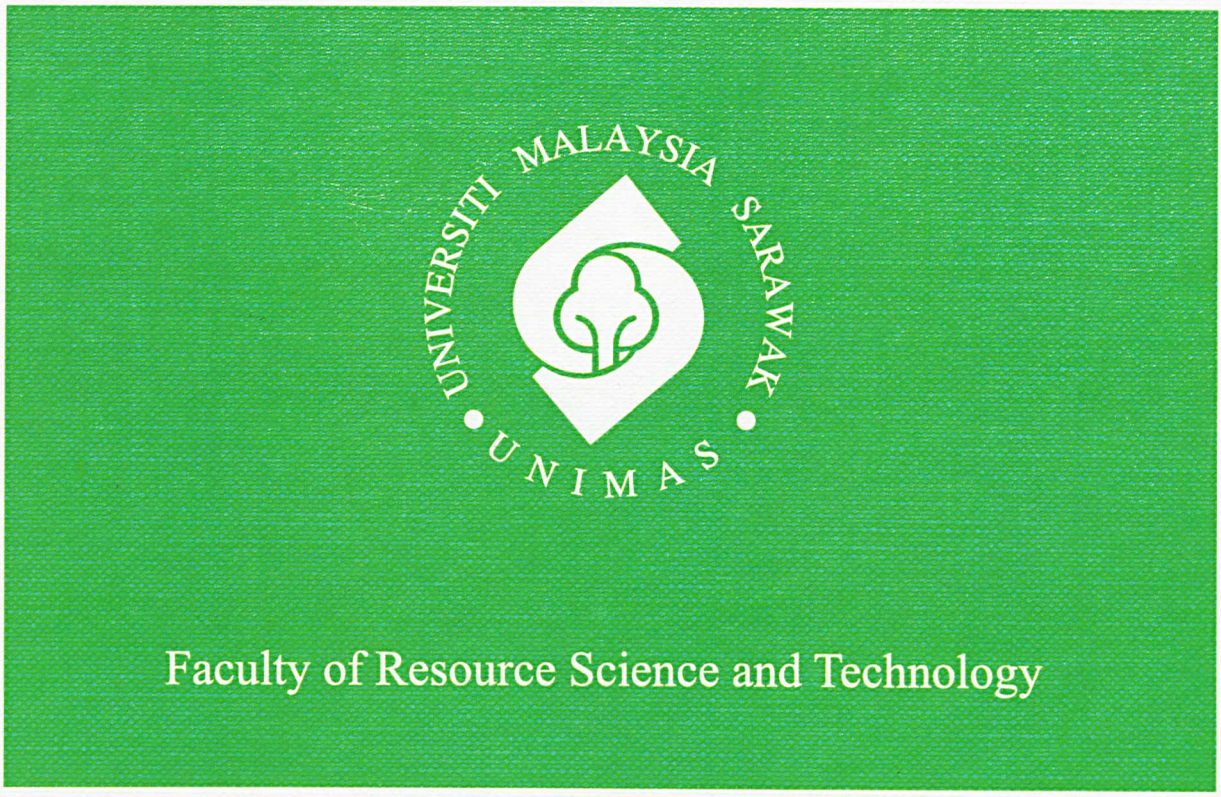

Post Toxicity Screening of Dipropyl Phthalate on Early Vertebrate Development

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Bachelor of Science with Honours
(Resource Biotechnology)
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Post Toxicity Screening of Dipropyl Phthalate on Early Vertebrate Development

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A thesis submitted in partial fulfilment of the requirement of
the Degree of Bachelor of Science with Honours (Resource Biotechnology)

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2017

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Post Toxicity Screening of Dipropyl Phthalate on Early Vertebrate Development

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ABSTRACT

Dipropyl phthalate (DPrP) is one of the synthetic phthalate esters (PAEs) used as a plasticiser. Due to its weak bonding, it leaks out easily from the matrix of products, found in the consumer products and the environment. Certain family members of PAEs have been proved to be carcinogens and endocrine disruptors. Several studies showed that it could be estrogenic, antiestrogenic, or neither both. DPrP toxicity and toxicogenomic of vertebrate development are not well-known. The zebrafish was chosen as the animal model. The fertilised eggs were exposed to nine tested concentrations of DPrP from 4-hour post fertilisation (hpf) to 96 hpf. The median lethal concentration (LC_{50}) was 5.16 mg/L, between 5.03 mg/L and 5.30 mg/L with 95% confidence level. The lowest observed effect concentration (LOEC) was 4.86 mg/L while the no observed effect concentration (NOEC) was 4.65 mg/L. The adverse effects of DPrP on morphology were uninflated swim bladder, pericardial oedema, and yolk oedema. Other effects included craniofacial deformation, blood island, bent spine, shortened body, stretched heart, microphthalmia, and yolk opaque. The hatching rate of zebrafish was reduced with the increasing in the concentration of DPrP. The results suggest that environment especially aquatic ecology could be affected by DPrP. Transthyretin gene (*ttr*) was the main study gene to discover DPrP impact on thyroid hormone. The expression level of *ttr* was examined at the transcriptional level. Total RNA was isolated from 96 hpf zebrafish embryos followed by semi-quantitative RT-PCR and agarose gel electrophoresis. Three concentrations of DPrP were tested for effect on *ttr*, which are 4.65 mg/L, 5.07 mg/L, and 5.70 mg/L. The transcriptional level of *ttr* reduced with the increasing of concentration of DPrP. Therefore, it is expected that DPrP could be endocrine disruptor through the downregulation of *ttr*.

Keywords: Dipropyl phthalate, *Danio rerio*, LC_{50}, transthyretin

ABSTRAK

Dipropil ftalat (DPrP) merupakan sejenis ester ftalat (PAEs) sintetik yang digunakan sebagai plasticiser. Oleh sebab ikatannya yang lemah, DPrP boleh keluar daripada matriks produk dengan senang. DPrP dijumpai dalam produk pengguna dan alam sekitar. Sesetengah jenis PAEs telah dikesan bahawa mereka ialah karsinogen dan pengganggu endokrin. Beberapa kajian menunjukkan bahawa DPrP mungkin bersifat estrogenik, anti-estrogenik atau keduanya tidak. Ketosikan dan toxicogenomik DPrP dalam perkembangan vertebrata tidak dikenalkan. Zebrafish telah dipilih sebagai model haiwan. Telur yang disenyawakan terdedah kepada sembilan kepekatan dari 4 jam selepas persenyawaan (hpf) sehingga 96 hpf. Kepekatan maut 50% (LC_{50}) ialah 5.16 mg/L, antara 5.03 mg/L dan 5.30 mg/L dengan 95% tahap keyakinan. Kepekatan terendah kesan (LOEC) ialah 4.86 mg/L manakala kepekatan tiada kesan dicerap (NOEC) ialah 4.65 mg/L. Kesan buruk pada morfologi ialah kandung kemih yang kurang berkembang, edema pericardium, dan edema kuning telur. Kesan lain termasuk deformasi kraniofasial, pulau darah, tulang belakang bengkok, badan pendek, keregangan jantung, microphthalmia, dan kuning telur legap. Kadar penetasan juga semakin berkurangan dengan peningkatan kepekatan DPrP. Keputusan ini mencadangkan bahawa alam sekitar terutamanya ekologi akuatik mungkin terjejas oleh DPrP. Gen transthyretin (ttr) merupakan gen utama untuk dikaji supaya kesan DPrP pada hormone tiroid boleh dikesan. Tahap ekspresi gen ttr diperiksa pada tahap transkripsi. Semua RNA telah diasingkan daripada embrio zebrafish yang 96 hpf. Selepas itu, RT-PCR kuantitatif separuh dan elektroforesis gel telah dilaksanakan. Tiga kepekatan DPrP telah diperiksa iaitu 4.65 mg/L, 5.07 mg/L, dan 5.70 mh/L. Tahap transkripsi ttr telah dikurangkan dengan penambahan kepekatan. Oleh itu, DPrP disangka sebagai penggangu endokrin melalui penurunan tahap transkripsi ttr.

Kata Kunci: Dipropil ftalat, Danio rerio, LC_{50}, transthyretin

Table of Content

Declaration	i
Acknowledgements	iii
Abstract	iv
Abstrak	iv
Table of Content	v
List of Tables	vi
List of Figures	vii
List of Abbreviations	ix
1.0 INTRODUCTION	1
2.0 LITERATURE REVIEW	3
2.1 Phthalate Ester	3
2.1.1 Dipropyl Phthalate	5
2.2 Developmental Toxicity Study	8
2.3 Endocrine Disruption	9
2.4 Transthyretin	11
2.5 Zebrafish	12
3.0 MATERIALS AND METHODS	15
3.1 Fish Maintenance	15
3.2 Egg Production and Collection	15
3.3 DEPC Treatment	15
3.4 Preparation of Chemicals and Test Solutions	16
3.5 Dipropyl Phthalate Exposure	17
3.6 RNA Extraction	17
3.7 Reverse Transcription	18
3.8 Polymerase Chain Reaction	19
3.9 Agarose Gel Electrophoresis	20
3.10 Data Analysis	20
4.0 RESULTS & DISCUSSION	22
4.1 Concentration-Mortality Rate and Hatching Rate	22
4.2 Microscopic Observation	27
4.3 RNA Quantification	43
4.4 PCR Optimisation	44
4.5 Transthyretin Transcriptional Level	46
5.0 CONCLUSION AND RECOMMENDATION	50
6.0 REFERENCE	51
7.0 APPENDICES	59

List of Tables

Table 1. Summary of DPrP effects from previous studies 7

Table 2. The ingredients of E3 medium 16

Table 3. The volume of DPrP used and their respective concentration 17

Table 4. The volume of each component used per reaction 18

Table 5. The primer used in this study 19

Table 6. The PCR master mix composition 19

Table 7. The concentration-mortality rate of dipropyl phthalate (DPrP) 22

Table 8. The ANOVA result. 22

Table 9. The hatching rate of treated embryo of each concentration 26

Table 10. The cumulative frequency of morphological deformations 42

Table 11. The quality of extracted RNA 43

Table 12. The ratio of RNA and ultrapure water to make a final concentration of 50 ng/μL 44

List of Figures

Figure 1. The general structure of PAEs 3

Figure 2. The male zebrafish (lower individual) and female zebrafish (upper individual) 12

Figure 3. The graph of concentration-mortality rate 23

Figure 4. The graph of mortality rate against concentration 23

Figure 5. The graph of hatching rate of treated embryos at 48 hpf, 72 hpf, and 96 hpf 25

Figure 6. The unhatched embryo and coagulated embryo 26

Figure 7. The graph of occurrence of uninflated swim bladder in treated embryos at 96 hpf. 27

Figure 8. The microphthalmia observed in different concentrations at 96 hpf 28

Figure 9. The occurrence of microphthalmia and yolk oedema observed in different concentrations at 96 hpf 31

Figure 10. Pericardial oedema and stretched heart were found in various tested concentrations 33

Figure 11. The occurrence of pericardial oedema and stretched heart observed in different concentrations at 96 hpf 34

Figure 12. Normal heart shape was found in the treated embryo with pericardial oedema at 6.34 mg/L 35

Figure 13. The occurrence of yolk opaque and shortened body observed in different concentrations at 96 hpf. 35

Figure 14. The shortened body and yolk opaque observed in different concentrations at 96 hpf 36

Figure 15. The blood island observed 38

Figure 16. The craniofacial deformation, bent spine and tail curvature detected on treated embryos 38

Figure 17. The minor morphological defects observed in DPrP treated embryos at 96 hpf 39

Figure 18. The major morphological defects observed in DPrP treated embryos at 96 hpf. 41

Figure 19. The gel electrophoresis result 43

Figure 20. The gel electrophoresis result of gradient PCR of ttr with an annealing temperature range of 53 to 58 °C. 44

Figure 21. The gel electrophoresis result of gradient PCR of ttr with an annealing temperature range of 60 to 65 °C. 45

Figure 22. The gel electrophoresis result of gradient PCR of ttr with an annealing temperature range of 58 to 60 °C 46

Figure 23. The gel electrophoresis result of normalisation by using β-actin2 as internal reference 47

Figure 24. The gel electrophoresis result of normalisation 47

Figure 25. The gel electrophoresis result of ttr transcriptional level between control and treatments 48

List of Abbreviations

DPrP	Dipropyl phthalate
EC_{50}	Toxicity concentration determination
ER	Oestrogen receptor
EU	European Union
EURL-ECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
hpf	Hour post fertilisation
LC_{50}	Median lethal concentration
NOEC	No observed effect concentration
LOEC	Lowest observed effect concentration
OECD	Organisation for Economic Co-operation and Development
OSHA	Occupational Safety and Health Administration
PAEs	Phthalate esters
TD_{Lo}	Toxic dose low

1.0 INTRODUCTION

Phthalate esters (PAEs) are a family of the artificial organic compound of ester that widely used as plasticizers to manufacture variety commercial products, including sealants, paints, adhesives, cosmetics and food packaging (Xu *et al.*, 2013). They can be found in the product as an additive to enhance the products in term of flexibility, transparency, durability and lifespan (Barlow *et al.*, 2007; Chan, 2012). As PAEs are not chemically bound to the polymer, they are easily leaked to the environment (Zou & Cai, 2013).

Certain PAEs were found to be harmful and listed as pollutants such as bis(2-ethylhexyl) phthalate and bis(2-ethylhexyl) adipate (Peñalver *et al.*, 2000). They have been detected in food, air, water source, soil and sediments (Magdouli *et al.*, 2013). Human may be exposed to PAEs as Guo *et al.* (2011) found that phthalate metabolites were detected from human urine samples across seven Asia countries. PAEs could serve as endocrine disruptors, carcinogens, developmental and reproductive toxicants, and immunotoxicants (Barlow *et al.*, 2007; Milla *et al.*, 2011). Some of the family members have been reported acts as oestrogenic endocrine disruptor and thyroid endocrine disruptor (Chen *et al.*, 2014; Zhai *et al.*, 2014).

Dipropyl phthalate (DPrP) is used to produce fuel cells and plasticisers (Gangolli, 1999). Yuan *et al.* (2002) found that several rivers in Taiwan were contaminated with DPrP such as Zhonggang (Yuan *et al.*, 2002). Although certain PAEs have been characterised, DPrP toxicity and endocrine disruption properties remain unknown. The results from different studies contradict each other too. 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 with zebrafish as the model organism. This is crucial to regulate the usage of DPrP as certain PAEs have been restricted with limited concentrations used for manufacturing by the European Union (EU) (Zou & Cai, 2013). Selderslaghs *et al.* (2009) suggested that *in vitro* testing should be supported by *in vivo* assays to be more accurate.

Hazard assessment of industrial chemicals requires acute fish toxicity data in Europe (European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), 2014).

Zebrafish has multiple advantages for being an animal model. It requires low cost, has high fecundity and external fertilisation with transparent egg since fertilisation until beyond pharyngulation (Selderslaghs *et al.*, 2009). Zebrafish embryo test has been well-developed to be an animal model and widely used in many studies including other PAEs effects evaluation (Selderslaghs *et al.*, 2009; Xu *et al.*, 2013). Other than that, 70% of human genes have at least one zebrafish orthologue (Howe *et al.*, 2013). The result obtained from zebrafish may be extrapolated to humans

The objective of this study is to evaluate the effects of DPrP, on both lethal and sublethal effects on early vertebrate development. The zebrafish embryos were exposed to nine different concentrations of DPrP and examine under the microscope at every 24-hour post fertilisation (hpf) until 96 hpf. The mortality and morphological deformation were recorded and analysed. The DPrP treated embryos from three different concentrations were prepared for RNA extraction followed by semi-quantitative reverse-transcription PCR to identify whether the *ttr* is affected by DPrP. The result suggested that *ttr* was downregulated directly proportional to the concentration of DPrP. It is expected that DPrP is correlated to *ttr*.

2.0 LITERATURE REVIEW

2.1 Phthalate Ester

Phthalate esters (PAEs) originate from alcohol and phthalic anhydride (Barlow *et al.*, 2007). They are dialkyl or alkyl aryl esters of 1,2-dibenzenedicarboxylic acid with general structure shown below, where R^1 and R^2 can be any alkyl chain or aryl group (Adewuyi & Olowu, 2012).

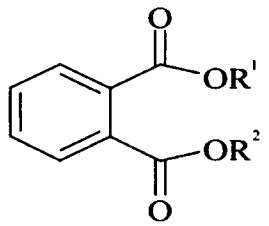

Figure 1. The general structure of PAEs. Adapted from Adewuyi & Olowu, 2012.

Oily, colourless, odourless, lipophilic and non-volatile are their characteristics (Barlow *et al.*, 2007). They are commonly used in plastic manufacturing as plasticisers to increase products' flexibility, transparency, strength, and lifespan (Chan, 2012). PAEs represent 82% of plasticiser use in United States, Western Europe and Japan (Johnson *et al.*, 2010). They have been used extensively in multiple industries, including electronics, medical devices, toys, paints, baby products, cosmetics and food packaging (Chan, 2012; Xu *et al.*, 2013; Zou & Cai, 2013). However, PAEs are no longer used for children toys since 1999 in the European Union (Johnson *et al.*, 2010). They can be used to enhance penetration and make the scent and colour last longer too (Barlow *et al.*, 2007). In the paper of Zou and Cai (2013), PAEs are intermolecular "lubricants", added as an additive instead of reagents, therefore, they can leach from the matrix. This is because the weak covalent bond is formed between the PAEs and the plastic matrix (Chan, 2012).

Owing to leaching, PAEs were detected in the aquatic environment, including China, Taiwan, and Spain (Domínguez-Morueco *et al.*, 2014; Xu *et al.*, 2013; Yuan *et al.*, 2002). As a result, it could harm the aquatic organisms and accumulate in them. Significant level

on bioaccumulation has been found in aquatic organisms which are fishes, crustacean, mollusca, and algae (Net *et al.*, 2015). Moreover, humans may be exposed from ingestion (including breast milk), inhalation, intravenous injection tubing and solutions, and skin absorption (Barlow *et al.*, 2007). PAEs could cross the placenta too (Barlow *et al.*, 2007). Generally, PAEs are metabolised to monophthalate which could be more harmful and then into oxidative metabolites to be glucuronidated before excretion (Josh *et al.*, 2014). Their metabolites were found in human urine across several Asia countries (Guo *et al.*, 2011). The concentration of monophthalates in the environment may be increased as microbes could degrade PAEs into monophthalates (Josh *et al.*, 2014).

Phthalates could activate peroxisomes and induce cytochrome P450 activity, resulting in oxidative stress imbalance, for example, diethylhexyl phthalate (DEHP) could induce the peroxisomal proliferation (Mathieu-Denoncourt *et al.*, 2015). The critical mechanisms of toxic action of DEHP and diethyl phthalate are oxidative stress with the increasing products of lipid peroxidation in the membranes (Mankidy *et al.*, 2013). Zhou *et al.* (2011) found that peroxidase level and malondialdehyde increased at higher doses, concluded that PAEs are potential oxidative stressors. Apoptosis could be triggered due to oxidative stress, leading to mortality (Mankidy *et al.*, 2013).

PAEs are also endocrine disrupters and they have oestrogenic effects but some PAEs show anti-oestrogenic effects while certain PAEs exhibit anti-androgenic effect (Barlow *et al.*, 2007). In fish, PAEs are linked to reproductive abnormalities and embryonic toxicity (Xu *et al.*, 2013). They are also suspected to cause early onset of puberty in girls, resulting in premature breast development (Barlow *et al.*, 2007). Male genital development may be affected too (Barlow *et al.*, 2007). PAEs may inhibit the retinoic acid synthesis during early development of the male reproductive system, causing the malformations owing to low testosterone levels (Chen & Reese, 2016). They could influence sperm cells, sperm mobility,

cryptorchidism and hypospadias (Adewuyi & Olowu, 2012). Other than endocrine disrupters, PAEs have possible teratogenic and carcinogenic effects, including breast and liver cancer (Adewuyi & Olowu, 2012; Barlow *et al.*, 2007). A total of six PAEs have been banned from utilizing in toys and childcare products for more than 0.1% (w/w) by the European Union (EU) in 1999, while any phthalate cannot more than 1 part per million (ppm) in China and Taiwan (Chan, 2012; Zou & Cai, 2013).

2.1.1 Dipropyl Phthalate

Dipropyl phthalate (DPrP) or $C_{14}H_{18}O_4$ (molecular weight: 250.29) is also known as dipropyl 1,2-benzenedicarboxylate, involved in polymerisation of alkenes and production of fuel cells and plasticisers (Gangolli, 1999). Since it has a shorter alkyl chain, the retention time is shorter, easier to leach out from the product (Mathieu-Denoncourt *et al.*, 2015). Although DPrP is a synthetic substance, it is biodegradable in both aerobic and anaerobic conditions (Yuan *et al.*, 2002). However, it still can be found in packaged food, milk formulae, vegetable oil, house dust and air (Biomonitoring California, 2015; Saillenfait *et al.*, 2010).

Like the other PAEs, DPrP is suspected to be a carcinogen and having reproductive hazard (Thermo Fisher Scientific, 2015). 2012 OSHA Hazard Communication Standard considers it as hazardous (Thermo Fisher Scientific, 2015). It may cause cancer and damage fertility or foetus (Thermo Fisher Scientific, 2015). DPrP could be the substitute for more harmful phthalates but it still has an anti-androgenic effect (Dodson *et al.*, 2012). It had weak binding on both oestrogen receptor (OR) and androgen receptor but showed no transactivation activity or vitellogenin (VTG) induction (Organisation for Economic Co-operation and Development (OECD), 2010). Male offspring may show female-like symptoms if affected by anti-androgens, including reduced anogenital distance,

undescended testes and vaginal pouch (Gray *et al.*, 2001). Similar effects were observed by Heindel *et al.* (1989) and Saillenfait *et al.* (2010). Mathieu-Denoncourt *et al.* (2015) stated that DPrP could inhibit the oestrogen from binding to OR, thus, it is said to be anti-oestrogenic which is not supported by Okubo *et al.* (2003). Hong *et al.* (2005) postulated that the PAEs may be metabolised *in vivo*, thus, showing conflicting result between *in vitro* and *in vivo*. Table 1 on the next page shows the summary of several works on DPrP effects.

Table 1. Summary of DPrP effects from previous studies.

Species	Effect	Reference
MCF-7 cell (human breast cancer cell)	Neither oestrogenic nor anti-oestrogenic properties were found	Okubo *et al.*, 2003
Human neuroblastoma SH-SY5Y cell	Human nicotinic acetylcholine receptors (nAChRs) inhibited	Lu *et al.*, 2004
Rat liver cell	Cytochrome P450 activity was inhibited	Ozaki *et al.*, 2016
Gymnodinium breve (dinoflagellate species)	Growth reduced to half at 33 ppm	Gangolli, 1999
Green neon shrimp (*Neocaridina denticulate*)	Short-term increased in susceptibility to pathogen was observed	Sung *et al.*, 2011
Giant freshwater prawn (*Macrobrachium rosenbergii*)	Mortality rate at about 20% Hemocytes damaged with reduced immunity Other physiological responses may be affected which are related to immunity	Chen & Sung, 2005
Medaka (*in vitro*)	Binding affinities was weak (0.0018%-0.024%) No transactivation activity was observed on oestrogen receptor No transactivation activity was observed on androgen receptor No induction of vitellogenin activity	OECD, 2010
Sprague-Dawley rat	No significant effects on the weight and histopathology of the testis Maternal body weight gains and foetal body weight reduced Anogenital distance of male foetus decreased Undescended testis occurred Ossification delayed	Foster *et al.*, 1980 Saillenfait *et al.*, 2010
CD-1 Swiss mouse	Fertility reduced at 2.5% dose while infertility observed at 5% dose Body, testis and epididymis weight reduced but liver weight increased Epididymal sperm concentration decreased Seminiferous tubular atrophy occurred Lesions observed (mainly in the testis and epididymis) Interstitial cell hyperplasia occurred No significant histopathologic effects in reproductive system, liver or kidneys of female	Heindel *et al.*, 1989

DPrP has been found to be able to bind human peroxisome proliferator-activated receptors (hPPAR) although it is relatively low compared to other phthalates such as didecyl phthalate and diisononyl phthalate (Josh *et al.*, 2014). Josh *et al.* (2014) also found that it has less affinity than natural ligand of hPPAR which are retinoic acid, natural ligands-conjugated linoleic acids, and linoleic acid. However, due to its shorter alkyl chain, it could be more potent in affecting PPARs (Mathieu-Denoncourt *et al.*, 2015). Prolonged exposure may result in thyroid hormone axis disruption and different chronic diseases, including obesity (Shaz *et al.*, 2011). Toxicity and liver cancer were observed in rodents through the activation of PPARs due to internal exposure of PAEs as PPARs could modulate carcinogenesis too such as hepatocarcinogenesis in rodents (Bility *et al.*, 2004). Activation of PPARs owing to PAEs lowers the concentration required for PPAR activation (Bility *et al.*, 2004).

2.2 Developmental Toxicity Study

According to Ward (2014), developmental toxicity is the negative effects during pregnancy which could be due to parental exposure to chemicals. Embryotoxicity is the developmental toxicity on foetus without symptom on the mother while teratogenicity is similar to embryotoxicity but more on the structural development (Nakamura *et al.*, 2011). Haldi *et al.* (2012) explained that the teratogens are those chemicals that can cause abnormalities in embryo morphology or organ function before the embryo dead. Developmental toxicity study involves embryogenesis which is very susceptible to toxicant exposure, thus providing a better safety data for human daily intake or consumption (Selderslaghs *et al.*, 2009). Median lethal concentration (LC_{50}) explains the lethality of the chemicals during the development (Haldi *et al.*, 2012).

There are several types of testing for developmental toxicity study that are *in vivo* test methods which introduce the test chemical to pregnant animals to identify the effects on them and their offspring (Selderslaghs *et al.*, 2009). To reduce the usage of animals and time consumption, non-mammalian species or invertebrates can replace the mammalian species such as chicken, fruit flies, fish and frogs (Seldeslaghs *et al.*, 2009). In Europe, acute fish toxicity data that came from developmental toxicity test are required to determine the hazardous of industrial chemicals (European Union Reference Laboratory for Alternatives to Animal Testing (EURL-ECVAM), 2014).

There are advantages using fish as a test subject for toxicity study. Fish such as zebrafish are well-developed animal model used for experimental purposes. Besides, since many chemicals could accumulate in the aquatic environment, the aquatic organisms are at a higher risk compared to the others (Lammer *et al.*, 2009). It could also be used as a bioindicator to monitor the water source that serves for recreational and drinking purposes. The fish is important as a human food resource and in aquatic food webs for the regulation of nutrient and energy flow (Lammer *et al.*, 2009).

2.3 Endocrine Disruption

Endocrine disruption chemicals (EDCs) change the adaptation of organism by interfering the endocrine system which involves in many vital functions (Shaz *et al.*, 2011). They may cause epigenetic imprinting, neurologic development deficits, obesity and even diabetes mellitus (Shaz *et al.*, 2011).

DPrP could be both oestrogenic and anti-oestrogenic based on Table 1 on page 7. On the other hand, Nishihara *et al.* (2000) stated that DPrP is oestrogenic with 1×10^{-3} of 10% relative effective concentration of 10^{-7} M 17β-estradiol (REC_{10}). Begum *et al.* (2004) found

that DPrP is one of the metabolites of dibutyl phthalate (DBP) by *Bacillus natto*. Thus, the study on DBP may help in hypothesising the effect of DPrP.

DBP showed anti-androgenic activity in lower concentration but exhibited androgenic activity in higher concentration *in vitro*, this may explain the reason of DPrP was found to be oestrogenic, anti-oestrogenic and neither oestrogenic nor anti-oestrogenic (Shen *et al.*, 2009). However, DBP had a weak estrogenic effect (Shen *et al.*, 2009). Bhatia *et al.* (2013; 2014) found that DBP exhibits anti-oestrogenic effects in female Murray rainbowfish but oestrogenic effects in the male. The oestrogenic activity of DBP may be similar to the androgenic activity that it varies with different conditions although it is within the similar species. Eye development of Medaka embryos was affected by DBP. This could be related to the oestrogen receptor activity (Tang, 2012). Chen *et al.* (2014) observed that the PAEs caused several symptoms on zebrafish which are death, tail curvature, necrosis, pericardial oedema and no response on touch. The cholesterol transport and steroidogenesis were influenced by DBP through the downregulation of expression of the related genes (Li *et al.*, 2016).

Thyroid hormones (TH), T_3 (triiodothyronine) and T_4 (thyroxine) are present in both eggs and embryos, whereby T_4 is the precursor of T_3 (Funkenstein *et al.*, 2000). THs are vital to regulate the development, somatic growth, energy provision, reproduction in vertebrates, metabolism, and heart function (Mathieu-Denoncourt *et al.*, 2015; Shen *et al.*, 2011). They are needed for the embryonic and larval development to regulate the organogenesis as well (Liu & Chan, 2002).

Endocrine disruptor or goitrogen may disturb the synthesis, secretion, transport, binding, action, or elimination of THs (Zhai *et al.*, 2014). Moreover, it could affect the downstream gene regulation through the disruption of co-repressor assembly and functions, including DNA methylation and histone modification (Shen *et al.*, 2011). In addition, they

could act as a competitive inhibitor of OR as excess liganded TH receptors may bind to the same DNA sequences in oestrogen-regulated genes, resulting in oestrogen inhibition (Lechan & Fekete, 2005). Oestrogen not only works as sex hormones but also influence multiple tissues including eye development, cardiovascular and skeletal systems (Ahi *et al.*, 2016; Hamad *et al.*, 2007). ORs could be found in liver, pancreas, heart, muscle and brain of the embryo (Bondesson *et al.*, 2016).

It was found that PAEs could increase the T_3 and T_4 level but the mechanism of action remains unclear, possible through the induction of hyperactivity of the thyroid gland or the enhancement of iodide intake (Mathieu-Denoncourt *et al.*, 2015). Unlike the other PAEs, dibutyl phthalate could act as an anti-thyroid hormone and antagonise T_3 action at the transcriptional level (Shen *et al.*, 2011). Therefore, it is possible that DPrP could have the similar effect on thyroid hormone.

2.4 Transthyretin

Transthyretin (TTR) is one of the TH-binding proteins in the bloodstream, playing a role in THs transport and metabolic processes related to the thyroid axis through the regulation of free plasma levels of THs (Morgado *et al.*, 2007). Other than TH, it can bind retinol-binding protein (RBP) to aid in retinol transport (Funkenstein *et al.*, 2000). In mammals, as in response to inflammation or infection, TTR is depressed (Morgado *et al.*, 2007). It also can be used to indicate the malnutrition or stress (Morgado *et al.*, 2007). THs regulate the TTR synthesis (Morgado *et al.*, 2007). As the TTR of teleost fish response to the THs similar to mammals, it can play its role as TH-binding proteins to transport the T_4, yet, the retinol-binding ability is unclear (Morgado *et al.*, 2007).

Oestrogen downregulates the liver *ttr*, by injecting oestrogen to the adult fish, whereby the untreated females have a lower *ttr* level compared to untreated males (Funkenstein *et al.*, 2000).

Mono-(2-ethylhexyl) phthalate could reduce the *ttr* transcription, thus, more free THs are unbound and eventually the excess T_4 will be eliminated via hepatic catabolism, leading to a reduction in circulating TH concentration (Zhai *et al.*, 2014). In turn, this will change the transcriptional level of the genes involved in the hypothalamic-pituitary-thyroid axis (Zhai *et al.*, 2014).

2.5 Zebrafish

Zebrafish (*Danio rerio*) originates from India, Burma, Malacca and Sumatra (Braunbeck & Lammer, 2006). It is a small fish that can survive in both soft and hard water at 26 °C (Braunbeck & Lammer, 2006). Male zebrafish has a slimmer body shape and reddish tint in its silver bands while the female has a swollen belly, making the sexes can be distinguished easily (Braunbeck & Lammer, 2006). Figure 2 shows two zebrafishes with different sexes. Egg spawning rate is 50 to 200 per days (Braunbeck & Lammer, 2006).

Figure 2. The male zebrafish (lower individual) and female zebrafish (upper individual). Adapted from Braunbeck & Lammer, 2006.

Zebrafish is a popular animal model for scientific study purpose (Braunbeck & Lammer, 2006). Besides, it outstands the mice as an animal model for as it has several

advantages which are lower cost, external fertilisation, egg transparency ease for observation and high fecundity (Braunbeck & Lammer, 2006). The embryo does not require external feeding (Lantz-McPeak *et al.*, 2014). 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). It has a thyroid system that is similar to the mammalian and amphibian thyroid system (Zhai *et al.*, 2014). Therefore, it can be used to extrapolate the findings to other vertebrates and humans in thyroid endocrine system disruptor.

The zebrafish toxicity assay is meant to determine lethality and toxicity curves (Haldi *et al.*, 2012). Many studies have been done on zebrafish to determine the toxicity endpoints or mechanisms of a chemical, vary from medium to high throughput experiments (Lantz-McPeak *et al.*, 2014; Selderslaghs *et al.*, 2009). This is because multi-well plates can be used for high-throughput experiments without harming the embryos (Lantz-McPeak *et al.*, 2014).

By 24 hpf, the cerebellum could clearly mark the hindbrain/midbrain boundary which assists in observation (Kimmel *et al.*, 1995). Besides, at this stage, the tail detachment endpoint can be determined as well (EURL-ECVAM, 2014). The heart is also formed and visible at 24 hpf (Kimmel *et al.*, 1995). At 48 hpf, heartbeat should be visible (Kimmel *et al.*, 1995). As the cartilage development in jaws begins during hatching period (48-72 hpf), craniofacial morphology could be checked at 72 and 96 hpf (Kimmel *et al.*, 1995).

One of the reasons that the test ends at 96 hpf is that the zebrafish embryo relies on maternal TH from the yolk until around 72 hpf when the embryonic thyroid is activated (Bagci *et al.*, 2015). Moreover, the zebrafish does not require feeding within this period (EURL-ECVAM, 2014). This can avoid the ethical issue as it does not violate the rule of protection of laboratory animals (EURL-ECVAM, 2014). The data are also more comparable and reproducible for other laboratories at 96 hpf (EURL-ECVAM, 2014). Other