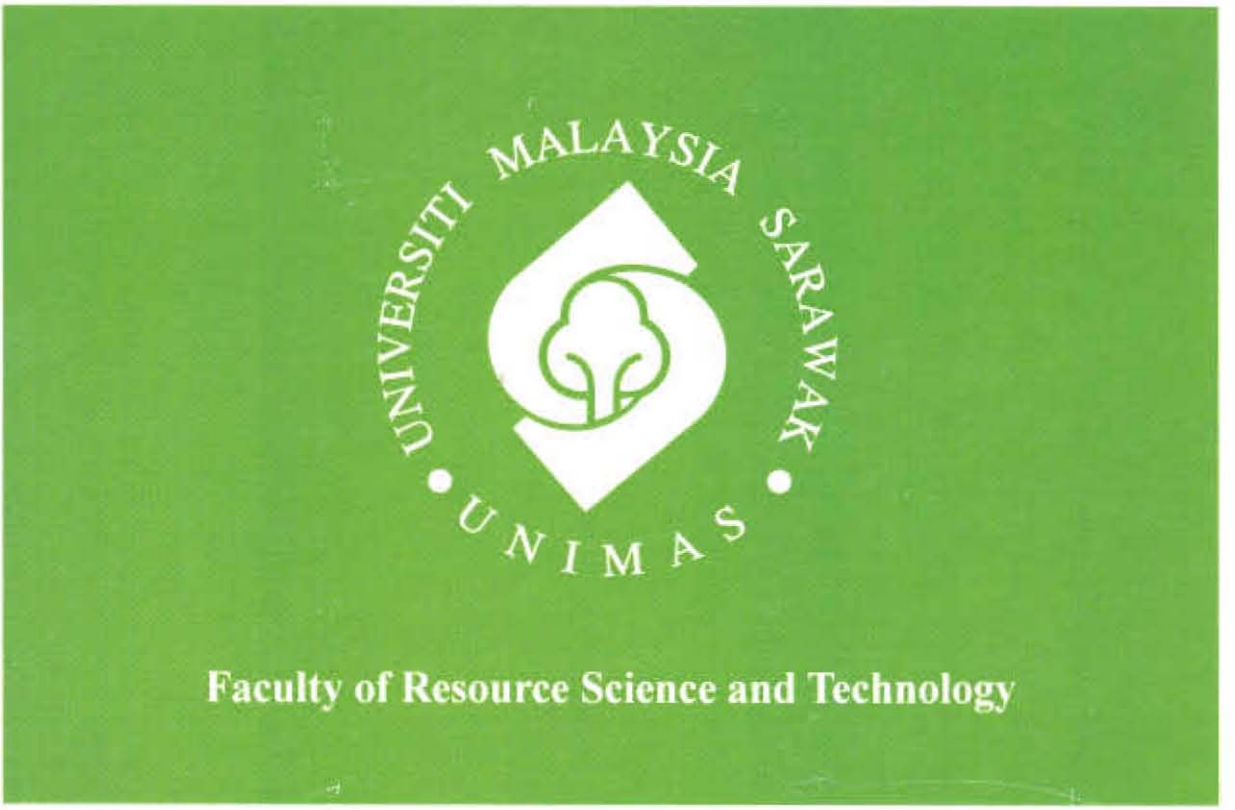



# Effect of Toxicity of Phthalate Esters during Early Vertebrate Development of Zebrafish (*Danio rerio*)

**Mohamad Shamil Faris Bin Mohamad Khalid**

**Master of Science**
**2018**

Pusat Khidmat Maklumat Akademik
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Mohamad Shamil Faris Bin Mohamad Khalid

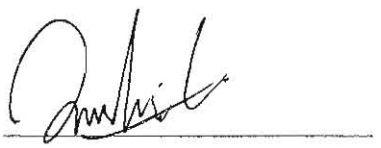
A thesis submitted

In fulfilment of the requirements for the degree of Master of Science (Biochemistry)

Faculty of Resource Science and Technology
UNIVERSITI MALAYSIA SARAWAK
2018

## DECLARATION

The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.



Mohamad Shamil Faris Bin Mohamad Khalid

Matric No: 15020157

Date: 18th March 2018

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# ABSTRACT

Diallyl phthalate (DAP) and dipropyl phthalate (DPP) belong to a group of chemicals called phthalate esters (PAEs) which are commonly used as plasticisers. However, higher temperature of the environment is able to break the weak bond of the weak bonding between these chemical with polyvinyl chloride (PVC) allow it to leak into the environment. These two chemical was listed as carcinogenicity and endocrine disrupting potentials. This study was set out to study the $LC_{50}$ and the morphological defect of DAP, DPP and DPP+DAP chemical on zebrafish embryonic development. Toxicity of the chemicals is determined by using probit analysis. The $LC_{50}$ are measured at 96 hpf. Morphological defect such as unhatched, pericardia edema, yolk sac edema, uninflated swim bladder and tail deformation, which were measured at 96 hpf. The $LC_{50}$ was determined to be at 6.58 mg/l for DPP, 5.12 mg/l for DAP and 1.71 mg/l for DAP+DPP with 95% confidence level. The $LC_{10}$ was determined to be at 3.80 mg/l for DPP, 3.60 mg/l for DAP, 0.92 mg/l for DAP + DPP with 95% confidence level. The thyroid hormone (TH) gene marker used in this study are; diodinase 1(*dio_1*), diodinase 2(*dio_2*), TH receptor alpha (*trα*), TH receptor beta (*trβ*), thyroid stimulating hormone beta (*tsh_β*) and transthyretin (*ttr*). This study demonstrated that DPP, DAP, DPP+DAP induced developmental toxicity, impaired the structure and functions of developing organ, and altered the expression of the thyroid hormone gene marker.



**Keyword**: Dipropyl phthalate, diallyl phthalate, *Danio rerio*, $LC_{50}$

*Kesan Ketoksikan Ester Ftalat semasa Pengembangan Awal Vertebrata Ikan Zebra* **Danio rerio**

## ABSTRAK

*Dialil ftalat (DAP) dan dipropil ftalat (DPP) tergolong di dalam kumpulan bahan kimia yang dipanggil ester ftalat (PAEs) sangat dikenali sebagai bahan pemplastik. Walaubagaimanapun, suhu panas persekitaran mampu untuk meleraikan ikatan yang lemah hasil daripada ikatan bahan ini dengan polivinil klorida (PVC) menyebabkan ianya mengalir ke persekitaran. Dua bahan kimia ini telah diklasifikasikan sebagai kasinogenik dan juga berpotensi untuk merencat endokrin. Kajian ini dibuat untuk mengkaji kesan $LC_{50}$ dan kesan morfologi daripada DAP, DPP dan DPP+DAP tersebut semasa pengembangan embrio (ikan zebra). Ketoksian bahan kimia ini diukur dengan mengunakan analisis probit. $LC_{50}$ diukur pada 96 jam selepas pesenyawaan (jsp). Kesan morfologi seperti tidak menetas, bengkak perikardia, bengkak kantung telur, bengkak pundi udara, ekor tidak sempurna, yang akan diukur pada 96 jsp. $LC_{50}$ untuk DPP telah ditentukan pada 6.58 mg/l, 5.12 mg/l untuk DAP dan 1.71 mg/l untuk DAP + DPP dengan tahap kenyakinan pada 95%. $LC_{10}$ ditentukan pada 3.80 mg/l untuk DPP, 3.60 mg/l untuk DAP, 0.92mg/l untuk DAP+DPP dengan tahap kenyakinan pada 95%. Gen hormon tiroid (TH) seperti diodinase 1 (dio_1), diodinase 2 (dio_2), reseptor TH alpha (trα), TH beta reseptor (trβ), hormon beta merangsang tiroid (tsh_β) dan transthyretin (ttr) digunakan sebagai gen penanda. Kajian ini menunjukkan DPP, DAP, DPP+DAP menyebabkan ketoksikan perencatan pengembangan, menjejaskan struktur dan fungsi pengembangan organ dan juga mengubah ungkapan penanda gen bagi gen hormon tiroid.*

***Kata kunci**: Dipropil ftalat, dialil ftalat,* Danio rerio, $LC_{50}$

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| DAP | Diallyl Phthalate |
| DPP | Dipropyl Phthalate |
| hpf | Hour Post Fertilization |
| EU | European Union |
| ECPI | European Council for Plasticisers |
| PVC | Polyvinyl Chloride |
| LOQ | Limit of quantification |
| LOD | Limit of detection |
| LOL | Limit of linearity |
| LOAEL | Limit observe adverse effect level |
| NOAEL | No observe adverse effect level |
| TDI | Tolerable daily intake |
| GC/MS | Gas Chromatography/Mass Spectrometers |
| GC/FID | Gas Chromatography/Flame Ion Detector |
| HPLC | High-Pressure Liquid Chromatography |
| LLE | Liquid – Liquid Extraction |
| UV-VIS | Ultraviolet-Visible spectrophotometry |
| $LC_{50}$ | Median Lethal Concentration |
| $LC_{100}$ | Absolute Lethal Concentration |

# CHAPTER 1

# INTRODUCTION

## 1.1 Introduction

Ecological pollution is a difficult issue that had been connected to the improvement, extension of modern culture, and to raise the population density during 20$^{th}$ century. Ecological defilement is escalated by the colossal generation of anthropogenic chemicals with properties great for a particular reason. In the past 80 years, the production of chemicals had increased from 1 to more than 400 million tonnes annually (Vogelgesang, 2002). Also, various chemicals are much of the time introduce in consumer things, which we reliably keep running over in the regular day to day existence. Besides that, the main problem is the presence of these chemicals into the environment, particularly in our aquatic ecosystem.

It is intuitively obvious that aquatic organism such as fish is continuously exposed to number of waterborne toxicants from different sources. The presence of unknown toxic chemical can be detected by measuring the effect on the physiological mechanism in an aquatic organism (Scott & Sloman, 2004). Therefore, these have been traditionally regarded as an important component of toxicity testing strategies. A variety of potential sources and routes of toxicants exposure exits in an aquatic system, some distinctly different from those terrestrial animals. Sea-going life form assume a critical part as early cautioning and observing framework for poison trouble in nature. Fish show a huge decent variety spoke to by more than 20,000 species and crossing more than 400 million years of (Marshall, 1996).

Unlike terrestrial animals, fish live in atmosphere virtually devoid of oxygen. Consequently, fish extract volumes of water weighing thousands of times of their body weight to stratify their oxygen demand. The integument and gills of an oceanic life form, which contrasts in penetrability as indicated by species, are imperative destinations of contaminant take-up. In fish, the effective circulatory framework permits the quick appropriation of contaminants crossing the hindrances.

Aquatic organism may amass chemicals specifically from the water. Bioaccumulation appraisal is especially essential for understanding the potential danger of contaminants, their destiny, and progression of sea-going species. The harmfulness of a synthetic is reliant on the degree of its bioaccumulation in a living being and especially the amount achieving the objective organ or tissue to animate a reaction.

Beginning times of advancement are thought to be extremely touchy to substance introduction, particularly for an oviparous creature like fish and fledgling (Von Westernhagen, 1988; Russell, Gobas & Haffner, 1999; Nagel, 2002). "Developmental toxicity" is a fairly more extensive term than "teratology" covering embryo toxicity, trade off development, practical insufficiency in the posterity (larval stage) and morphological variations from the norm. Toxicant applies their impact especially amid the basic times of advancement. Escalated explore has been directed on endocrine disturbances, concentrating on interruption of sex hormones, sexual separation and regenerative framework (Vinggaard, Hnida, Breinholt & Larsen, 2000; Orn, Holbech, Madsen, Norrgren & Petersen, 2003; Meucci & Arukwe, 2005; Andersen et al., 2006).

A few testing rules have been produced by United State Environmental Protection Agency (US-EPA) and the Organization for Economic Co – operation and Development (OECD), European Union - Registration, Evaluation, Authorizations and Restriction of Chemicals (EU-REACH) and other administrative bodies. Persistent endeavors are being made to refine and increment the affectability and specificity of the test strategies, by adding extra sub deadly endpoints to address the impacts on improvement, sensory system, physiology, conduct, and proliferation, all of which may be similarly vital in natural significance over the long haul, however happen at prior circumstances and lower introduction levels (Darland & Dowling, 2001; Orn, Holbech, Madsen, Norrgren & Petersen, 2003; Peitasaro, Kaslin, Anichthik & Panula, 2003; Fort et al., 2004a; Fort et al., 2004b; Holbech et al., 2006; Hori et al., 2006 ; Gunnar & Quevedo, 2007).

This lethality testing is imperative in the hazard evaluation to recognize an issue with a specific end goal to build up a documentation for the hazard appraisal by distinguishing what is the worry that should be tended to, how the worry was raised and whether the issue is agreeable to chance appraisal (enHEALTH, 2004). The issue that has been recognized may have identified with discernments, science, financial aspects and social variables. For an illustration, it might be accounted for that a toxicant could be available in a water supply. This could be sporadic or be a customary event.

Sometimes, the reason might be known and along these lines been overseen. If the pollution cannot be identified by chemical analysis then an investigation is required by using investigative monitoring methods. So as to examine this issue further, the organic testing (bioassays) that are focused towards recognizing perils to human and additionally

biological wellbeing can be utilized as a component of a coordinated procedure of examination.

There is a lack of knowledge on phthalate esters compound despite their wide distribution in our daily products. Most of the PAEs chemical have been restricted for more than a particular concentration in consumer products by the European Union (EU) (Zou & Cai, 2013). Phthalates have been detected in many body tissues including urine, blood, semen, amniotic fluid, and breast milk (National Research Council, 2008). In Malaysia, the metabolite of the phthalates ester can be found in the range of 94.9 ng/mL in human urine (Guo et al., 2011). These chemicals have been rated as Category 2 for both carcinogenicity and reproductive toxicity due to lack of evidence (Thermo Fisher Scientific, 2010).

Besides that, the possible negative effects of phthalate esters compound on human health and development still remain largely unknown. The phthalate esters is suspected to be a carcinogenic, mutagenic and endocrine disruptor toward human (Fatoki et al., 2010). There is less knowledge regarding the use of zebrafish as a model animal to evaluate the phthalate esters compound in term of teratogenic and toxicogenomic study. Zebrafish is a model organism that had been recommended as the model organism for toxicology study by the European Scientific community and OECD (OECD, 1992; Nagel, 2002; Braunbeck et al., 2005). Moreover, zebrafish is vertebrate animal that have 76 % genome similarities to human genome and also have fast life cycle from embryo to juvenile fish.

The Ministry of Health has risen their concern on the obesity issue among the children in Malaysia. The obesity is not only related to the diet of the person but it also causes by

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other factor such as disruption of endocrines system in human body. The endocrines system also involve in the maintaining of our metabolism system that indirectly control of our body system react towards obesity diseases. Based on the previous study, obese children have higher detection of phthalates compare to non-obese children, the phthalates compound was detected in their body fluid (Rabasseda, 2012).

## 1.2 Objectives

The general objectives of this study are to identify the phthalate esters (PAE) effects on early vertebrate development in zebrafish (*Danio rerio*).

The main objectives are :

I. To determine the $LC_{50}$ of phthalate esters on the zebrafish embryonic development;

II. To characterise the morphological defects of phthalate esters exposure on the development of the organs throughout the zebrafish embryonic development;

III. To analyse the 6 endocrine disruptors gene marker expression of zebrafish when exposed with phthalate esters.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Phthalate Esters

Registration, Evaluation, Authorization of Chemical (REACH) in the EU have listed about 15 phthalate esters as Substances of Very High Concern (SVHC). This SVHC list is from the REACH Community Rolling Action Plans (CoRAP Reviews) and was published in October 2011. Six phthalates have been included in the CoRAP Reviews and were suspected to be Carcinogenic, Mutagenic, and Reproductive (CMR), Persistent, Bioaccumulative and Toxic (PBT) substances or Endocrine Disruptors.

Since the year 1950, these chemicals were widely used and phthalates usually released into the atmosphere, food or liquid. Other than that, phthalates are found amazingly in varies of products include toys, tubing, blood bags and polish. According to the European Council for Plasticizers and Intermediates (ECPI) (2014), phthalate is formed due to the product of the chemical reaction between acid and alcohol. Phthalate esters are derived from the phthalic acid, by the reaction of phthalic anhydride with alcohol(s) that range from the methanol up to tridecyl alcohol either as a straight or with some branching chain. Phthalate families formed based on the chemicals depend on the number of carbon atom at their molecular backbone. A phthalate is a group of chemical which is also known as phthalate ester that is used as a plasticiser to impart more flexibility and strengthen the structure of the plastic.

The plasticiser is synthetics ingredient used to promote flexibility and durability in plastic found in medical products, floors, and food processing materials (Hubinger, 2010). Phthalates are also used as gelling agents in many of cosmetic and beauty products: shampoo, hairspray, hair gel, deodorant, nail polish, and body lotion. Phthalates are used in fragrances because they are responsible for making the scent last longer.

The world consumption of plasticiser in 2014 is approximately at 6.4 million tonnes; more than 95 % of plasticisers that are used in flexible PVC. 70 % of the plasticisers are from the phthalates ester and 30 % of non-plasticizers are used in the world (Cullen, 2012). In 2014, the most regional demand in the world come from the China with the cumulative 43% from the whole world usage of phthalate ester in Figure 1. The world distribution of the phthalates and non – phthalate plasticisers in Figure 2. This non – phthalates plasticisers chemical have emerged because of the awareness of dangers of this chemical to the environment and human health. Plasticiser molecules are effectively dispersed throughout the PVC and attached themselves to the surfaces of the polymer particles by physisorption. A Van der Waals interaction takes place between the plasticiser and polymer particles (Shashoua, 2001).

Human can be exposed to phthalates chemical by digesting foods that have been in contact with containers and products containing phthalates. The use of plastic containers has been a constant increase in all fields of human activities, resulting in the portion of plastic in total solid waste growing at a rate of 1 % per year. Plastic solid waste, especially which from plastic food containers, poses a great problem due to both its volume and relative non-biodegradability.

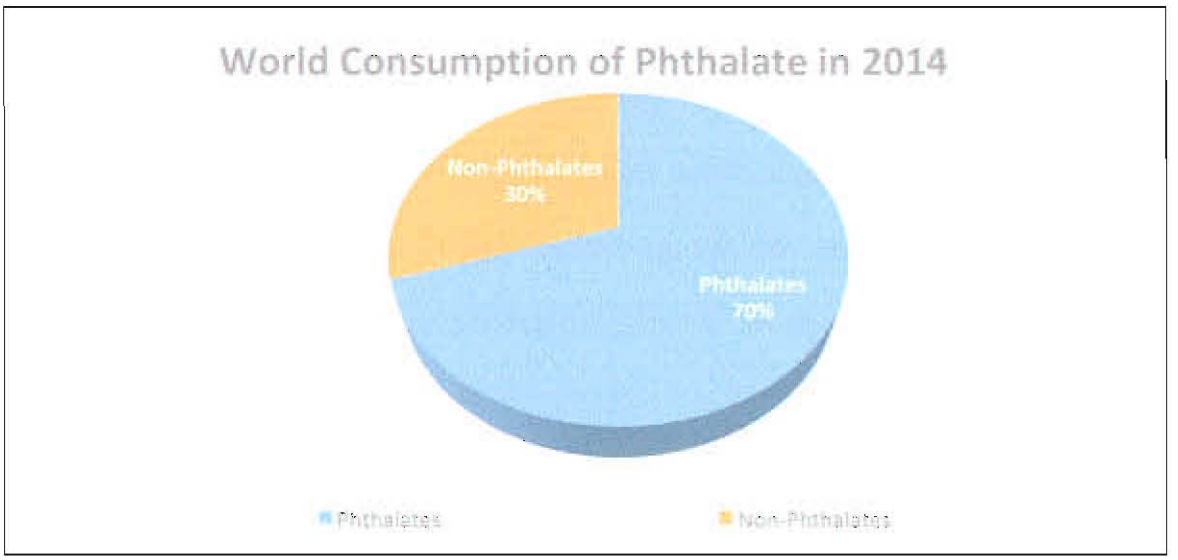



**Figure 1:** Regional Demand for Plasticizers – 2014 (Cullen, 2012).

Plasticisers can be leak out from plastic as phthalates when it is exposed to weather conditions undergoes degradation thus it is released migrate to the waters intended for human use

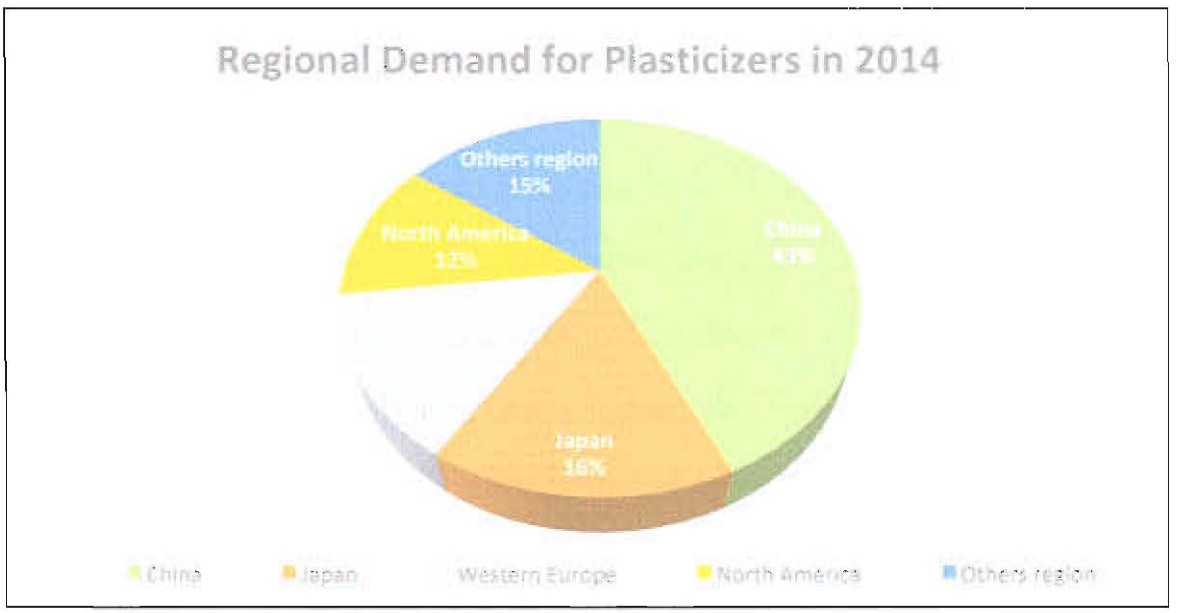



**Figure 2 :** Phthalates and Non - Phthalates world distribution - 2014 (Cullen, 2012)

Based on research conduct by (Bosnir et al., 2007), they have discovered that the phthalate particles can be migrated from a plastic container into soft drink and mineral water. The migration of phthalate particles into the soft drink is based on the preservative used in the soft drink. The most common of preservatives used in the soft drink is orthophosphoric acid, Na-benzoate, and K-sorbate; the highest phthalate levels were measured in soft drink is K-sorbate which is at 759.80 of 819.40 μg/l. The dimethyl phthalate has the higher chance to be migrated into the soft drink. The phthalate particles transfer from the plastic container to soft drink is 5 – 40 times higher compared to migration into the mineral water. Dibutyl phthalate showed the highest level of migration into mineral water compare to others phthalates which are at 11.33 of 20.22 μg/l (Bosnir et al., 2007). It is also showed that dibutyl phthalate has the higher risk to be transferred into the mineral water compare to a soft drink.

The plastic containers that are used in microwave ovens are usually made of polypropylene (PP), where the use of plasticisers is not essential and its addition should not be intentional. Therefore, phthalate migrations test are often not required for PP packaging. In 2005, Shen reported that the presence of phthalates at concentrations of 1.44 mg/kg for dibutyl phthalate (DBP), 0.10 mg/kg for benzyl butyl phthalate (BBP), and 8.72 mg/kg for di-(2-ethylhexyl) phthalate (DEHP) in plastic containers that were being used in microwave ovens. Most of the containers used for microwave operations are inert, however, studied done by (Moreira, Andre & Cardeal, 2014), have shown that the migration of various chemical substance may occur during microwave heating. DBP was found in a concentration ranging from < LOQ to 7.5 μg/l.

If the container were used for a prolonged time, the migration will be increased which correlated with increasing heating time. The (Moreira, Andre & Cardeal, 2014) has established the tolerable daily intake (TDI) for phthalates at 0.01 mg/kg body weight per day for DBP based on toxicological studies which regard to health effects, many types of food can be contaminated by phthalates during the manufacturing process, resulting in an even higher concentration of phthalates in food.

Lesser extent exposure can also occur from breathing in air that contains phthalate vapours or dust contaminated with phthalate particle. Once phthalates enter a person's body, they are converted into breakdown product (metabolite) that passes out through urine. In the Fourth National Report on Human Exposure to Environmental Chemicals (Health, 2009) CDC Scientists measured 13 phthalate metabolites in the urine of 2,636 or more participant aged six years and older who took part in the National Health and Nutrition Examination Survey (NHANES) during 2013-2014. By measuring phthalate metabolites in urine, Scientists are ahle to estimate the amount of phthalate that has entered people's bodies. Research has found that adult women have higher levels of urinary metabolites than men for those phthalates that are used in soaps, body washes, shampoos, cosmetics and similar personal care products. In 2009, CDC also found that because of hand-to-mouth behaviours of young children, they have a higher risk of being exposed to phthalate particles in dust than adults. Phthalates are also found in both inert and active ingredients in some pesticide formulations.

Phthalate metabolites have been detected in many body tissues including urine, blood, semen, amniotic fluid and breast milk (National Research Council, 2008). There are two types of phthalates which include high-molecular-weight and low-molecular-weight. Low molecular weight phthalate consist of three to six carbon atoms in their backbone whereas high molecular weight phthalate has seven or more atom in the backbone. Human exposure to phthalate can occur through inhalation, ingestion, and dermal contact. Once it is absorbed, they are rapidly metabolised to monoesters, and the high-molecular-weight monoesters can undergo further oxidation to form oxidative metabolites. The higher amount of low-molecular-weight phthalate metabolite concentration were associated with poorer scores on the aggression, attention problems, conducts problem, and depression all of these data were statistically significant (Engel et al., 2010).

In Europe, high molecular weight phthalate is found to be safe in current application (ECPI, 2016). In 2015, Chemical and Engineers News stated that five carbon chains will disrupt the sexual development three times as DHEP. Other than that, the length of linear chain phthalate needed to be four to six carbon atoms to produce testicular toxicity. In the study by Lovekam Harrison (2010) mentioned scientists have begun to suspect that phthalates might be connected to a massive drop in male fertility globally over the past few decades. This is due to the repeated studies have shown sperm counts have decreased by about 50 percent in the past half century including with the sexual development of boys in the womb.

Development of fetal rats was being used to investigate the effect of exposed phthalate and found that this chemical interferes with the sexual development (Harrison, 2010). In 2013, Lovekamp-Swan & Davis study mentioned short or long chains are not reproductive toxicant. Besides, in the past few years, there are many diseases that researchers linked to the phthalates exposure that included asthma, attention-deficit hyperactivity disorder, breast cancer, obesity and type II diabetes, low IQ, neurodevelopmental issues, behavioural issues, autism spectrum disorders, altered reproductive development and male fertility issues (CDC, 2015).

### 2.1.1 Dipropyl phthalate

Dipropyl phthalate is a phthalate metabolite of dibutyl phthalate and is the dipropyl ester of benzene – 1, 2 – dicarboxylic acid. Dipropyl phthalate is a clear colourless liquid that is mostly used in organic intermediates and chemical reagent. This chemical is used to make plasticisers and polymer additive. The molecular formula of this chemical is $C_{14}H_{18}O_4$ and the structural formula in on Figure 3. Based on Table 1, dipropyl phthalate is classified under low molecular weight phthalate and believed to be dangerous to living things and shows degradation with potential genotoxic effect.

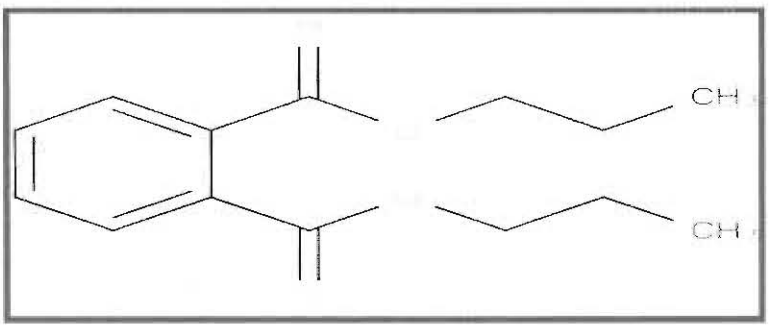



**Figure 3:** Aromatic hydrocarbon structure of dipropyl phthalate.

**Table 1:** General properties of dipropyl phthalate.

| **Molecular Formulae** | $C_{14}H_{18}O_4$ |
|---|---|
| Molar Mass | 250.29 |
| Density | 1.082 g/cm3 |
| Flash Point | 166.289 °C |
| Boiling Point | 317.28 °C at 760 mmHg |
| Solubility in Water | Slightly soluble |
| Solubility | soluble in alcohol |

Dipropyl phthalate is an ester since ester reacts with acids in order to liberate heat along with acids and alcohols to interact each other. Furthermore, in order to ignite the reaction product, strong oxidising acid is used and cause a vigorous reaction. This chemical is combustible and fires hazard when exposed to heat and flame. The flammable hydrogen is generated by the reaction of esters with alkali metals and hydrides.

The interaction between esters with caustic solutions will generate the heat. Since there are lacks of knowledge on this chemical, Acros Organics (2015) conducting their studies and researches and they suspected this chemical may cause cancer and damage fertility or the unborn child. DPP is one of the metabolites from the dibutyl phthalate (DBP). These chemical have been found in the daily product with the various concentration as stated in Table 2 and Table 3. There are several studied had been done to study the effect of this chemical toward animal model as stated in Table 4.

**Table 2:** Various concentrations of phthalate chemical in the daily product.

| Compound | Concentration (µg/kg) | Sample | Instrumental | LOD | LOQ | References |
|---|---|---|---|---|---|---|
| DBP | 7.30 to 50.3 | Commercial whole milk | GC/MS | 0.09 | - | Casajuana & Lacorte, (2004) |
| DBP | <LOD to 490 | Olive Oil | GC-MS/MS | - | - | Cavaliere, Macchione, Sindona & Tagarelli, (2008) |
| DBP | 10.2 to 142.8 | Meat based, fish based, dairy, vegetables, condiments, fresh fruit, bread | LLE | - | - | Cirillo, Fasano, Castaldi, Montuari & Coccheiri, (2011) |
| DBP | <LOD to 244 (µg/L) | Wines | SPE | 18 | 29 | Del Carlo et al., (2008) |
| DBP | 4.07 to 9.79 | Cow milk | GC-MS | 0.02 a 072 | - | Feng, Zhu & Sensentein, (2005) |
| DBP | 0.001 to 0.003 | Water (food simulant) | GC-MS,HPLC/UV-VIS and HPLC fluorescence | 2.0 X 10-3 | 5.0 X 10-3 | Gonzalez-Castro et al., (2011) |
| DBP | 32 and 34 (µg/L) | Water released from disposable PP soup bowl and PVDC wrap film | HPLC/UV | 0.40 (µg/L) | - | Jen & Liu, (2010) |

*LOQ: Limit of Quantitation
*LOD: Limit of Detection

**Table 3:** Phthalate concentrations that have been reported.

| Compound | Concentration (µg/kg) | Sample | Instrumental | LOD | LOQ | References |
|---|---|---|---|---|---|---|
| DBP | 22 to 360 | Commercial vegetables Oils | GC-MS | 10 | 40 | Nani et al., (2011) |
| DBP | <LOD to 175 | Olive Oil | GC/MS/MS | 30 | - | Rios, Morales & Marquez-Ruiz, (2010) |
| DBP | <LOD to 9,840 | Plastic containers | GC/MS | 10 | - | Shen, (2005) |
| DBP | 0.17 mg/L | Bottled mineral water | GC-FID | 0.5 mg/L | 1.7 mg/L | Kanchanamayoon, Prapatpong, Chumwangwapee & Chaithongrat, (2012) |
| DBP | 5 to 32.01 µg/L | Bottled mineral water | GC/MS | 0.01 µg/L | 6.6 ng/L | Dumitrascu, (2012) |
| DBP | 0.2 to 7.5 | Water (food simulant) | GC/MS | 0.08 µg/L | 0.2 µg/L | Moreira, Andre & Cardeal, (2014) |

*LOQ: Limit of Quantitation
*LOD: Limit of Detection

**Table 4:** Acute toxicity of dibutyl phthalate.

| Route | Species (strain) | Endpoint | Dose | Effect | References |
|---|---|---|---|---|---|
| Inhalation | Rat | $LC_{50}$ | 4250 mg/m3 | Lung and brain. | ATSDR, (2001) |
| Oral | Rat (Ficher-344) | LOAEL | 1540 mg/kg | Hypospermia of the epidedermis (in male) | NTP, (1995) |
| | Rabbit | $LC_{50}$ | 100 mg/kg | | Clearsynth, (2015) |
| | | | 3200 mg/kg | Eyes | |
| | Mouse | $LD_{50}$ | 300 mg/kg | | |
| | Rat | | 980 mg/kg | Reproductive | |
| Dermal | Rabbit | $LD_{50}$ | > 20,860 mg/kg | Reproductive organ | Megaloid, (2012) |
| Intraperitoneal | Mouse | | <1.39-5.76 g/kg | Kidney, liver | Nematollani, Guess & Autian, (1967) |
| | p53-null mice | $LD_{50}$ | | presence of MNGs centers of seminiferous cord cross-sections | Safarini et al., 2011 |
| | Fish carp (*Cyprinus carpio*) | 96 h-$LC_{50}$ | 16.30 mg/L | liver | Zhao, Gao & Qi, (2014) |

*LD: Lethal Dose
*LC: Lethal concentration
*LOAEL: Lowest observed adverse effect level

#### 2.1.1.1 Toxicity study of dipropyl phthalate

When an organism reacts with PAEs, it does not cause an immediate toxic effect. Tsai and Sung (2013) had studied the effect of multiple teeth new rice shrimp (*Neocaridina denticulate*) due to the exposure to a sublethal dose (50 mg/ L) of phthalate esters, PAEs, the dipropyl phthalate. In their study, exposure to high concentration of shrimp dipropyl phthalate, DPP (50 mg/ L) will cause 6 mRNA gene expressions to decrease (Tsai & Sung, 2013). These 6 mRNA genes include three of immune and metabolic genes and three genes of unknown function. On the other hand, when the shrimp are exposed to the lower concentration of DPP (0.5 mg/ L), only four genes affected including the three well-known functions. On the other hand, Tsai and Sung also suggested that non-lethal dose of PAEs may cause potential water ecosystems crisis. In their studies, they conclude that DPP is mainly producing rice shrimp ecotoxigenicity. Ecotoxicogenomics is the study of gene and protein expression in the organisms that are important in responses to environmental toxicant exposures (Snape, Maund, Pickford & Hutchinson, 2004).

Besides, Lovekamp-Swan and Davis (2003) had studied about the mechanism of phthalate ester toxicity in the female reproductive system. They concluded the experiment on the female because they believed that female or woman have unique exposure profile to phthalate that is very harmful to such exposures. In their initial reproductive and developmental toxicity studies on phthalate, they used female rodent as the model organism and this rodent involves *in vivo* exposure during organogenesis. Phthalates that have very short (diethyl) or very long (dioctyl) side chains are not going to affect the reproductive system (Lovekamp-Swan & Davis, 2003).

Moreover, in their studies, they explained that DEHP is the most potent reproductive toxicant among the phthalates, followed by dihexyl phthalate (DHP), dibutyl phthalate (DBP), and dipropyl phthalate (DPP). Furthermore, studies on Pregnant Sprague-Dawley rat to the exposure on DPP at doses of 0.0 (olive oil), 0.5, 1.0 and 1.5 g/kg per day, by gavage had been made on gestation days of the rat (6-20 day). This study had been done by Saillentfait, Roudot, Gallissot, Sabate & Chagnon, (2011) also calculated the effects of toward fetal weight and anogenital distance of the male fetuses via their studies. This chemical has a low potential to adversely affect prenatal development in the rat. Fetal effects, including developmental delay, evidence of alteration of male sexual differentiation and increase in skeletal variants were observed at 1g/kg per day and above. The no observed effect level (NOEL) for embryo-fetal toxicity was found to be 0.5 g/kg per day.

### 2.1.2 Diallyl phthalate

Diallyl phthalate has IUPAC name as bis (prop-2-enyl) benzene-I, 2-dicarboxylate and its molecular weight 246.26 g/mol. The molecular formula for this type of chemical is $C_{14}H_{14}O_4$ (PubChem, n.d) as in Figure 4. Physical properties of diallyl phthalate are detailed in Table 5.

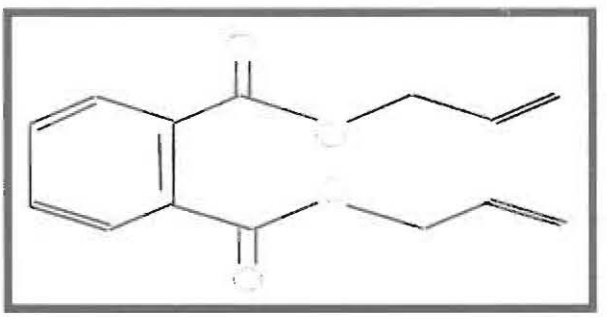


**Figure 4:** The structural formula of diallyl phthalate

**Table 5:** The summary of physiochemical properties

| Property | Value |
|---|---|
| Boiling point | 290°C |
| Melting point | -70°C |
| Relative density (water = 1) | 1.1 |
| Solubility in water, g/100ml at 20°C | 0.015 |
| Vapour pressure, Pa at 25°C | 0.02 |
| Relative vapour density (air = 1) | 8.3 |
| Flashpoint | 166°C (closed cup). |
| Auto-ignition temperature | 385°C |

Diallyl phthalate is one of the compound listed under phthalate ester category. DAP is produced either by an esterification reaction between allyl alcohol and phthalic anhydride or by the condensation between allyl chloride and disodium phthalate. The two highly reactive allyl groups in the molecule attribute to the functionality of DAP as cross-linking agents. The allyl groups are able to react with other unsaturated organic chemicals to form C-C covalent bonds.

#### 2.1.2.1 The general uses of DAP in daily life

Diallyl phthalate is used as a plasticiser in the industry and as a monomer in the processing of thermosetting plastics, polyester resins, and varnishes. It is added to polymer and prepolymer systems to make them easily moulded during the curing process and then it binds covalently into the polymer matrix to produce rigid thermoset plastics. The usage of this chemical may end up to discharge to the environment through various waste streams (PubChem, n.d).

Diallyl phthalate is widely used in electrical and electronic products, painting and coating as well as plastic and rubber products. This chemical is used as an additive in plastic productions because the addition of this chemical to rubbers and resins assist flexibility, workability, and stretch ability. The physical characteristics of diallyl phthalate as plasticisers give the polymer the desired flexibility and softness and it takes up to 40 % of the final weight of the material (Autian, 1973).

DAP is also used as a crosslinking agent during the manufacture of other polymers such as polyvinyl chloride (PVC) and unsaturated polyesters (UP). Such polymers are used for finished consumer products such as window frames, insulating varnish for coil and wire, and sheet moulded compound for ship body (OECD SIDS, 2004).

## 2.3 The hazard effects of DAP

If diallyl phthalate is released to air, an estimated vapour pressure of $1.2X10^{-3}$ mm Hg at 25 °C then it will exist solely as a vapour in the atmosphere. Vapor-phase diallyl phthalate will be degraded in the atmosphere by reaction with photochemically-produced hydroxyl radicals and ozone; the half-lives for these reactions in the air are estimated to be 6.9 and 11 hours, respectively. The UV spectrum of diallyl phthalate in water was found to have absorbance near 275.5 and 281 nm and is expected to have absorption shoulders which extend beyond 290 nm and therefore may be susceptible to direct photolysis by sunlight. If released to soil, diallyl phthalate is expected to have low mobility based upon an estimated $K_{oc}$ of 1,360 (OECD SIDS, 2004)

Volatilization from moist soil surfaces is not expected to be an important fate process based upon an estimated Henry's Law constant of $3.9X10^{-7}$ atm-cu m/mole. Biodegradation at 92 % of its theoretical Biological Oxygen Demand (BOD) in 4 weeks using an activated sludge inoculum in the Japanese MITI test suggests that biodegradation will be an important environmental fate process. If released into water, diallyl phthalate is expected to hold molecules of gas or liquid as a thin layer on the internal surface below the suspended solids and sediment based upon the estimated $K_{oc}$ (OECD SIDS, 2004).

Volatilization from water surfaces is not expected to be an important fate process based upon this compound's estimated Henry's Law constant. Hydrolysis is expected to be an important process based on estimated hydrolysis half-lives of 1.0 year and 37 days at pH 7 and 8, respectively. Occupational exposure to diallyl phthalate may occur through inhalation of aerosols and dermal contact with this compound at workplaces where diallyl phthalate is produced or used. Use data indicate that the general population may be exposed to diallyl phthalate via dermal contact with products containing diallyl phthalate (OECD SIDS, 2004).

The potential risks of exposure to DAP give rise to concern about the safety of human health and the environment. Workers may get exposed to DAP occupationally during printing, polymer, electronics, and stone, clay and glass industries. The Japanese Industrial Safety and Health Association had conducted an occupational exposure assessment of a factory in Japan producing DAP during operations (OECD SIDS, 2004). The survey found that workers were exposed to DAP at concentrations of $\leq 0.11$ mg/m$^3$ during manufacture of DAP and 0.02 - 0.96 mg/m$^3$ during manufacture of the polymer. In all cases, the

exposure was below the following Occupational Exposure Limit, Time-Weighted Average (OEL-TWA) values. In addition, exposure to consumers is through dermal routes and inhalation. Exposure of DAP was expected to be low in the range of 0 - 134.5 ng/m$^3$. DAP is mixed with raw materials and little remains intact in the consumer products because of a chemical reaction.

Approximately half of DAP produced is used as a monomer for the preparation of DAP prepolymer, which is partially polymerised soluble resin. The prepolymer contains up to 2 % unreacted DAP monomer. The prepolymer is an intermediate and becomes covalently bound into the matrix of products. The DAP monomer content in the final product is expected to be much lower than in the prepolymer due to further reaction of the allyl groups. In addition, DAP prepolymer content in UV curable ink used in printing paper is estimated to be 10 wt %, and DAP content in the DAP prepolymer is about 2 wt %. About 4 mg/m$^2$ of DAP is loaded in the paper printed with UV-ink including DAP prepolymer (NICNAS, 2008). It is expected that the net content of DAP in the printed paper is very low level prior to its use by consumers.

### 2.4 Toxicology of diallyl phthalate (DAP) in other animals

In the study by Eigenberg, Carter, Schram & Sipes (1986), the investigation was focused on excretion, distribution and pharmacokinetic in male Fischer-344 rats and male B6C3F1 mice using 14C-DAP. In the excretion and distribution studies, 14C-DAP was administered by gavage to rats and mice at 1, 10 or 100 mg/kg. The results for rats were 25 – 30 % of the DAP was excreted as $CO_2$, and 50 – 70 % appeared in urine within 24 hours.

Whereas in mice, 6 – 12 % of the DAP was excreted as $CO_2$, and 80 – 90 % was excreted in the urine within 24 hours. Tissue distribution and pharmacokinetic studies were conducted in rats and mice dosed via the tail vein with 10 mg/kg/bw of 14C-DAP.

However, no DAP was found in blood, liver, kidney, muscle, skin or small intestine in both species. Furthermore, DAP was found more hepatic toxicity to rats than to mice. The same species but difference effect was observed in toxicity for allyl alcohol (AA). The doses of AA (25, 50 and 75 mg/kg bw) were not hepatic toxicity in mice. While in rats, periportal necrosis was evident in 9 of 12 animals in a dose-dependent manner. The differential hepatotoxicity of DAP was related to the toxicity of AA (NICNAS, 2008).

Apart from that, DAP was found to be genotoxicity *in vitro*. It did induce a number of chromosome aberrations in mice (OECD TG 475 as cited in OECD SIDS, 2004). For the carcinogenic potential of DAP in mice and rats, the observed evidence of lymphoma (mice) and mononuclear cell leukaemia (rat) in aged animals is considered to be equivocal evidence of carcinogenicity (Cadwell, 1999). Another evident is retarded ossification in forelimb and hind limb phalanges, metatarsals, and caudal vertebrae were observed in rats and also causes fetal toxicity at doses which also produced maternal effects (Saillenfait, Gallissot & Sabate, 2008). Besides that, there are a lot of animal model have been tested with the Diallyl phthalates chemical as listed in Table 6.

**Table 6:** Acute toxicity of diallyl phthalate.

| Route | Species (strain) | Endpoint | Dose | Effects | NOEL/LOEL | References |
|---|---|---|---|---|---|---|
| Oral | Mouse (B6C3F1) | $LD_{50}$ | 1,070 mg/kg (M)<br>1,690 mg/kg (F) | Death at 400, 600 mg/Kg; absence of chemical induced lession at necropsy | NOEL = 200 mg/kg/day (M,F)<br>LOEL = 400 mg/kg/day (M,F) | NTP, (1983) |
| | Rat (Fischer 344) | | 891 mg/kg (M)<br>656 mg/kg (F) | Periportal lesions of hepatic lobules, necrosis, fibrosis, bile duct hyperlasia, hepatocellular hyperplasia, multifocal renal cortical tubular necrosis | NOEL = 50 mg/kg/day (F)<br>:undetermined (M)<br>LOEL = 100 mg/kg/day (F); 50 mg/kg/day (M) | NTP, (1985) |
| | Rat (Wistar) | | 896 mg/kg (combined) | (no further details available) | | FMC, (1978) |
| | Dog | | ~800 mg/kg (combined) | Caused hepatic toxicity | | OECD SIDS, (2004) |
| | Rabbit | | 1,700 mg/kg | (no further details available) | | ChemID Plus, (2015) |
| Dermal | Rabbit | $LD_{50}$ | 0.5 g/kg | No skin and eye irritation | | OECD SIDS, (2004) |
| | Rabbit | | 3,360 mg/kg | Mortality | | OECD SIDS, (2004) |
| | Rabbit | $LD_{Lo}$ | 3,140 mg/kg | Changes in lungs, thorax, and respiration and diffuse hepatocellular necrosis | | OECD SIDS, (2004) |
| | Mouse (CBA/CaBkl) | | (not stated) | Skin sensitisation | 3.23 at 5% w/v and 10.74 at 50% w/v | NICNAS, (2008) |

Table 6 continued.

| Route | Species (strain) | Endpoint | Dose | Effects | NOEL/LOEL | References |
|---|---|---|---|---|---|---|
| Subcutaneous | Rabbit | $LD_{Lo}$ | 1,000 mg/kg | Changes in lungs, thorax, and respiration and diffuse hepatocellular necrosis | | ChemID Plus, (2015) |
| Inhalation | Rat (Sprague Dawley) | $LC_{50}$ | 8,300 mg/m$^3$ (combined); 10,310 mg/m$^3$ (M); 5,200 mg/m$^3$ (F) | Immediate signs of irritation, Discolorration of the nasal turbinates and lungs. | | FMC, (1982) |
| Gavage | Rat (Sprague Dawley) | $LC_{100}$ | 4470 mg/m$^3$, 16.7, 50, 150 mg/kg bw/day | Mortality Periportal hepatocyte necrosis, enlargement and basophilia, bile duct proliferation and periportal fibrosis, stomach ulceration | NOEL = 50 mg/kg/day (M,F) | FMC, (1980) |

## 2.2 Embryo toxicity test

Fish traditionally have been considered as a key component in chemical toxicity strategies in the aquatic life (Braunbeck et al., 2005). Fish are exposed to the toxicants throughout their life cycle, and the fish acute toxicity testing (OECD, 1992) has been of importance. However, increased animal welfare considerations have motivated the search for alternative assays to the use of adult fish. The embryos was choose as an alternatives for adult fish because it is more sensitive as compared to adult fish, by showing it several sublethal effect endpoints in the early development (Dahl, Gorolhova & Breitholtz, 2006).

The early life stages of the fish have long been recognised as very sensitive in responses (Marchetti, 1965). In most cases, embryo-larval and early juvenile stage sensitivities are also comparable to those of full life cycle test within a factor of two (Mckim, 1977). Long term toxicity can be predicted by testing the developmental stages of fish and another freshwater organism (Mckim, 1977; Munley, Brix, Panlilio, Deforest & Grosell, 2013). The guidelines for testing the embryos and larvae of fish have been standardized (ISO, 1999; OECD, 2013), so that the traditional adult fish acute toxicity test has been replaced; (OECD, 1992; ISO, 1999; Braunbeck et al., 2005; Braunbeck & Lammer, 2005; OECD, 2013) with *Danio rerio* embryo test (DarT) set-up by Nagel (2002).

In the zebrafish embryo test, the eggs are exposed in a static system from fertilisation to the completion of embryogenesis, in a 24-well polystyrene in a small volume of the exposure media. All the responses of the individual organisms are recorded at certain time points during the exposure (Schulte & Nagel, 1994). The effect, lethal and sublethal

developmental endpoints are selected and monitor are recorded (Nagel, 2002). Coagulation of the egg, non-detachment of the tail from the yolk, lack of somite and heart beat represents the lethal endpoints. Developmental sub lethal endpoints that might indicate the mode of action of the toxic response can include completion of gastrulation, eye development, spontaneous movement, heart rate/blood circulation, pigmentation and edema (Nagel, 2002). Teratogenic effects are investigated following any malformation in aforementioned sub lethal endpoints. Implementation of sublethal effects makes the testing more compatible with ethical and animal welfare legislation (Lammer et al., 2009).

Monitoring certain morphological changes or physiological responses might indicate some modest action to identify and classify some unknown chemical compounds of the exposure media. The test can be expanded to cover other related effects by extending the time of exposure as well as other endpoints like deformation of the spine and locomotors activity. The zebrafish is a popular model to be used in the toxicology screening because of it has a full recorded data for its development from embryo to the adult fish by (Kimmel, Ballard, Kimmel, Ullmann & Schilling, 1995).

## 2.3 Zebrafish as model organism

The zebrafish (*Danio rerio*) is a tropical freshwater cyprinid fish originating from South Asia, especially in Bangladesh, India, Nepal and Pakistan, among others. This fish has become a model organism in ecotoxicology and environmental sciences, with a range of prospective applications in integrative risk assessment of chemicals (Holbech et al., 2001; Schirmer et al., 2008; Coe et al., 2009; Ali, van Mi & Richardson, 2011; Brown et al.,

2012). This is because, it possesses sterling qualities such as small size, cheapness, easy husbandry and superb fertility. In captivity, it breeds all year round, at a frequent interval of 1 – 6 days and eggs are not sticky and can be collected easily in large quantities.

The zebrafish genome is fully sequenced; therefore it can be used for molecular and genetic analyses. There are hundreds of establishing biomarker genes that can be used to narrow down the affected genes in the toxicogenomic test. Its life cycle is rapid under ideal rearing conditions. These traits have led to the wide – spread use of this species in standardised testing protocols for evaluation of drugs and chemicals. In addition, *ex Utero* exposure will avoid maternal influence. Besides that, it also has a transparent body in the early development. This will help in the observation of affected phenotypes of the embryo during early development.

Zebrafish physiology and neuroanatomy are parallel to those of humans (Panula et al., 2006; Panula et al., 2010). A previous study conducted by the Ali, van Mi & Richardson, in 2011, stated that 60 chemical compounds have been tested on zebrafish and rodent the results showed most of the compound had a similar toxicity level in both species, some compounds caused a stronger effect in zebrafish than in rodents, or vice versa. In zebrafish larvae (> 3 days post fertilisation) the majority of the organs and locomotors response are developed, suggesting that the function of behaviour are in place (Kimmel, Ballard, Kimmel, Ullmann & Schilling, 1995).

The zebrafish nervous system becomes functional within days and all major component of the brain is present at 5 dpf (Nusslein-Volhard & Dahm, 2002). The rudimentary forms of

locomotors activity begin to develop in early life stage in zebrafish. Thus zebrafish have emerged as powerful model to study the development and function of the nervous system (Strahle & Korzh, 2004) and the behaviour, genetic and biochemical aspects of locomotion (Drapeau et al., 2002; Guo, 2004; Fetcho, 2007; Fetcho, Higashijima & McLean, 2008). The zebrafish behaviour repertoire is robust, conserved and similar to that of mammals and has high throughput due to the powerful video tracking tools developed recently (Spence, Gerlach, Awrence & Smith, 2008; Stewart et al., 2012; Champangne, Hoafnagels, deKloet & Richardson, 2010; Mathur & Guo, 2010).

The zebrafish is a well-characterized model for testing the toxicological effects on reproductive capability (Laan, Richmond, He & Campbell, 2002; Spitsbergen & Kent, 2003). Since the zebrafish genome is sequenced, a variety of biomarkers is available to measure. The molecular mechanism of the zebrafish endocrine and hormonal signalling pathways are highly similar to those of other vertebrates, allowing for extrapolation of data from this model to other species (Laan, Richmond, He & Campbell, 2002; McGonnell & Fowkes, 2006; Segner, 2009). Endocrine disrupting biomarkers are equally sensitive in zebrafish as in other species (Holbech et al., 2001; Holbech et al., 2006; Hutchinson, Ankley, Segner & Tyler, 2006).

### 2.4 Thyroid hormone (TH) gene

These two phthalates ester chemical have been included in the CoRAP Reviews and were suspected to be Carcinogenic, Mutagenic, and Reproductive (CMR), Persistent, Bioaccumulative and Toxic substances (PBT) or Endocrine Disruptors. Endocrine

disrupting chemicals (EDCs) are a compound that interferes with endocrine (or hormone system) in both wildlife and humans. EDCs can modulate the endocrine system through multiple mechanisms of action and possibly cause cancerous tumours, birth defect, and other developmental disorders. Animal studies have indicated that many of the adverse effects were often permanent and some EDCs could act as epigenetic modulators which led to potential transgenerational effects (Anyway, Cupp, Uzumcu & Skinner, 2005; Waring & Harris, 2011). The adverse effects of EDCs which have been observed in many vertebrate species cause growing concern among researchers and policy makers.

It also had been found to affect the 3.5.3'-triiodothyronine ($T_3$) and thyroxine ($T_4$) level but the mechanism still remains unclear (Mathieu-Denocourt, Wallace, de Solla & Langlois, 2015). Dibutyl phthalate (metabolised into DPP by *Bacillus natto*) act as an anti-thyroid hormone and antagonise $T_3$ action at the transcriptional level (Begum, Katsumata, Kaneco, Suzuki, & Ohta, 2004). In humans, the thyroid hormone thyroxine ($T_4$) and 3.5.3'-triiodothyronine ($T_3$) play an important role in regulating development and growth in target tissues (Chan et al., 2003; Johnson & Lema, 2011).

This is especially important in regards to the development of the central nervous system (CNS), which begins in utero reference (Fisher, 1997; Kilby, Gittoes, McCabe, Verhaeg & Franklyn, 2000; Calvo et al., 2002). Mild maternal hypothyroidism in early pregnancy has been associated with adverse neuropsychological outcomes, demonstrating that changes in maternal thyroid status affect fetal CNS development (Pop et al., 2003).

In most vertebrates (particularly mammals, fish and amphibians), $T_4$ is considered a prohormone, and must first be metabolized to $T_3$ in peripheral tissue by the selenocysteine containing enzymes deiodinase 1 (*dio_1*) or deiodinase 2 (*dio_2*) in order to bind to thyroid hormone nuclear receptors (Kohrle, 2000). *dio_1* catalyses both outer ring deiodination (ORD) and inner ring deiodination (IRD), while *dio_2* only catalyses ORD. In contrast, deiodinase 3 (*dio_3*) only catalyses IRD and metabolises $T_4$ into the biologically inactive reverse triiodothyronine ($rT_3$) (Darras, Hume & Visser, 1999).

Thyroid hormone (TH) plays an essential role in vertebrate development – as has been shown for fish (Power et al., 2001), amphibian (Tata, 2006), birds (McNabb, 2007) and mammals (Bernal, 2007). The life cycle of teleost fish is typically defined according to the following stages: zygote, embryo (pre hatch and post hatch), larval, juvenile and adult. A multitude of data is now available showing a stimulatory effect of TH treatment on larval and juvenile teleost development, while goitrogen treatment induces adverse effects (Power et al., 2001; Blanton & Specker, 2007).

The role of TH in the prehatch stages have long remained scarce, although it was known that both TH receptor alpha and beta (*tra* and *trβ*) are present in pre hatch fish embryos and are responsive to TH (Essner, Breuer, Essner, Fahrenkrug & Hackett , 1997; Liu, Lo & Chan, 2000; Jones, Rogers, Kille & Sweeney, 2002; Raine, Cameron, Vijayan, Lamarre & Leatherland, 2004). Only more recently it was shown also in these early stages that TH treatment accelerates while phenylthiourea treatment delays embryonic development and hatching (Elsalini & Rohr, 2003; Walpita, Van der Geyten, Darras & Rurangwa, 2007) .

In zebrafish, the embryonic thyroid gland begins to develop quite early but it only becomes active around or after hatching (Wendl et al., 2002; Alt et al., 2006). Therefore, pre hatch embryos rely on TH taken up from the maternal deposit in the egg yolk. As is the case in many other teleost species studied (Power et al., 2001), $T_4$ is the predominant TH present in zebrafish eggs with a ratio of $T_3/T_4$ between 1/5 and 1/10. Since most of the action of TH is mediated via binding of $T_3$ may be a crucial step in zebrafish.

Three types of iodothyronine deiodinases (*dio_1 – 3*) have been described in the teleost, each with a structure and function similar to what has been described in mammals and other vertebrates. It is mean that both *dio_1* and *dio_2* are capable of converting $T_4$ into $T_3$ while *dio_3* is purely inactivating the enzyme. Injection of two different *dio_2* antisense morpholino for the knockdown study has resulted in each case in a clear delay in embryonic development as demonstrated by three different morphological indices; otic vesicle length, heat to trunk angle and pigmentation index (Walpita, Crawfor, Janssens, Van der Gerten & Darras, 2009).

However, a previous study showed that knockdown of *dio_1* alone does not change zebrafish embryonic development rate but a combined knockdown of *dio_1* and *dio_2* severely disturbs the development to a greater extent than *dio_2* knockdown alone. Thyroid stimulating hormone (*tsh*) secretion functions as a common regulators of the hypothalamic-pituitary-thyroid (HPT) axis in fish are triggered by changes in the concentration of circulating (TH) (De Groef, Van der Geyten, Darras & Kuhn, 2006), evaluation of thyroid stimulating hormone beta (*tsh_β*) can be used to determine whether environmental chemicals caused thyroid dysfunction (Yu et al., 2011).

Transthyretin (*ttr*) binds TH and transport them to various target tissues (Morgado, Santos, Jacinto & Power, 2007b). Several environment contaminants that share same structural similarity to the TH may interfere with TH homoeostasis by binding *ttr* in vertebrates, thus disrupting the TH axis (Morgado, Hamers , Van de Ven & Power , 2007a). Therefore, *ttr* gene was used as an indicator for thyroid system disruption in several studies (Li et al., 2009; Yu et al., 2011). TH may directly or indirectly regulate plasma *ttr* (Morgado, Santos, Jacinto & Power, 2007b). The biological activity of TH is predominantly mediated by two thyroid hormone receptor (TR), thyroid hormone receptor alpha (*trα*) and thyroid hormone receptor beta (*trβ*) (Yang et al., 2007). Chen, Yu, Yang & Zhou, (2012), found that the upregulation of TR might due to the increased $T_3$ levels and would influence the transcription of other gene involved in thyroid function. Abnormal *trα* and *trβ* might lead to the failure of TH bind and activate the appropriate post-receptor response cascade (Liu, Chang, Zhao & Zhu, 2011). This showed that the *trα* and *trβ* had indirectly or directly related to the regulation of $T_3$.

# CHAPTER 3

# METHODOLOGY

## 3.1 Fish maintenance

The animal model *Danio rerio* at the age of 3 months was bought from the supplier in Kuala Lumpur. After that the animal was kept in the aquarium at the Animal Biotechnology Laboratory, Lower Ground Floor, Faculty of Resources science and Technology, Universiti Malaysia Sarawak (UNIMAS). The aquarium was used "overflow tank system" as shown in Figure 5. This system had a centralized water filter tank. All the water in the separated aquarium above it has flown to the sump tank at the bottom of the aquarium for the filtration and nitrification process to take placed. The filtered water was pumped back into the aquarium by using pump at the end of the sump tank. Every single aquarium had 15 pieces of adult zebrafish male and female with the ratio of 1:1. All the fish had to go through acclimation period for 1 month or 3 cycle of breeding.

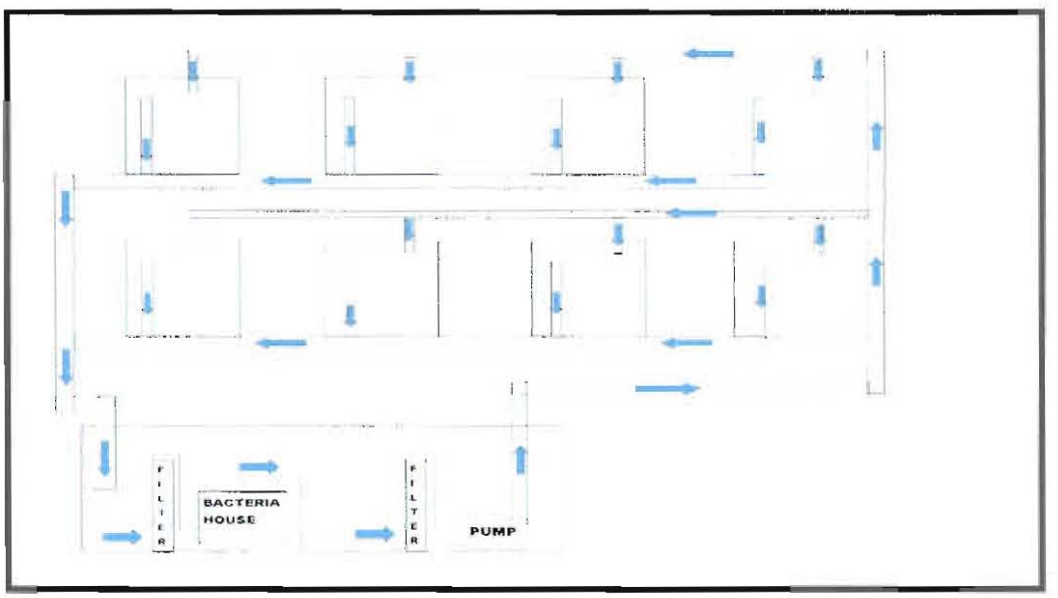



**Figure 5:** Diagram of overflow tank system.

The fish facility and the water temperature were maintained at 27 °C. The zebrafish were kept constant for 14 hour light and 10 hours dark cycle. The water use for the aquarium was pre-filtered with the water filter (Panasonic PP-123) to removed small particle and chlorine. The fish was feed twice per day at 9.00 a.m and 6.00 p.m with commercial flake food (Pro Bits, Italy). The amount of food feed to every tank was a full volume of spatula then after 30minutes the leftover food was collected and removed from the tanks. The water used in the aquarium was change regularly once a month. The aquarium aeration was maintained for 24 hours.

### 3.2 Fish breeding

The fish breeding tanks were set up late in the evening before the feeding time. The breeding tank was set up as shown in the Figure 6. Each of the breeding tanks was set up with 3 pieces of adult zebrafish. In addition, the ratio of female to male zebrafish that was applied in one breeding tank was 2:1 to ensure a large number of fertilised eggs produced (OECD, 2013). The fishes used for breeding was not feed in the evening before mating to reduce the debris in the breeding tanks in the next morning. The mating process was taken placed at dark cycle at night, so it was left overnight in the fish facility room.



**Figure 6:** Breeding tank. Source: https://outlook.wustl.edu/archive-assets/img/stories/secondary/2012-aug-zebrafish_fish-tank.png

The next day morning, one hour after the light switch on the breeding tanks were checked to observe the presence of the eggs. These breeding tanks consist of a 2 – liter acrylic box assembled with a removable inner sieve to separate fishes from freshly laid eggs (Kent & Varga, 2012).

### 3.3 Embryo collection

The Embryos were then collected from the bottom of the acrylic box and staged according to Kimmel, Ballard, Kimmel, Ullmann & Schilling, (1995) before further analysis. Only 4 hour post fertilization (hpf) embryo was selected to be used for toxic effect analysis. All embryos will be cultured in the Embryo Medium (EM). According to Cold Spring Harbour Protocol, the ingredients for EM were shown in Table 7. Generally, embryo medium consists of sodium chloride (NaCl), potassium chloride (KCl), calcium chloride ($CaCl_2$) and magnesium chloride ($MgCl_2$). This chemical was added into 1 L glass bottle. Double distilled water was used and added in the glass bottle until the water level reaches 1 L by using measuring cylinder. The solution was stirred to allow the salts to dissolve completely.

**Table 7:** List of the components for 1 liter for 10X EM stock solution preparation

| Component | 1X (g) | 10X (g) |
|---|---|---|
| NaCl | 0.2900 | 2.9000 |
| KCl | 0.0133 | 0.1330 |
| $CaCl_2$ | 0.0484 | 0.4840 |
| $MgSO_4$ | 0.0816 | 0.8160 |

The embryo were removed from the breeding tanks by using a syphon and filter with the tea filter then washed with running water to remove any debris from the eggs. Unfertilized eggs which appear in white opaque were discarded immediately as they will contaminate the healthy eggs and allow the growth of bacteria or mould. Furthermore, according to Selderslaghs, Van Rompany, De Ceon & Witters, (2009), 0.0002 % of methylene blue (MB) was mixed with the eggs which were at 0 hour post fertilisation (hpf) since the chemical served as a mild fungicide. Moreover, the viable eggs were sorted out using a dissecting microscope (Olympus SZ51, New York). Petri dish (90 mm diameter) was used as a container to hold up to 30 embryos during the development of zebrafish (Westerfirld, 1995).

### 3.3 Fish embryo acute toxicity (FET) test

This test was designed to determine acute toxicity of chemicals on embryonic stages of fish. This method test were carried out according to the OECD guideline test no. 236:fish embryo acute toxicity test on embryos with a few adjustment (OECD, 2013). The FET test was performed on newly fertilized zebrafish eggs and exposure started before the embryo reached 8-cell stage. The duration of the chemical exposure in the FET test was set at 96 hpf. Every 24 hours, the observation was done to recorded one apical point as an indicator of lethality; coagulation of fertilised eggs. At the end of the exposure period, acute toxicity was determined based on the observation that had been recorded, and the $LC_{50}$ will be calculated with the probit analysis. The early preparation process had been done as shown in the Figure 7.

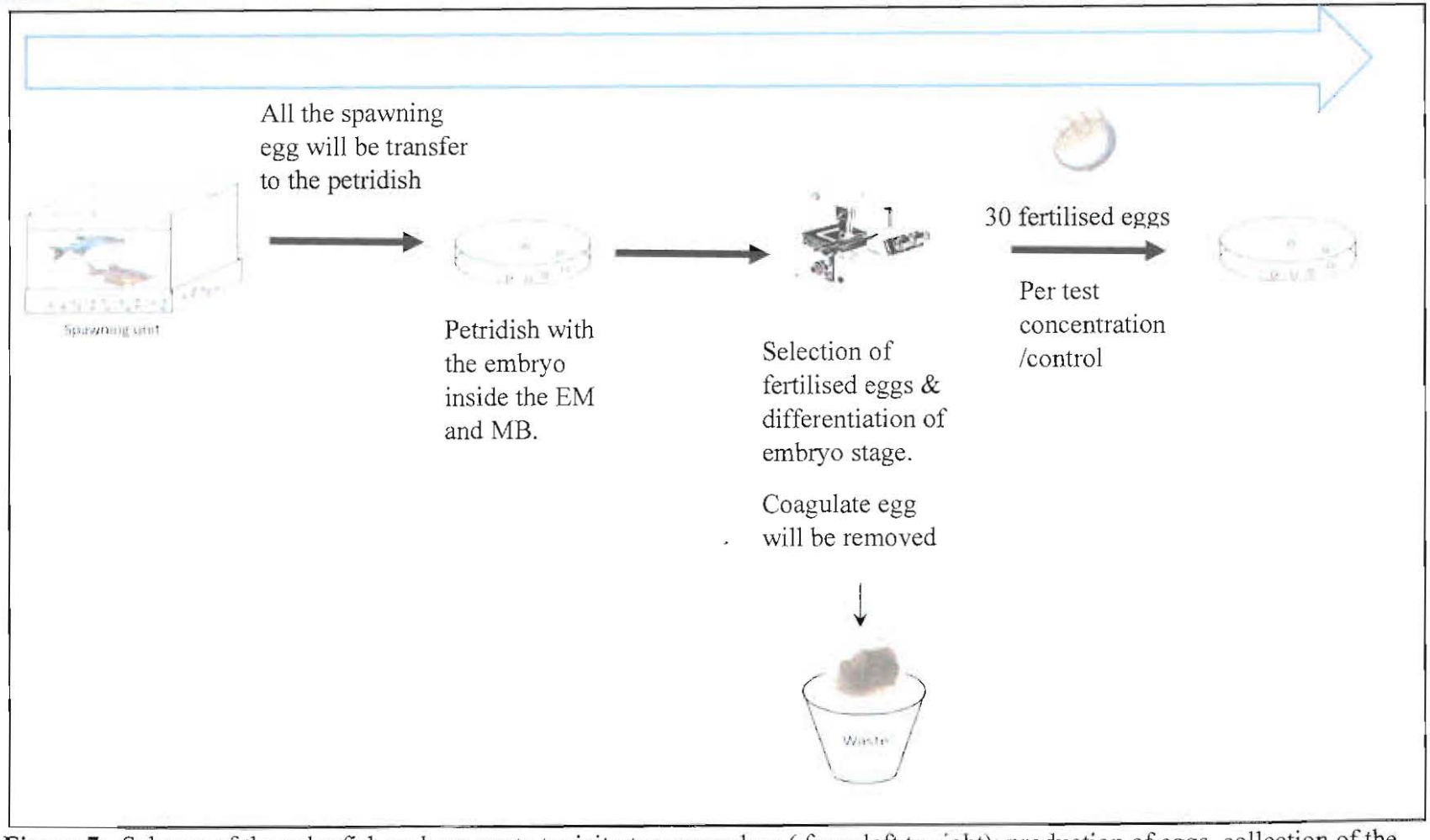



**Figure 7:** Scheme of the zebrafish embryo acute toxicity test procedure ( from left to right): production of eggs, collection of the eggs, all the embryo will be transfer into petridish containing EM and MB, selection of fertilised eggs with an inverted microscope and distribution of fertilised egg into petridish according to their hpf stage and concentration.

### 3.3.1 Validity of the FET test

For the result to be valid a few criteria was followed;

a. The overall fertilisation rate of all eggs collected was more than 70% in the batch tested.
b. The water temperature was maintained at 27 °C in the incubator (Thambi-50, Malaysia) at any time during the test.
c. The overall survival of embryo in the negative (embryo medium) control was more than 90% until the end of the 96 hrs exposure.
d. The exposure to the positive control using 4.0 mg/L 3, 4-dichloroaniline for zebrafish had a minimum mortality of 30% at the end of the 96 hrs exposure.
e. The hatching rate in the negative control was more than 90% at the end of the 96 hrs exposure.

### 3.3.2 Chemical exposure for FET test

The test chemical was set up with 30 embryos for Petri dish and 3 Petri dish was used for every concentration. All test was carried out in 90 mm petri dish in triplicates per each concentration. The test was carried out in semi-static renewal interval conditions with the test solution was changed every 24 hours. All the treated embryo was kept inside the incubator at the temperature of 27 °C while the pH was maintained at 6.8 - 7.2 throughout the experiment. Upon renewal, the embryo was covered by a small amount of old test solution to avoid drying. The coagulated eggs was removed every time the solution was changed. The extra care was taken to minimise any stress or technical error to the embryos.

There are only one type of control was done; it is the negative control by using the embryo medium and the methylene blue solution. The diallyl phthalate (DAP) and Dipropyl phthalate (DPP) was supplied from the Sigma-Aldrich, USA. These chemicals was delivered in the solution form which the purity of DAP at 97% and the purity of the DPP at 98 %.All the solution was stored in the cool, dry, well-ventilated area. The calculation of conversion was calculated based on the density of the chemical and the dilution factor. As shown in the Figure 8 and Figure 9.

$$x\ mg/l = \frac{\frac{y}{1000}\ ml \times 1.078\frac{g}{ml} \times 1000\ mg \times 98\%}{\frac{500}{1000}\ l}$$

*where* $x$ = *Concentration of DPP;*
$y$ = *Volume of DPP*

**Figure 8:** Formula to calculate DPP in mg/L.

$$x\ mg/l = \frac{\frac{y}{1000}\ ml \times 1.121\frac{g}{ml} \times 1000\ mg \times 97\%}{\frac{500}{1000}\ l}$$

*where* $x$ = *Concentration of DAP;*
$y$ = *Volume of DAP*

**Figure 9:** Formula to calculate DAP in mg/L.

Different concentration of test solution were prepared by pipetting the DPP and DAP into media storage bottle filled with 500 mL of EM and MB. The concentration of DPP and DAP was converted using their respective formula from µl/500mL into mg/L as stated in the Table 8.

**Table 8:** Chemical concentration of each chemical for FET test.

| DPP (mg/L) | DAP (mg/L) | DAP + DPP (mg/L) |
|---|---|---|
| 4.31 | 3.36 | 1.10 |
| 5.39 | 4.04 | 1.50 |
| 6.47 | 4.84 | 1.85 |
| 7.01 | 4.93 | 2.00 |
| 8.30 | 5.61 | 3.00 |
| 9.60 | 6.52 | 4.00 |

### 3.4 Morphological observation

The experiment was repeated exactly the same as the FET test. However, the only changes part was the endpoint of this experiment; uninflated swimbladder, yolk sac edema, pericardial edema, unhatched and tail deformation. Besides that, the endpoints for this experiment are the sub lethal effects and all of the endpoints were observed and compared at each stage and concentrations. A compound microscope (LEICADM 750, New York) was used to observe and capture the images for the development of embryos at each stage at a different time; 4 hpf, 24 hpf, 48 hpf, 72 hpf, and 96 hpf.

### 3.5 Data collection and statistical analysis

All the observations and data were collected and documented. The software Minitab version 17 was used to perform the biological analysis. The no observed effect concentration (NOEC) will be replaced with low effect concentration (LEC) as suggested by the Warne & van Dam, (2008), because of lack statistic evidence to support for NOEC

value. Mortality rate was calculated based on the percentage of the dead embryo in each treatment and control. The mortality rate was then harmonised by using the formula in Figure 10.

$$Mortality\ rate = \frac{Death\ per\ replicate\ (Treatment)}{total\ number\ of\ all\ replicate} - \frac{Total\ Death\ (Control)}{total\ number\ of\ all\ replicate}$$

**Figure 10:** Calculation for mortality rate.

For calculation of $LC_{50}$, the number of dead embryos was counted and expressed in percent mortality then analysed by using the probit analysis formula. The calculation of probit analysis was using 2nd edition software. Firstly, all the concentration was converted to log (10) concentration as showed in the Table 9. Then all the mortality was converted to proportions.

**Table 9:** The chemical concentration was converted to log (10) concentration from mg/L.

| DPP (mg/L) | Log (10) DPP | DAP (mg/L) | Log (10) DAP | DAP + DPP (mg/L) | Log (10) DAP + DPP |
|---|---|---|---|---|---|
| 4.31 | 0.63 | 3.36 | 0.53 | 1.10 | 0.04 |
| 5.39 | 0.73 | 4.04 | 0.61 | 1.50 | 0.18 |
| 6.47 | 0.81 | 4.84 | 0.68 | 1.85 | 0.27 |
| 7.01 | 0.85 | 4.93 | 0.69 | 2.00 | 0.30 |
| 8.30 | 0.92 | 5.16 | 0.71 | 3.00 | 0.48 |
| 9.60 | 0.98 | 5.61 | 0.75 | 4.00 | 0.60 |

The proportions were corrected for control mortality if it is more than 10% using Schneider-Orelli's (1947) formulas stated in the Figure 11. After that, the corrected proportion or uncorrected proportions will be converted to the empirical probit. A dose response curve is drawn using the log (10) doses and empirical probit and the regression equation is derived.

$$\text{corrected mortality (p)} = \frac{\%\ \text{Responded} - \%\ \text{Responded in Control}}{100 - \text{Responded}\ \%\ \text{in Control}} \times 100$$

**Figure 11:** The calculation of corrected mortality (p)

Empirical probit less than 1 and more than 7 was ignored as they have little and no significance in the estimation of LD or LC (Hayes, 2014). The empirical formula was shown in the Figure 12. The equation of the curve and log10 doses were used to derive expected probit.

$$\text{emperical probit (y)} = 5 + (x - \mu)/\sigma$$

**Figure 12:** The calculation of empirical probit (y).

The original mortality (Observed) and derived mortality (Expected) was used to calculate the Chi-Square test with (No. of log doses used -2) degrees of freedom. If the Chi-square test is non-significant, it indicates good curve fitting. The Z value was derived by using the formula shown in the Figure 13.

$$z = \frac{1}{(\sqrt{2\pi})e(-1/2(Yi - 5)^2)}$$

Yi = Expected probit

**Figure 13:** The calculation of the Z value.

The weighting coefficients (W) were derived by using this formula; W=Z^ (2)/PQ, where P = expected proportion and Q = (1-P). Besides that, the weighted coefficients were used to calculate the standard error. The formula used was shown in the Figure 14.

$$SE = \sigma/\sqrt{\Sigma nW}$$

$\sigma$ = Standard deviation (1/slope)
n= number of animals in each group
W= Weighting coefficient

**Figure 14:** The calculation of standard error.

The LC values were derived from the curve drawn by using working probit and log concentration. Antilog of the concentration was corresponded to respective probit value. 95% fiducial confidence limits were calculated by using the formula in the Figure 15.

$$\text{Fiducial Limits} = \text{Antilog}\,(\text{Log10 Dose} \pm 1.96\,(\text{SE}))$$

**Figure 15:** The formula of the 95% ficudial confidence limit.

### 3.6 Molecular analysis

Before the molecular analysis was done some precauntion step were taken such as to sterilice the apparatus and the place of experiments. Apparatus including all glassware, plastic wares including scalpel and forceps were autoclaved at 121 °C for 15 mins at 15 psi prior to use. Reused apparatus were disinfected with Decon 40 % before further cleaning using detergent and rinsed under distilled water. Apparatus were dried in 105 °C oven before wrapping in aluminium foil and subjected to autoclaving. To create an RNase-free

environment, 0.1 % (v/v) of DEPC was prepared by mixing the diethyl pyro carbonate (DEPC) with distilled water. This was used to prepare the solutions for RNA extraction and autoclaved for 15 minutes per litre to sterilise and remove DEPC (Sigma-Aldrich, 1999). The protocol suggested by Teakle et al. (2002) was used for DEPC treatment. 0.1 % DEPC was prepared to soak plastic wares and glass wares for at least 2 hours. Then, they were autoclaved to be sterile and DEPC free.

### 3.6.1 Total RNA (TRNA) extraction

Total RNA was extracted from the 96 hour post fertilization (hpf) zebrafish that had been exposed to the toxic chemical by using Tri Reagent®. The embryos were selected and water was removed via pipetting, then, they were being transferred to a 1.5 ml microfuge tube. Embryo or tissue weighing 50 – 100 mg were homogenised in a 1 ml of Tri Reagent® according to manufacturer protocols (Molecular Research Center, Inc.). Homogenate was then incubated at room temperature for 5 minutes to allow complete dissociation of nucleoprotein complex and subsequently centrifuged at 13,000 rpm for 10 minutes at 4 °C. After centrifugation, the supernatant was transferred to a new tube and 200 µl of chloroform was added. The samples were shaken for 15 seconds and left at room temperature for approximately 5 minutes. The samples were centrifuged at 12,000 rpm for 15 minutes and the upper phase was transferred to a new tube. 500 µl of ice-cool isopropanol was added to each sample and incubated at room temperature for 10 minutes. The samples were centrifuged at 12,000 rpm for 10 minutes to precipitate the RNA. The RNA pellet was washed with 75 % ethanol and centrifuged again at 7,500 rpm for 5 minutes. The supernatant was removed and the RNA pellet was allowed to air dry before

added with 10 µl of RNase-free water or in DEPC water and incubated at 55°C for 10 minutes.

### 3.6.2 Quantification of RNA

For spectrophotometric measurement, 3 µL of DNA aliquots were diluted with 2997 µl of distilled water in the cuvette and quantified using a spectrophotometer. The absorbance values of 260 nm, 280 nm, and 320 nm were recorded. The purity of the RNA sample was calculated by using ratios of $A_{260}/A_{230}$ and $A_{260/280}$ as shown in Table 10 (Cirera, 2013; Greco et al., 2014). The concentration of RNA was calculated by the formula: The purity of the sample was determined by calculating the ratio of $A_{260\text{-}320}/A_{280\text{-}320}$ and the concentration of RNA was calculated as shown in Figure 16.

RNA (ug/µL) = [A260 x (40 ug RNA/ml)/ (1 A260) x (dilution factor)] /1000

**Figure 16:** RNA formula for quantification.

**Table 10:** The optimum reading of UV spectrophotometer

| Ratio | Purpose | Optimum Reading |
|---|---|---|
| $A_{260}/A_{280}$ | Used to access the purity of DNA and RNA from protein contamination | ~ 1.8 – 2.0 |
| $A_{260}/A_{230}$ | Used to access the integrity of nucleic acid from organic contaminants | ≥ 1.8 |

### 3.6.3 Agarose Gel Electrophoresis (AGE)

10x TAE buffer was prepared by using 48.4 g of Tris base buffer, 3.7 g EDTA, 11.4 ml acetic acid, mixing with 1 L of water. Then, the 10X TAE buffer was autoclaved. Before using the 10X TAE buffer, 200 mL of 10X TAE buffer was diluted with distilled water to a

final volume of 1 L. A 1% (w/v) agarose gel was prepared by using 0.5 g of agarose powder into 50 ml of 1X TAE buffer. The mixture was heated in the microwave for 1.5 minutes to dissolve the powder. The gel solution was cooled to about 60 °C and 2 µL of Ethidium Bromide (10 mg/ml) was added and the gel was poured into the moulding tray with the comb in the position. After the gel solidified, it was submerged in the 1X TAE buffer to 2 to 5 mm depth and ready for use. 1 µL of loading dye and (6X) and 4 µL of distilled water were mixed with 2 µL sample before loading to the gel. The gel was run at 100V for approximately 30 minutes and was viewed under a UV Transilluminator.

### 3.6.4 cDNA Synthesis

The cDNA synthesis was carried out by using SuperScript™ III First-Strand cDNA Synthesis System (Invitrogen) according to the manufacturer's instructions. First, the concentrations of template RNA was diluted to final concentration of 0.05 µg/µL by adding ultra pure water. The volume of ultrapure water was added to template RNA according to the ratio as shown in Table 11. A mixture component of 1 µl of Oligo $(dt)_{20}$ primer, 1 µL of RNA, 1 µL of 10mM of NTPs mix and 7 µL of Nuclease-Free Water as shown in Table 12, was incubated for 5 minutes at 65 °C and then at least 1 minute on ice before spun for 10 seconds in a micro centrifuge to collect the condensate. The diluted sample RNA (< 5 µg for optimum result) was added to the mixture component and mixed thoroughly followed by vortex for less than 5 seconds and briefly centrifugation

**Table 11:** First mixture for cDNA synthesis component.

| Component | 1X Reaction | Master Mix (10X) |
|---|---|---|
| RNA sample | 1 μL | 10 μL |
| Oligo$(dt)_{20}$, primer | 1 μL | 10 μL |
| 10 mM dNTP mix | 1 μL | 10 μL |
| DEPC-treated water | 7 μL | 70 μL |
| Final volume | 10.00 μL | 100.00 μL |

The cDNA Synthesis Mix was added together with 10 μL of RNA and primer mix to each reaction tube on ice for a final volume of 20 μL as in Table 12. Then the tubes were incubated at 25 °C for 10 minutes and incubated again at 50 °C for 50 minutes before heat inactivated at 37 °C for 20 minutes. The reaction tubes were stored at -20 °C prior to PCR amplification. The negative control for cDNA synthesis the template RNA was replaced with the ultra-pure water.

**Table 12:** Second mixture for cDNA synthesis component.

| Component | 1X Reaction | Master Mix (10X) |
|---|---|---|
| 10X RT Buffer | 2.00 μL | 20.00 μL |
| 25 mM $MgCl_2$ | 4.00 μL | 40.00 μL |
| 0.1 M DTT | 2.00 μL | 20.00 μL |
| RNaseOut™ (40 U/μL) | 1.00 μL | 10.00 μL |
| Superscript™ Reverse Transcript (200 U/μL) | 1.00 μL | 10.00 μL |
| Final volume | 10.00 μL | 100.00 μL |

### 3.6.5 Primer design

Primers for the use in the RT-PCR were designed by using Primer3 (version 0.4.0) online program (http://bioinfo.ut.ee/primer3-0.4.0/). The parameters for primer design were fixed at 20 – 25 bp of primer length, 55 – 65 °C of annealing temperature, 40 – 60% GC content with a product size of 80 - 400 bp. Primers were designed according to the mRNA sequences available in the gene bank. The list of primer that used in this project was listed in Table 13.

**Table 13:** The primer sequences used in this project.

| Gene name | Primer sequence | Product size (bp) | GC% | Accession number |
|---|---|---|---|---|
| *β-actin* | F: TGC CCA TCT ACG AGG GTT AC | 341 | 55.00 | NM_181601.4 |
| | R: CTC GTG GAT ACC GCA AGA | | 55.65 | |
| *ttr* | F: AGC CCT TTC TCG TAC ACC AC | 153 | 55.00 | NM_001005598.2 |
| | R: ATG GCA ATC GAA TGA CTT GGT | | 42.86 | |
| *dio_1* | F: GTT CAA ACA GCT TGT CAA GGA CT | 142 | 43.48 | NM_001324404.1 |
| | R: AGC AAG CCT CTC CTC CAA GTT | | 52.38 | |
| *dio_2* | F: GCA TAG GCA GTC GCT CAT TT | 103 | 50.00 | NM_212789.4 |
| | R: TGT GGT CTC TCA TCC AAC CA | | 50.00 | |
| *trα* | F: CTA TGA ACA GCA CAT CCG ACA AGA G | 85 | 48.00 | NM_131396.1 |
| | R: CAC ACC ACA CAC GGC TCA TC | | 60.00 | |
| *trβ* | F: TGG GAG ATG ATA CGG GTT GT | 110 | 50.00 | NM_131340.1 |
| | R: ATA GGT GCC GAT CCA ATG TC | | 50.00 | |
| *tsh_β* | F: GCA GAT CCT CAC TTC ACC TAC C | 121 | 54.55 | NM_181494.2 |
| | R: GCA CAG GTT TGG AGC ATC TCA | | 52.38 | |

### 3.6.6 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

The aliquot from the finished reverse-transcription reaction was added to the PCR master mix. 5X Green GoTaq® Flexi Buffer (Cat. # M8291, Promega, USA) was used to conduct PCR with the thermal cycler (T100, Bio-Rad Laboratories, USA). The PCR master mix component was listed in Table 14. The initial denaturation was set at 95 °C in 2 minutes, with 36 cycles of denaturation at 95 °C in 30 seconds, annealing at 60 °C for 30 seconds,

extension at 72 °C for 8 seconds, and a final extension at 72 °C for 5 minutes. The composition of the mixture as suggested by Ishak et al., (2008) was modified:

**Table 14:** The PCR master mix component.

| **Component** | **1X (µl)** | **10X (µl)** | **Final concentration** |
|---|---|---|---|
| 5X Green GoTaq® Flexi Buffer | 5 | 50 | 1x |
| $MgCl_2$ solution, 25 mM | 2 | 20 | 2.0 mM |
| dNTP, 10 mM each | 0.5 | 5 | 0.2 mM each dNTP |
| Forward primer, 100 µM | 0.1 | 1 | 0.2 µM |
| Reverse primer, 100 µM | 0.1 | 1 | 0.2 µM |
| GoTaq® DNA Polymerase (5 u/µL) | 0.2 | 2 | 1 u |
| Ultrapure water | 16.1 | 161 | - |
| cDNA | 1 | 10 | - |
| Total volume | 25 | 250 | |

*β-actin gene* was used as the internal reference for normalisation First, *β-actin* was amplified and loaded on the 2% (w/v) agarose gel for gel electrophoresis. After visualisation with UV transilluminator (UltraSlim UV transilluminator, MaestroGEN, TW), the band intensity was compared and the volume of the cDNA used for PCR was adjusted according to the band intensity. This process was to normalize the concentration of cDNA used in the PCR sample. Samples were then proceeded to thermal cycling by using PCR machine (T100 Thermal Cycler, Bio-Rad, USA) with the setting used as shown in Table 16 and 17.

**Table 15:** Thermal cycling parameters for *β-actin*

| Stage | Number of Cycles | Temperature (°C) | Duration |
|---|---|---|---|
| Initial Denaturation | 1 | 95 °C | 2 min |
| Denaturation | 35 | 95 °C | 30 s |
| Annealing | | 60 °C | 30 s |
| Extension | | 72 °C | 30 s |
| Final Extension | 1 | 72 °C | 5 min |

**Table 16:** Thermal cycling parameters for others gene.

| Stage | Number of Cycles | Temperature (°C) | Duration |
|---|---|---|---|
| Initial Denaturation | 1 | 95 °C | 2 min |
| Denaturation | 35 | 95 °C | 30 s |
| Annealing | | 59 °C | 30 s |
| Extension | | 72 °C | 6 -10 s |
| Final Extension | 1 | 72 °C | 5 min |

The agarose gel was placed into the gel box. The gel box was filled with 1X TAE buffer until the gel was covered for 2 – 5 mm. The loading buffer was added to each sample. 100 bp DNA ladder was loaded into the well of the gel. The samples were loaded into the remaining wells. The voltage was set at 95 V for 75 minutes. Then, the result was visualised under UV light after removal of gel from gel box.

# CHAPTER 4

# RESULTS AND DISCUSSION

## 4.1 The Lethal Concentration (LC) and morphological defect for each of chemical

### 4.1.1 Dipropyl phthalate (DPP)

The mortality rate of dipropyl phthalate (DPP) treated embryos was shown in Figure 17. The mortality rate increased which is the concentration of the DPP increased. As shown by probit analysis in Figure 18, DPP is a dose-response relationship chemical.

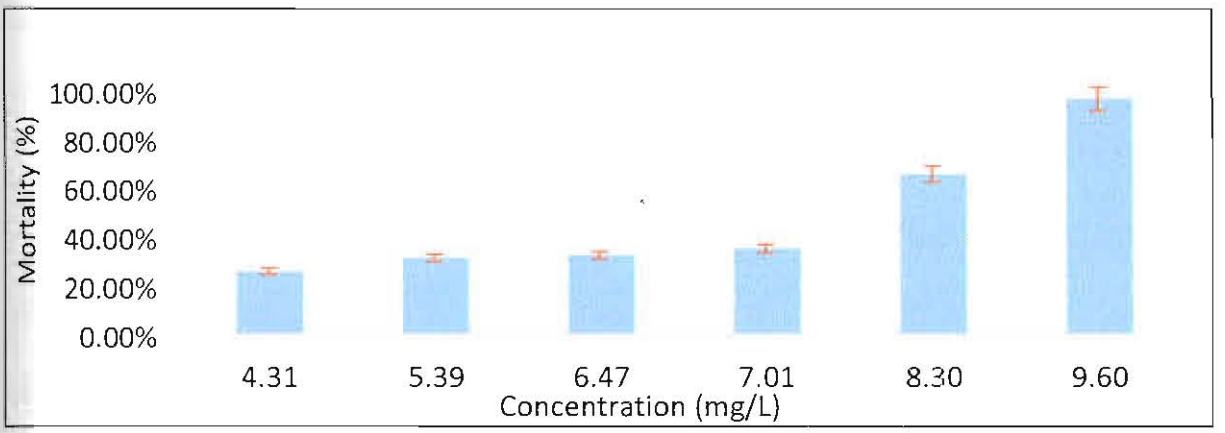



**Figure 17:** Mortality rate for dipropyl phthalate.

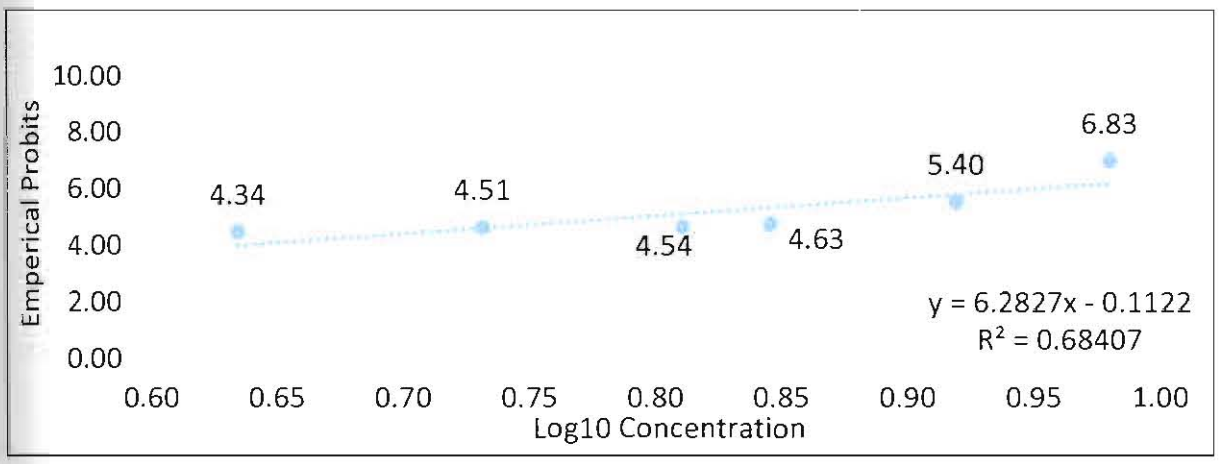



**Figure 18:** The Probit Analysis of dipropyl phthalate.

In total, 540 embryos were used for the 6 DPP concentration, with 350 embryos being analysed for organ malformation. Fish embryo test (FET) test control; negative and positive showed a result that were based on the validity of the test, with the negative control mortality being less than 9 % with a standard error of 2.3% and the positive control mortality more than 30 % with a standard error 1.8%. The $LC_{50}$ obtained from the embryos exposed to DPP was 6.577 mg/L with the range of 95% fiducial CI from 5.792 to 7.467 mg/L. The $LC_{10}$ for the DPP was 3.799 mg/L with the range of 95% fiducial CI from 3.346 to 4.313 mg/L. These result suggest that DPP in the aquatic environment present low teratogenic risk, as embryo exposed to DPP in the current study were at greater concentration than those noted in surface water and sediment as the average of detection is 0.1 µg/L (Yuan et al., 2002).

Other studies also indicated a low teratogenic risk from exposure to DPP. Saillenfait et al., (2010) found that in pregnant rats given PP at doses 0.5, 1.0, 1.5 g/kg/d in olive oil by gavage on gastrulation days 6 – 9 a significant difference in body masses of male and female foetuses at the 1.5 g/kg dose was noted and 3 of 75 males showed malposition testis at this dose. Besides that, the study done by Gardner at el., (2015), showed that the $LC_{50}$ of DPP at 14.7mg/L by using the *Xenopus laevis* embryos. There are a few malformation that can being seen in this study; abnormal gut coiling, edema and notochord abnormalities that were observed in *Xenopus laevis* tadpoles when exposed to DPP. In this study the morphological defect were unhatched embryo, uninflated swimbladder, yolk sac edema and pericardial edema as shown in Table 17.

**Table 17:** The morphological defects by Dipropyl Phthalate (DPP).

| Concentration (mg/l) | Unhatched (%) | Uninflated Swimbladder (%) | Yolk Sac Edema (%) | Pericardial Edema (%) |
|---|---|---|---|---|
| 4.31 | 0.00 ± 0.000 | 0.00 ± 0.000 | 18.44 ± 0.034 | 15.19 ± 0.000 |
| 5.39 | 5.36 ± 0.028 | 55.92 ± 0.018 | 39.58 ± 0.030 | 39.82 ± 0.028 |
| 6.47 | 7.12 ± 0.046 | 96.17 ± 0.019 | 30.16 ± 0.031 | 17.68 ± 0.021 |
| 7.01 | 11.55 ± 0.012 | 100.00 ± 0.024 | 100.00 ± 0.033 | 19.02 ± 0.029 |
| 8.30 | 51.77 ± 0.038 | 100.00 ± 0.023 | 91.11 ± 0.012 | 91.11 ± 0.012 |

*Standard deviation (SD)

In the negative control, embryo started to hatch between 48 to 72 hpf and the rate of hatching embryo was 100%. At low concentration, there was no unhatched defect was observed. At the concentration 5.39 mg/L, the unhatched defect was recorded at 5.36%. The percentage of unhatched defect was increased from 7.12% at 6.47 mg/L to 11.55% at 7.01 mg/L and 51.77% at 8.30 mg/L.

There are possibilities that DPP may affect the hatching mechanism in the embryo stages. As the hatching mechanism involves two process; digest chorion to weakening it by hatching enzyme and the movement of the larvae to break the chorion. Hatching enzyme is required to digest the chorion (Sano et al., 2008). The motility is needed for hatching too (Bagci et al., 2015). The hatching mechanism involved in two phases; zebrafish hatching embryo (ZHE) and physical movement by twitching inside the chorion. At the 48 hpf, the embryo will secrete the ZHE from the hatching gland at the yolk sac to digest the chorion.

The wall of the chorion already weakens then the wall of the chorion will be broken when there is a movement from the embryos. The larvae will hatch then swim free from the chorion.

However, there was no evidence of teratogenic risk, which is an agreement with data from the current study, although developing embryos in the current investigation were exposed to DPP. It is possible that the genetic differences in clutches may have attributed to this occurrence. Thus far, a threshold environmental concentration for DPP has not been determined although according to Gao et al. (2015), due to trends in toxicity values, data suggest that DPP may be conservatively estimated as similar to DBP.

### 4.1.2 Diallyl phthalate (DAP)

The mortality rate of diallyl phthalate (DAP) treated embryos was shown in Figure 19. The mortality rate increased which is the concentration of the DAP increased. As shown by probit analysis in Figure 20, DAP is a dose-response relationship chemical.

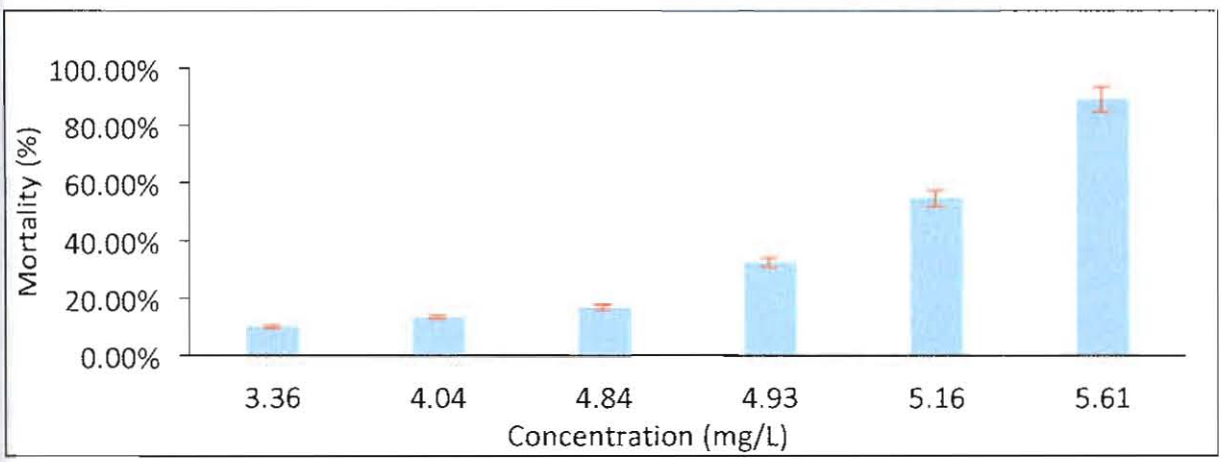



**Figure 19:** Mortality rate for diallyl phthalate.

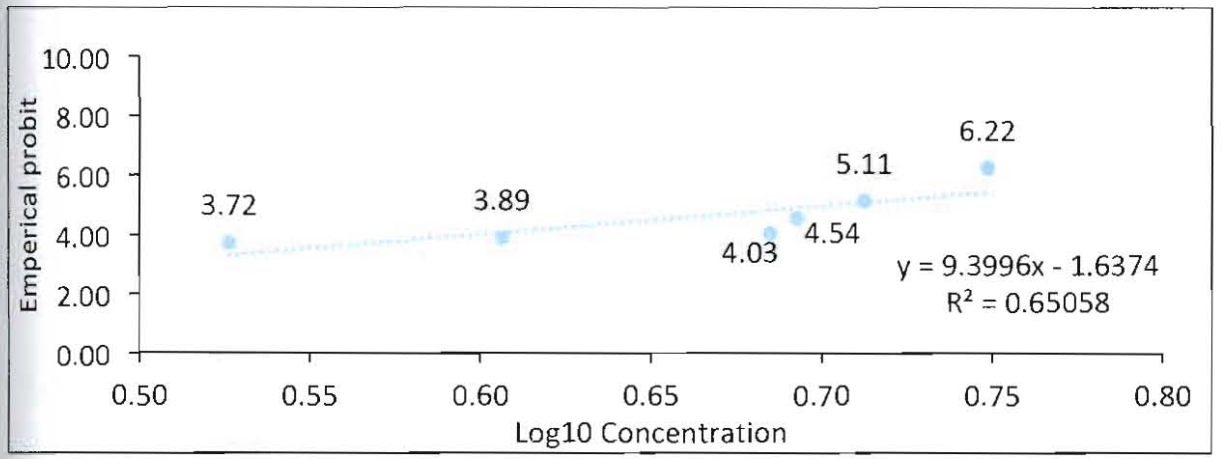



**Figure 20:** The Probit Analysis of diallyl phthalate.

The total number of embryo was used for 6 DAP concentration was 540 embryo. The $LC_{50}$ for DAP was at 5.122 mg/L with the range of 95% fiducial confidence interval from 4.703 – 5.579 mg/L. Meanwhile, the $LC_{10}$ for the DAP was at 3.593 mg/L with the range of 95% fiducial confidence interval from 3.299 – 3.913 mg/L. The result suggest that DAP in the aquatic environment present a teratogenic risk , as embryo exposed to DAP in the current study were much higher concentration that found in the sediment at the range 0.0018 – 0.0055 mg/L at Chester River, Maryland (Peterson & Freeman, 1984).

In a NTP study, female Fisher 344 rats were given DAP by gavage, for 13 weeks, at dose ranging from 25 to 400 mg/kg/day. Clinical signs were noted at 400 mg/kg/day, and macroscopic lesions of the liver at 200 mg/kg/day and higher. A NOAEL (no-observed – adverse-effect-level) was establish at 50 mg/kg/day, based on histopathological findings in the liver at 100 mg/kg/day (NTP, 1985). In the study perform by Saillenfait et al., 2008, the DAP showed that prenatal toxic effect at 200 and 250 mg/kg/day, as evidence by a decrease in fetal bodyweight. Additionally, developmental retardation was indicated to incomplete skeletal ossification of some bone at 250 mg/kg/day.

In the present study, the morphological test for DAP to zebrafish had 4 endpoint; uninflated swim bladder, yolk sac edema, pericardial edema and tail deformation as shown in Table 18. Out of 4 of this endpoint, the occurrence of tail deformation is the less severe as it percentage less than 10% either at low concentration or high concentration. Meanwhile, the percentage for the yolk sac edema and pericardia edema show a decreasing trend when the embryo was exposed from low concentration to high concentration. However, the uninflated swimbladder morphological defect show a stagnant percentage of occurrence at more than 95% despite of the exposure occur at low concentration or high concentration.

**Table 18:** The morphological defects of diallyl phthalate (DAP) chemical.

| Concentration (mg/L) | Uninflated swim bladder (%) | Yolk Sac Edema (%) | Pericardial Edema (%) | Tail deformation (%) |
|---|---|---|---|---|
| 3.36 | 96.00 ± 0.023 | 76.00 ± 0.035 | 42.30 ± 0.034 | 4.00 ± 0.000 |
| 4.04 | 97.160 ± 0.012 | 61.14 ± 0.018 | 33.40 ± 0.030 | 7.19 ± 0.028 |
| 4.48 | 96.54 ± 0.020 | 43.16 ± 0.039 | 29.65 ± 0.051 | 5.16 ± 0.021 |
| 4.93 | 97.36 ± 0.012 | 36.35 ± 0.024 | 25.35 ± 0.033 | 10.45 ± 0.029 |
| 5.16 | 98.36 ± 0.023 | 20.68 ± 0.053 | 23.00± 0.012 | 9.15± 0.012 |

* Standard deviation.

The uninflated swim bladder is one of the most severely defected morphological when treated with DAP as shown in Table 18. The defect will be increased as the concentration was increased. At the low concentration of 4.312 mg/L, there is none of this effect was observed. However, the occurrence starts to increase from the concentration of 5.39 to 8.30

mg/L. the percentage of the occurrence was at 55.92% to 100% and it drops to 33.33% at 10.78 mg/L because of mortality rate at 10.78 mg/L was 97.78%. This defect was observed at lower concentration when the embryo was exposed with the DAP in contrast to the DPP chemical. As shown in Table 19, the uninflated swim bladder defect showed a constant number of percentage from the concentration of 3.36 mg/L to 5.16 mg/L, the percentage was at the range 96.00% to 98.36%. At the concentration of 6.52 mg/L, the percentage was dropped to 50.54%., this was due to the number of mortality. At this concentration, most of the embryo was found dead before 48 hpf this might be the effect for the decreases of the morphological defect percentage.

Swim bladder started to inflate after 72 hpf, as none of the embryos inflates its swim bladder at 72 hpf. The swim bladder was believed to be homologous to the lungs of terrestrial vertebrates based on the morphological and molecular lines of evidence (Jonsson, Kubota, Timme-Laragy, Woodin & Stegeman, 2012). Although the mechanism of anterior chamber inflation is not known, its formation coincides with metamorphosis (Brown, 1997), linking this event to thyroid hormone (TH) known to drive dramatic morphological changes in the flounder (Inui & Miwa, 1985).

In humans, TH plays an important role during initial expansion of lungs at birth (Liggind, 1995). Hence, it is tempting to speculate that TH could regulate inflation of the anterior chamber of the swimbladder. The event of second chamber inflation could be coupled with other morphological changes which occur during this period time. At 15 – 20 dpf the hindgut elongates, shifts forward and makes the first gut loop followed by the second loop soon afterwards. These alterations in the distribution of the gut may be a principal factor in

shifting the position of the body's centre of gravity (Korzh et al., 2008; Wan et al., 2006). This, in turn, could necessitate an anterior expansion of the swim bladder to adjust buoyancy control.

### 4.1.3 Mixture of dipropyl phthalate and diallyl phthalate

The mortality rate of dipropyl phthalate and diallyl phthalate (DPP+DAP) treated embryos was shown in Figure 21. The mortality rate increased which is the concentration of the DPP+DAP increased. As shown by probit analysis in Figure 22, DPP+DAP is a dose-response relationship chemical.

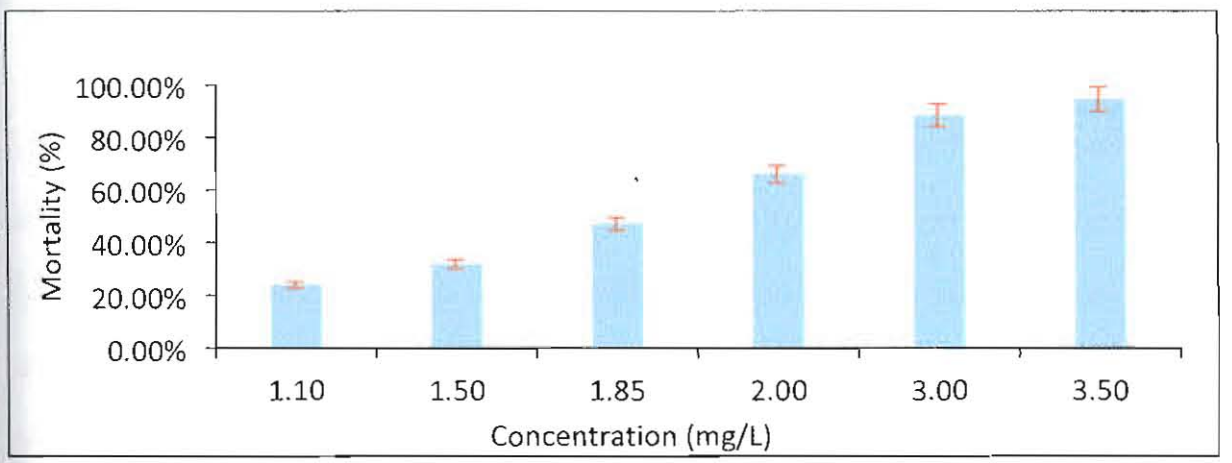



**Figure 21:** Mortality rate for combination dipropyl phthalate and diallyl phthalate.

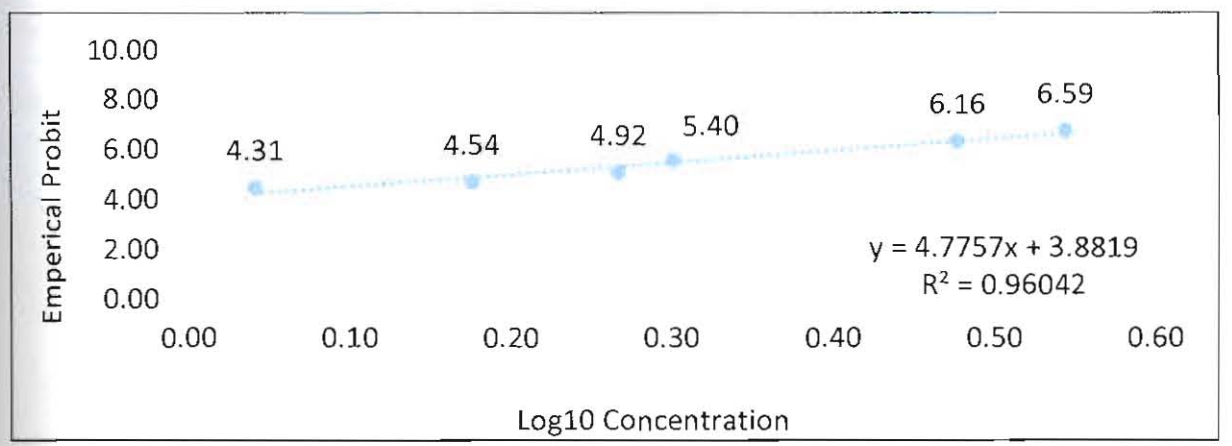



**Figure 22:** The Probit Analysis of mixture of dipropyl phthalate and diallyl phthalate.

The total number of embryo was used for 6 DPP+DAP concentration was 540 embryo. The $LC_{50}$ for DPP+DAP was at 1.709 mg/L with the range of 95% fiducial confidence interval from 1.438 – 2.032 mg/L. Meanwhile, the $LC_{10}$ for the DAP was at 0.915 mg/L with the range of 95% fiducial confidence interval from 0.770 – 1.088 mg/L. the combination of this two chemical had less data but based on the this study the $LC_{50}$ of this mixture was not alarming because it is still more than found in the environment. Although, the concentration that found in the environment is not as high as the $LC_{50}$ of this study but the concentration to cause half of the embryo population to dead is far less than the single chemical. Thus, the combination of this chemical had more toxic strength then the single chemical.

There were four endpoint for the morphological defect test for the DAP+DPP chemical; unhatched, uninflated swimbladder, yolk sac edema and pericardial edema as shown in Table 19. The unhatched embryo is more severe to occur at 24.06% compare to high concentration at 4.81%. However, uninflated swimbladder, yolk sac edema and pericardia edema shown a constant increasing in the percentage of morphological defect when the embryo were exposed to the DPP+DAP but the pericardial edema was the most severe morphological defect as it percentage was more than 67% despite of the exposure to the embryo at low or high concentration.

**Table 19:** The morphological defects of the mixtures of dipropyl phthalate (DPP) and diallyl phthalate (DAP) chemical.

| Concentration (mg/L) | Unhatched | Uninflated Swimbladder | Yolk Sac Edema | Pericardial Edema |
|---|---|---|---|---|
| 1.10 | 24.06 ± 0.006 | 49.12 ± 0.035 | 21.93 ± 0.023 | 80.78 ± 0.003 |
| 1.50 | 23.06 ± 0.031 | 65.83 ± 0.018 | 54.87 ± 0.012 | 66.97 ± 0.023 |
| 1.85 | 19.06 ± 0.048 | 76.61 ± 0.039 | 61.14 ± 0.020 | 81.45 ± 0.051 |
| 2.00 | 3.22 ± 0.039 | 80.54 ± 0.024 | 42.29 ± 0.012 | 79.21 ± 0.033 |
| 3.00 | 4.81 ± 0.017 | 85.14 ± 0.053 | 65.79 ± 0.023 | 85.99 ± 0.064 |

*standard deviation

The pericardial edema had involve in the heart structure. The heart is one of the primary organs to form and function firstly during zebrafish embryo development (Stainier et al., 1993, Lee et al., 1994). Zebrafish has become a model to study human cardiac diseases and is also commonly affected during toxicity studies (Hill et al., 2005). In this study, the DPP+DAP had impaired the heart morphology of zebrafish embryos and cause some characteristic cardiac defect; these defect become progressively more serious with increasing concentration. Heart function may be affected by the abnormalities of the pericardial and heart, there by resulting in irregular heartbeat and blood flow failure (Yamauchi et al., 2005). Previous work also demonstrated that phthalate had effect on the heart rate (Pfuderer & Francis, 1975; Rock et al., 1987). Dibutyl phthalate and Butyl benzyl phthalate had decrease the heart rate of goldfish (Pfuderer & Francis, 1975), and the cardiac arrested in rats after infusion of mono (2-ethylhexyl) phthalate (Rock et al., 1987).

## 4.2 Molecular analysis

### 4.2.1 RNA Quantification

The Total RNA (TRNA) quantification was listed in Table 20. The RNA integrity was run by using the agarose gel electrophoresis (AGE).

**Table 20:** The concentration of RNA.

| Concentration (mg/L) | Concentration of RNA (µg/µL) | Ratio (260:280) | Final concentration (µg/µl) |
|---|---|---|---|
| Control 1 | 5.44 | 1.60 | 0.5 |
| DPP, 3.80 | 4.20 | 1.38 | 0.5 |
| DPP, 6.60 | 6.40 | 1.72 | 0.5 |
| DAP, 3.60 | 2.32 | 1.54 | 0.5 |
| DAP, 5.10 | 3.56 | 1.68 | 0.5 |
| DAP+DPP, 0.90 | 3.08 | 1.65 | 0.5 |
| DAP+DPP, 1.70 | 3.26 | 1.61 | 0.5 |

A 260/280 ratio of ~1.80 is generally accepted as "pure" for DNA; a ratio of ~2.0 is generally accepted as "pure" for RNA. In Table 21, the ratio of OD 260/280 was calculated to determine the purity and the concentration of the sample. This ratio is to show is there any contamination in the sample when the RNA was extracted. The low OD260/280 ratio may be associated with the contamination from the residual of chemical that used in the extraction protocol.

All the RNA sample that extracted from the zebrafish larvae in this study are not pure, but good enough to do cDNA synthesis for PCR. There are few factor that contribute to the contamination of the sample. Small changes in the pH of the solution will also cause the 260/280 to vary (Wilfinger, Mackey & Chomczybski, 1997). It is important to ensure that the undiluted and diluted sample is at the same pH and ionic strength. The low OD260/OD280 could also be due to prolonged drying of RNA. Complete drying of RNA pellet will reduce the solubility, giving an OD260/OD280 ratio with less than 1.6. The low OD260/OD280 ratio may be associated with the pH used for RNA dilution as acidic pH decreases the ratio (Anusubel et al., 2003).

### 4.2.2 Temperature optimization of the targeted gene using Gradient PCR at 54 – 65 °C

The gradient PCR was done to analyse the optimum temperature for every targeted gene; *tsh_β, ttr, trα, trβ, dio_1, dio_2*. The gradient temperature was set between 54 – 65 °C. The optimum temperature for every targeted gene was analysed by using image J software based on the AGE gel result as in Table 21.

**Table 21:** The annealing temperature of all targeted gene.

| Gene name | annealing temperature | number of amplicon size |
|---|---|---|
| *tsh_β* | 60 °C | 121 bp |
| *ttr* | 58 °C | 153 bp |
| *trα* | 60 °C | 85 bp |
| *trβ* | 60 °C | 110 bp |
| *dio_1* | 59 °C | 142 bp |
| *dio_2* | 58 °C | 103 bp |

The primer length and sequence are critical in amplifying PCR products with specificity and efficiency (Dieffenbach et al., 1993). The stability of the primer-DNA template duplex is measured by its melting temperature (Tm), the temperature at which half of the primer-DNA duplex dissociates to become single-stranded DNA. The length of the primer and the G and C content of the primer-DNA template duplex play critical roles in determining Tm, which is calculated by the formula 4(G+C) + 2(A+T), and this is the rule of thumb for calculating annealing temperature. The observed annealing temperature is usually a few degrees below the calculated annealing temperature and is influenced by other variables of the PCR, such as Mg++ concentration and K+ concentration.

### 4.2.3 Direct sequencing

All the PCR sample were sent for sequencing to get the nucleotide sequence. The nucleotide sequence search were carried out using BLASTn through the NCBI server as shown in Table 22. The result shown the query coverage and the max identity. The query coverage mean the percentage of the nucleotide sequence by sequencing cover the sequence in the NCBI database. The max identity is the percentage of similarity of nucleotide sequence with the database of the gene in the NCBI server, the higher the percentage the higher the similarity of the gene with the database.

**Table 22:** The BLASTn result for targeted gene.

| Gene | Accession | Description | Query coverage | E-value | Max Identity |
|---|---|---|---|---|---|
| *tsh_β* | XM_021476793.1 | PREDICTED: *Danio rerio* thyroid stimulating hormone, beta subunit, α (*tsh_β*), transcript variant X2, mRNA | 96% | 0.0 | 99% |
| *ttr* | XM_009294552.3 | PREDICTED: *Danio rerio* transthyretin (prealbumin, amyloidosis type I) (*ttr*), transcript variant X1, mRNA | 97% | 0.0 | 99% |
| *trα* | XM_009299516.3 | PREDICTED: *Danio rerio* thyroid hormone receptor alpha a (*trα*), transcript variant X4, mRNA | 100% | 0.0 | 99% |
| *trβ* | XM_005159502.4 | PREDICTED: *Danio rerio* thyroid hormone receptor beta (*trβ*), transcript variant X3, mRNA | 98% | 0.0 | 99% |
| *dio_1* | NM_001324404.1 | *Danio rerio* deiodinase, iodothyronine, type I (*dio_1*), transcript variant 2, mRNA | 99% | 0.0 | 99% |
| *dio_2* | NM_212789.4 | *Danio rerio* deiodinase, iodothyronine, type II (*dio_2*), mRNA | 99% | 0.0 | 99% |

### 4.2.4 PCR of targeted for $LC_{50}$ treated sample

The $LC_{50}$ for every treated sample from three different chemicals (DAP, DPP and DAP + DPP) were run PCR with the targeted gene to observe the level of expression. The AGE picture of the targeted gene was analysed by using Image J software to get the qualitative data.

Studies exploring the association between DPP, DAP and DPP+DAP with the thyroid endocrine disrupting effect are limited, however, growing human studies have indicated that DPP and DAP has the potential to alter thyroid hormone level (Boas et al., 2010; Meeker & Ferguson, 2011). In the present study, developing zebrafish embryos/larvae were employed to assess effect of these chemicals on the mRNA expression related to thyroid hormone synthesis, secretion, transport and metabolism. The result showed that DPP, DAP and DPP+DAP change the expression of gene involved in the hypothalamic – pituitary – thyroid (HPT) axis, thus clearly demonstrating its thyroid endocrine disrupting activity. It has been suggested that DPP, the active metabolite of DBP, is related to the toxic effects of its parent compound (Heng, Anand-Ivell, Teerds & Ivell, 2011). In the previous study done by Aarab et al., (2006) DAP also suggested to be thyroid endocrine disruptor hormone.

The pituitary gland regulates thyroid activity through the secretion of TSH, and in turn TSH secretion is triggered by changes in the concentration of circulating THs via feedback mechanism (Ji, Jin, He & Yin , 2012). TSH is glycoprotein complex that composed of two subunits (α and β), and the β subunits (*tsh_β*) play pivotal roles in the HPT axis. The *tsh_β*

gene mRNA transcription level after exposed to the DPP, DAP and DPP+DAP as shown in Figure 23. In the present study, transcription of *tsh_β* gene was significantly down regulated after exposed to the DPP, DAP and DPP+DAP chemicals similar with the study done by Shen et al., (2011) the *tsh_β* gene transcription show decreases mRNA transcription when exposed with DBP, the gene was extracted from the head tissue of tadpole after reached stage 57. It has been proposed that assessment of *tsh_β* gene transcription can be used to determine whether environmental pollutants give rise to thyroid dysfunction.

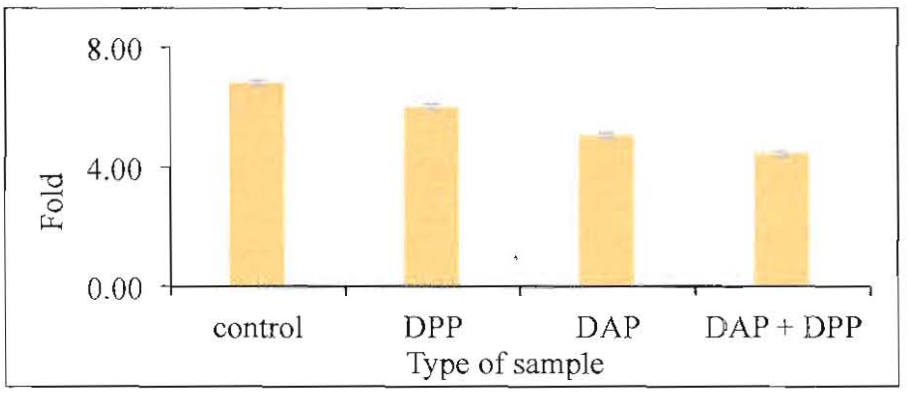



**Figure 23:** The PCR for treated sample for the *tsh_β* gene.

TTR, an important transport protein for THs, play an important role in the thyroid axis in fish by regulating the supply of the hormone to various tissues (Power et al., 2000). The *ttr* gene mRNA transcription level after exposed to the DPP, DAP and DPP+DAP as shown in Figure 24. In this study, DPP, DAP and DPP+DAP exposure led to decrease of *ttr gene* transcription, which may reduce the amount of TTR binding to free THs. Consequently, the excess uncombined $T_4$ would be more susceptible to hepatic catabolism, resulting in a greater elimination and a reduction in circulating TH concentration (Fernie et al., 2005).

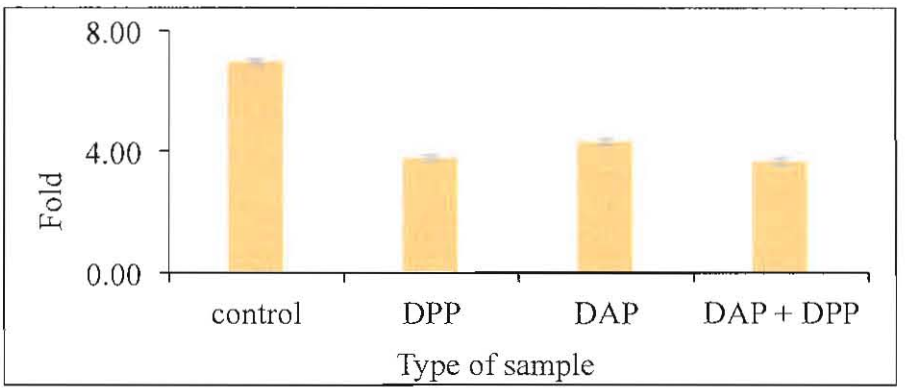



**Figure 24:** The PCR for treated sample for the *ttr* gene.

Deiodinase play a key role in the regulating of circulating and peripheral TH level in vertebrates. Deiodinase activity was critical for regulation of tissue TH concentration and strongly affected by serum TH concentration (Orozco & Valverde, 2005). Previous study had recommended a measure of deiodination gene expression as sensitive biomarker of thyroid disruption in fish upon exposure to environment (Li et al., 2009; Shi et al; 2009). Teleost, there are three type of deiodinases; type 1 (*dio_1*), type 2 (*dio_2*), type 3 (*dio_3*) and each of them has different functions. *dio_1* has a considerable influence on iodine recovery and thyroid hormone degradation (Geyten, Byamungu, Reyns, Kuhn, & Darras, 2005). *dio_2* exclusively catalyzes outer-ring deiodination of $T_4$ into active $T_3$, consequently controls the intracellular concentration of T3, and *dio_3* is purely inactivating enzyme (Orozco & Valverde-r, 2005). The *dio_1* and *dio_2* gene mRNA transcription level after exposed to the DPP, DAP and DPP+DAP as shown in Figure 25 and 26 .In this study, both *dio_1* and *dio_2* were significantly reduce after exposed with DPP, DAP and DPP+DAP, consistent with the previous study that showed *dio_1* may be more readily response to change thyroid levels than *dio_2* (Shi et al., 2009).

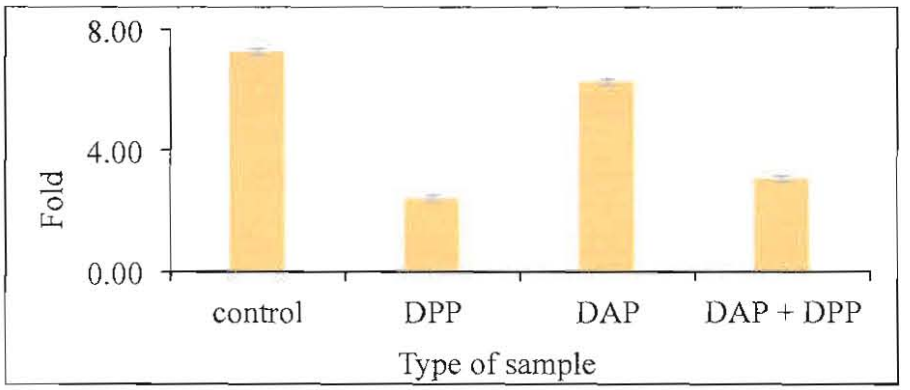



**Figure 25:** The PCR for treated sample for *dio_1* gene.

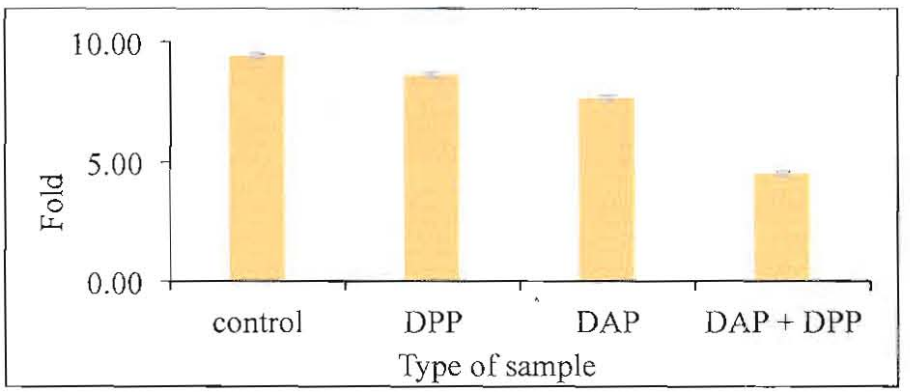



**Figure 26:** The PCR for treated sample for *dio_2* gene.

The action of thyroid hormones was mediated by their binding to nuclear receptor. In zebrafish, there were two type receptor (α and β), which was expressed in developing eggs prior to hatch and the two types had different functions (Power et al., 2001). The *trβ* and *trα* gene mRNA transcription level after exposed to the DPP, DAP and DPP+DAP as shown in Figure 27 and 28. In this study we found thyroid hormone receptor beta (*trβ*) mRNA expression was decreases compared to the thyroid hormone receptor (*trα*). Although, both of it mRNA expression was decreases compare to the control. Similar studies showed that the mRNA expression profiles of zebrafish thyroid hormone receptor beta could response to the presence to TH more efficiently than *trα* (Liu et al., 2009; Liu &

Chan, 2002). Abnormal thyroid hormone receptor (α and β) might result in the failure of THs to bind and activate the appropriate post-receptor response cascades. Thyroid hormone receptor beta (*trβ*) was largely regulated by $T_3$ at the level of transcription. In previous study, $T_3$ content was increased and thyroid hormone receptor beta (*trβ*) was significantly up regulated after exposure to PFOS (Shi et al., 2009).

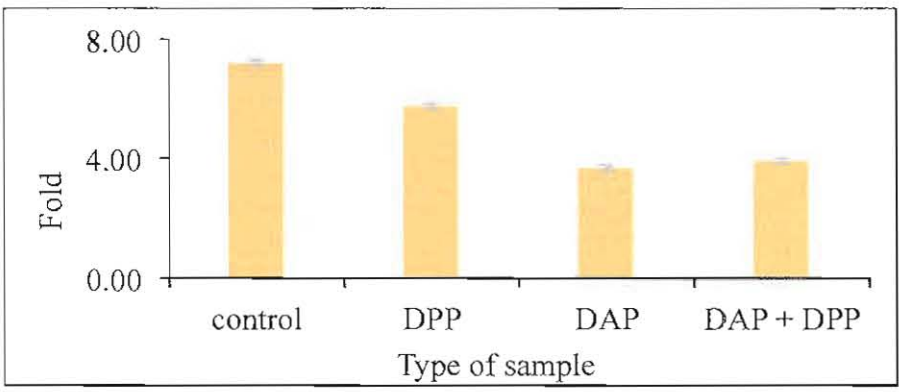



**Figure 27:** The PCR for treated sample for the *trα* gene

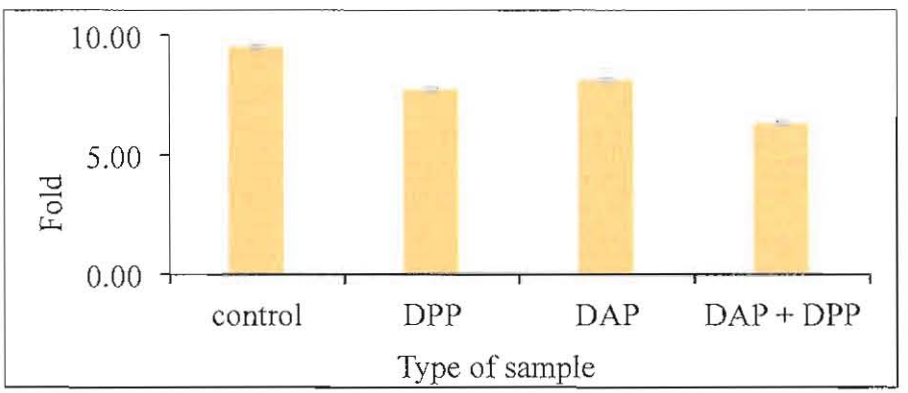



**Figure 28:** The PCR for treated sample for the *trβ* gene.

# CHAPTER 5

# CONCLUSION AND RECOMMENDATIONS

Throughout this project, it is clear that DPP, DAP and the DPP+DAP could induce both lethal and sublethal effect in the early vertebrate development of zebrafish. Although the concentration of the $LC_{50}$ for DPP, DAP and DPP+DAP were found at high concentration in this study compare to the concentration found in the environment, some action needs to take to make sure the concentration found in the environment won't be the same as the $LC_{50}$. The most morphological defect for DPP and DAP were uninflated swimbladder, and for DPP+DAP was pericardial edema. This study demonstrates that DPP, DAP, DPP+DAP induced developmental toxicity, impaired the structure and functions of developing organ, and altered the expression of the thyroid hormone gene marker; *tsh_β, ttr, trα, trβ, dio_1 and dio_2*. Although, the exact mechanisms of the DPP, DAP and DPP+DAP toxic effect towards the thyroid endocrine hormone still need further research. This research suggests that the expression change of the gene might be one of the main cause of zebrafish embryo morphological defect. The thyroid hormone gene can be the biomarkers for these chemicals as this study show that all 6 gene markers had a change in the mRNA expression after zebrafish embryos were exposed to these chemicals.

However, the result based on AGE was semi-quantitative. For a more precise data, qPCR is recommended. The membrane may be studied to identify the presence of membrane distortion, resulting in edema. Gene knockdown study needs to be carried out to give insight into the function of the targeted gene after the embryo treated with the chemical to understand the gene pathway that affected by this chemical.

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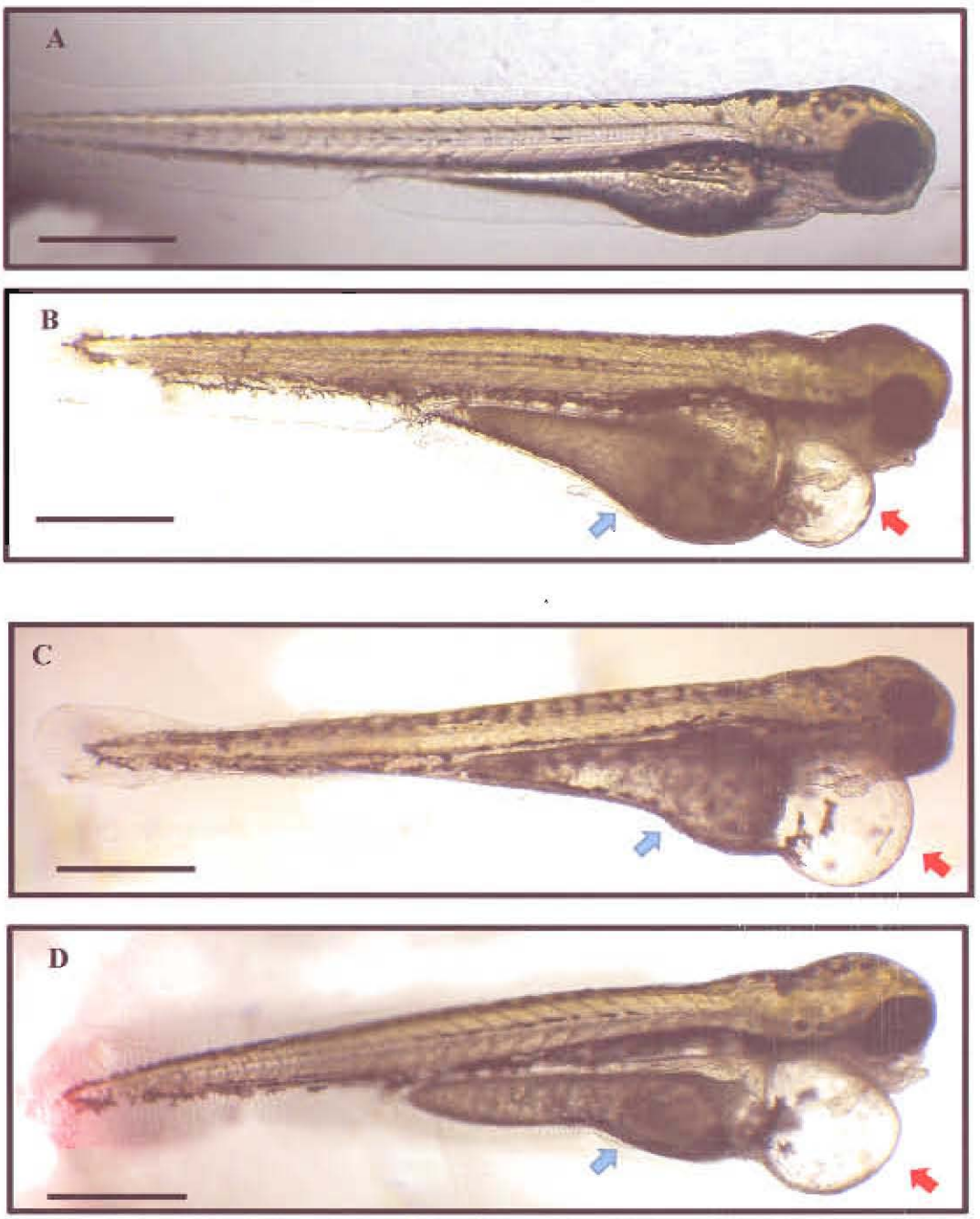
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# APPENDICES

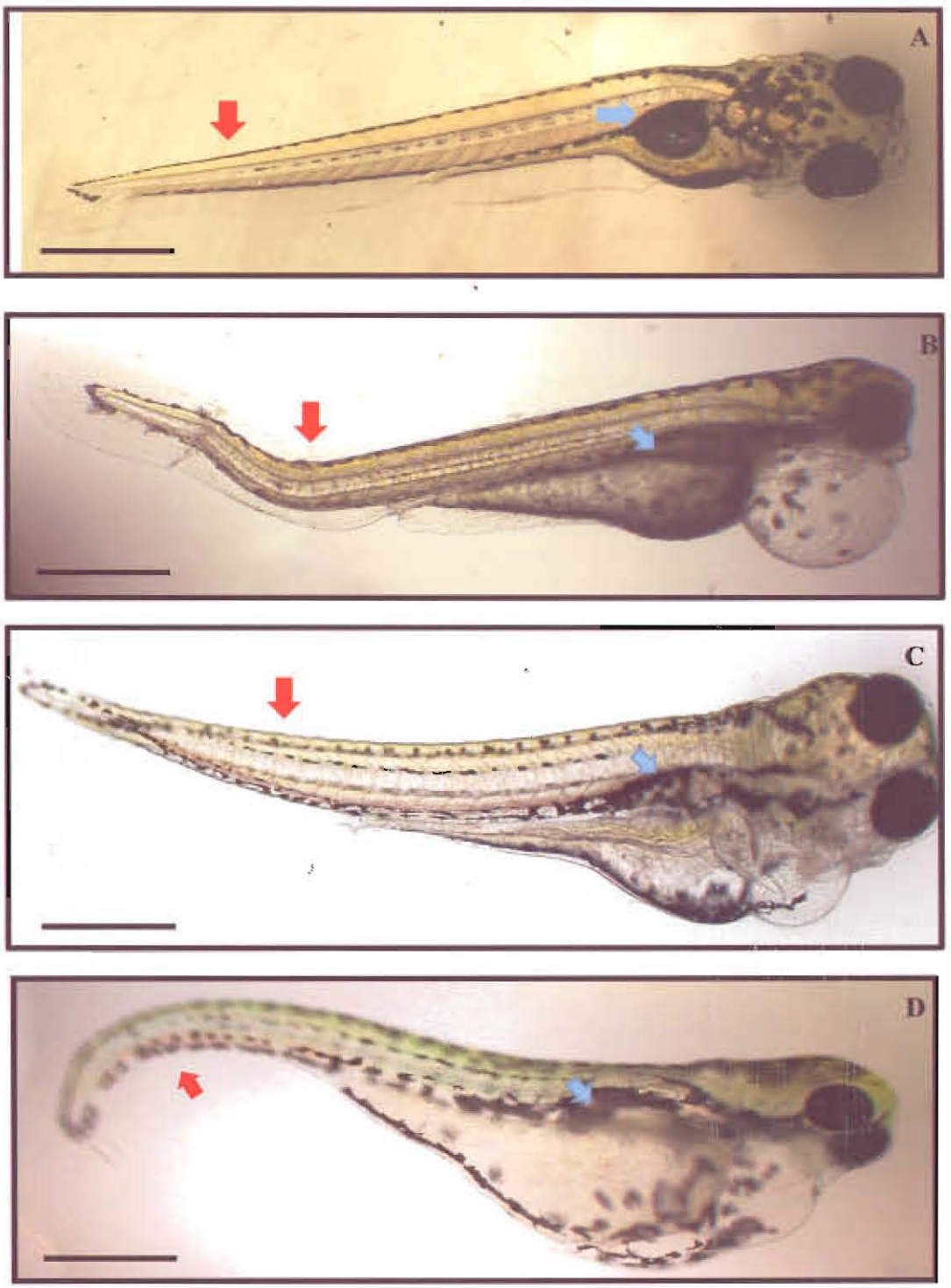
**Appendix 1: Morphological Defect of zebrafish embryo after Treatment.**

Pericardial edema and Yolk Sac edema at 96 hpf



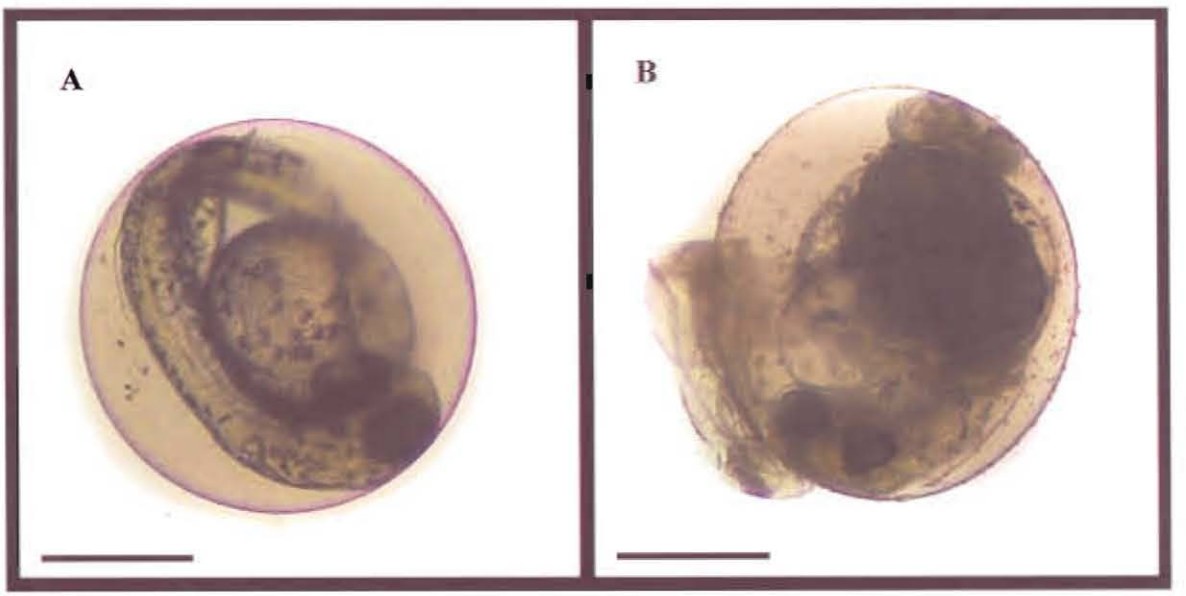

**Appendix 1-1:** Pericardial edema and Yolk Sac edema at 96 hpf. Control (A), Treated with DPP (B), Treated with DAP (C), Treated with DAP+DPP (D). Red arrowhead indicates pericardial edema while blue arrowhead indicates yolk sac edema at 96hpf. Scale bar = 1 mm

Uninflated Swimbladder and tail deformation at 96 hpf.




**Appendix 1-2:** Uninflated Swimbladder and tail deformation at 96 hpf. Control (A), Treated with DAP (B), Treated with DPP (C), Treated with DAP+DPP (D). Blue arrowhead indicates uninflated swim bladder while red arrowhead indicates tail deformation at 96hpf. Scale bar = 1 mm

Unhatched and Coagulation at 96 hpf.




**Appendix 1-3:** Unhatched and Coagulation at 96 hpf. Unhatched embryo (A) and Coagulate embryo (B). Scale bar = 0.5 mm

## Appendix 2: Gene used for the thyroid hormone (TH)

LOCUS NM_181601 1786 bp mRNA linear VRT 26-JUN-2016
DEFINITION Danio rerio actin, beta 2 (*β-actin*), mRNA.
ACCESSION NM_181601
VERSION NM_181601.4
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

>NM_181601.4 Danio rerio actin, beta 2 (***β-actin***), mRNA

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CCCCGTGCTGTCTTCCCATCCATCGTGGGTCGCCCCAGACATCAGGGTGTCATGGTTGG
TATGGGACAGAAAGACAGCTACGTTGGTGATGAGGCTCAGAGCAAGAGAGGTATCCT
GACCCTGAAGTACCCCATCGAGCACGGTATTGTCACCAACTGGGATGATATGGAGAAG
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TGCTCACAGAGGCCCCCCTGAACCCCAAGGCCAACAGGGAAAAGATGACACAGATCA
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TGTGCCCATCTACGAGGGTTACGCCCTGCCCCATGCCATCCTCCGTCTGGACTTGGCTG
GCCGTGACCTGACTGACTACCTCATGAAGATCCTGACCGAGAGAGGCTACAGCTTCAC
CACCACAGCTGAGAGGGAAATTGTCCGTGACATCAAGGAGAAGCTCTGCTATGTGGCC
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TCAACTCCATCATGAAGTGTGACGTCGACATCCGTAAGGACCTGTATGCCAACACTGT
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LOCUS NM_001324404 1464 bp mRNA linear VRT 09-MAR-2017
DEFINITION Danio rerio deiodinase, iodothyronine, type I (dio1), transcript variant 2, mRNA.
ACCESSION NM_001324404
VERSION NM_001324404.1
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

>NM_001324404.1 Danio rerio deiodinase, iodothyronine, type I (dio1), transcript variant 2, mRNA

GATTCTCCTGATTTCGCCGTGATATAATGTTGTGTTTTGGAGGATGGCACGCCTCTTTG
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LOCUS NM_212789 2219 bp mRNA linear VRT 09-MAR-2017
DEFINITION Danio rerio deiodinase, iodothyronine, type II (dio2), mRNA.
ACCESSION NM_212789 XM_697490
VERSION NM_212789.4
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

>NM_212789.4 Danio rerio deiodinase, iodothyronine, type II (dio2), mRNA

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NM_131340 2198 bp mRNA linear VRT 23-APR-2017
DEFINITION Danio rerio thyroid hormone receptor beta (thr-β), mRNA.
ACCESSION NM_131340 XM_687890
VERSION NM_131340.1
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

>NM_131340.1 Danio rerio thyroid hormone receptor beta (thrb), mRNA

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LOCUS NM_131396 2200 bp mRNA linear VRT 23-APR-2017
DEFINITION Danio rerio thyroid hormone receptor alpha a (thrα-a), mRNA.
ACCESSION NM_131396
VERSION NM_131396.1
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

>NM_131396.1 Danio rerio thyroid hormone receptor alpha a (thraa), mRNA

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TCTTCGTAAGGAAGTGGCAGTGGCGTCCTGTAACTGCTGAATGAGCGACCGTGAGCTG
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GAGAAGTGTCAGGAGATGTACCTGTTGGCATTCGAGCACTACATCAACCACCGCAAGC
ACAACATCTCCCACTTCTGGCCCAAGCTGCTGATGAAGGTGACGAACCTGCGCATGAT
CGGCGCCTGCCACGCCAGCCGCTTCCTGCACATGAAGGTGGAGTGTCCAACAGAACTG
TTCCCGCCGCTCTTCCTGGAGGTCTTCGAGGATCAGGAGGGAAGCACTGGAGTGGCAG
CACAGGAAGACGGTTCCTGCTTAAGGTGAACCTCGTTTCGGTCCCGCACCTCCAAACC
ACATCCATTCACGTGCATCACAAGAGGTTTACCTTGTTCTGCTGCTTGCCCAAAATAAA
AAGGACACAGCCAGACTCCCCCGAAAGCATCTTTTGGAATAGTTTAACCCGCTGGTTC
AGATGCATAGCTGGGTTTCAGCGTCCTTTTTGACCGATATTGTGTCATTATGGCTCCAG
GAGTTCGGGACGTGGTCTCCATTTAAAGTGACACAGAGGAACAAAAGGGCAAAAAGG
ACAAACTCACTGTGACTCGCTAATCTCAGCTCAATGCAACCCACCAGCACTGCAGACA
CACACTGCCCACAGACGCAATCTACAGCACCAGCACGGGGAATCAGGAAGACCTCTA
TTTTTTTTCTCTCTCTCTGGACGTACAGTACAGAACTGGTTTACAGCAGCACTTTTATTA
GAACAGAACATTCAGTTCTTTACACTTAGCCAGATGATGAATATGTTGATTGAGAAGT
TGGCTCTAAAATGGGTCATATTATAATGTTTTGAAATAAAAGTGCATTGTGCTATG

LOCUS NM_001005598 791 bp mRNA linear VRT 26-JUN-2016
DEFINITION Danio rerio transthyretin (prealbumin, amyloidosis type I) (ttr), mRNA.
ACCESSION NM_001005598 XM_002665652
VERSION NM_001005598.2
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

>NM_001005598.2 Danio rerio transthyretin (prealbumin, amyloidosis type I) (ttr), mRNA

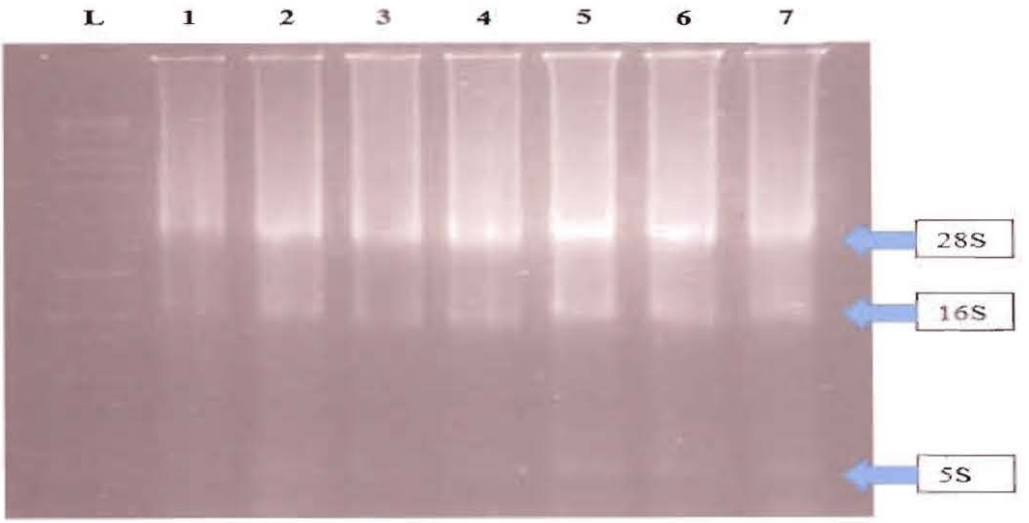
ATTTTGGGTGTTATCTCCCACAGTTCTGCTCGAACATGGCGAAAGAAGTGATTTGTGTG
CTTCTCGCATCTCTGTTTGCCCTCTGTAGATCTGCTCCAGTGGCTTTTCATGGTGGTTCA
GATGCTCACTGTCCTCTGACGGTAAAAATCCTGGATGCTGTCAAAGGGACGCCTGCTG
GAAATATAGCTCTGGATCTGTTTCGTCAAGATCAAGGTGGGACATGGGAAAAGATTGC
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CACTCCTGGTGTGTATCGGGTGGAGTTTGACACTTTAACCTACTGGAAGACAGAGGGT
CGCACACCTTTCCACCAGCTGGCTGATGTGGTGTTTGAAGCTCATGCAGAGGGACATC
GTCATTACACACTGGCTCTCCTTCTGAGCCCTTTCTCGTACACCACAACTGCTGTGGTC
GTCAAGGCACATGACTGACATCCTTTAAAAGATGGCCCATATCAACAACTTGAAACCA
AATACTGTACATCCCGAGTTCAAGATTACCTTCTTGCAGTAACCAAGTCATTCGATTGC
CATTTCAATAAGTATTTATGTAGGTATTTGTGGTAACTTTTTGATAGCTTTAAACATTTC
ATCACACCTGGTTTTCATAAATCTATTTACAAGTGAGGGACACTAGTTACGCTTCCAGC
CAAAGATGCGAATGTGACTTATGTGCGAAACTGGAATATCATTTAAAACATTTGCGAA
TAAAGCACCATTTCTATCCAATGAGCAAAA

LOCUS NM_181494 816 bp mRNA linear VRT 23-APR-2017
DEFINITION Danio rerio thyroid stimulating hormone, beta subunit, a (tshβa), mRNA.
ACCESSION NM_181494
VERSION NM_181494.2
KEYWORDS RefSeq.
SOURCE Danio rerio (zebrafish)
ORGANISM Danio rerio

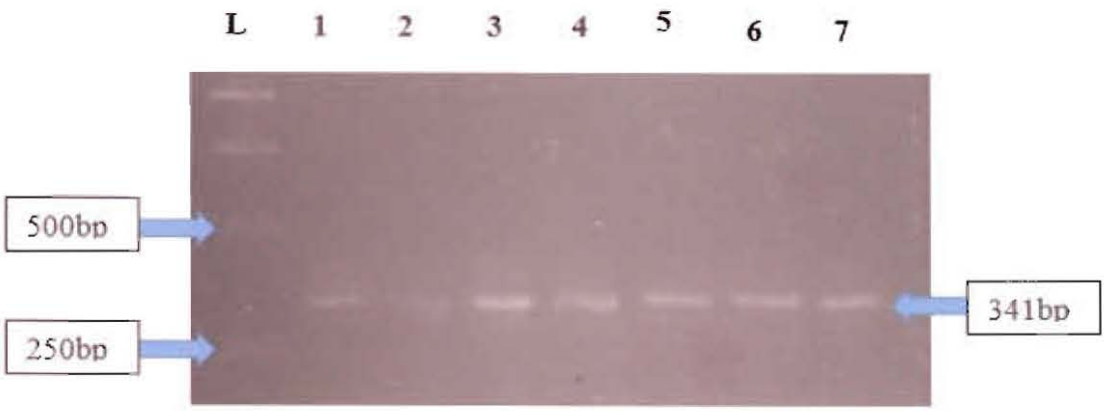
>NM_181494.2 Danio rerio thyroid stimulating hormone, beta subunit, a (tshβa), mRNA

AGGTTAATCCAGACCCTCCAGACAGACATCCTCATACACTGCCACCTATCATGTCTCTC
CTGTATGTTATTGGCATGCTGGGACTTTTAATGAAGGTTGCCGTGCCTATGTGTGCCCC
CACTGACTACACCATCTACATTGAGAGACAGGAGTGCAATTACTGTGTGGCTGTCAAC
ACCACCATCTGCATGGGCTTCTGTTTCTCCAGGGACAGTAACATAAAGGAGTTGGTGG
GTCCTCGTTTTATTGTTCAGAGGGGATGCACTTATCAGGAAGTTGAGTACCGGACAGC
CGTCTTGCCGGGATGCCCTTCACATGCAGATCCTCACTTCACCTACCCAGTGGCACTTA
GCTGCCACTGCAGCACCTGCAAAACCCACAGTGATGAATGTGCCCTTAGAACCCGCAG
CGCTGGGATGAGATGCTCCAAACCTGTGCATCATCTGTACCCCGAGGAGAACAACTAC
GCCCAAGCATATTGGGACCAGTATGAGTAATATTTGATTATTCCTTGGCTGAATCTAAA
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ATGTCATAAAGTTACCTAATCCATTACGGTTACTGGGTTTTATATCCAGCATCTGATGT
GTTTATTGAAGCCAAACATGATTTAAAAAGAAGGTGTGGTTTTAATAGGGTTCAAATA
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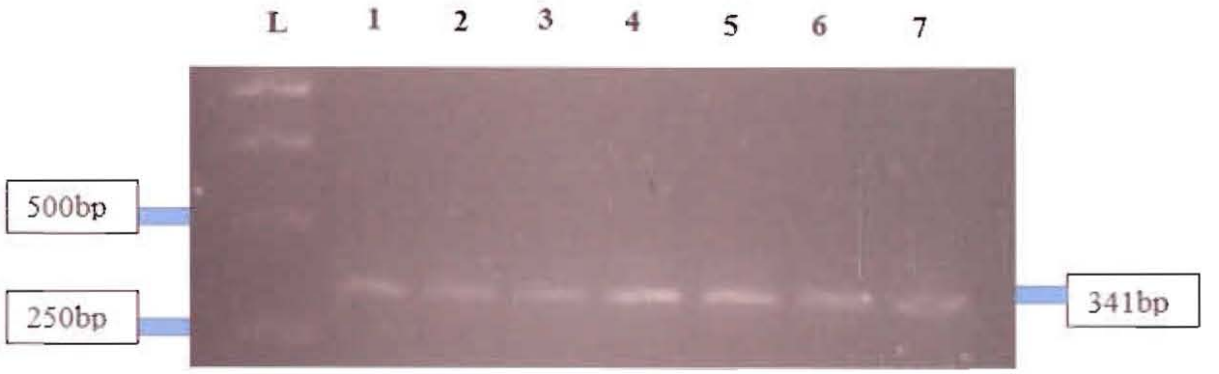
## Appendix 3: Agarose Gel Electrophoresis (AGE)



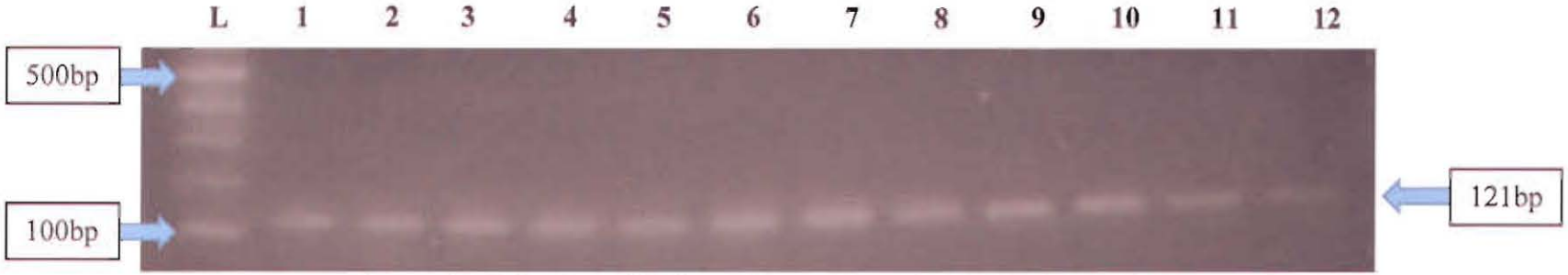

**Appendix 3-1**: The AGE for RNA. (L: DNA Ladder 1kb; 1: Control; 2: DPP 3.80 mg/l 3: DPP 6.60 mg/l; 4: DAP 3.60 mg/l; 5: DAP 5.10 mg/l; 6: DAP+DPP 0.90 mg/l; 7: DAP+DPP: 1.70 mg/l)



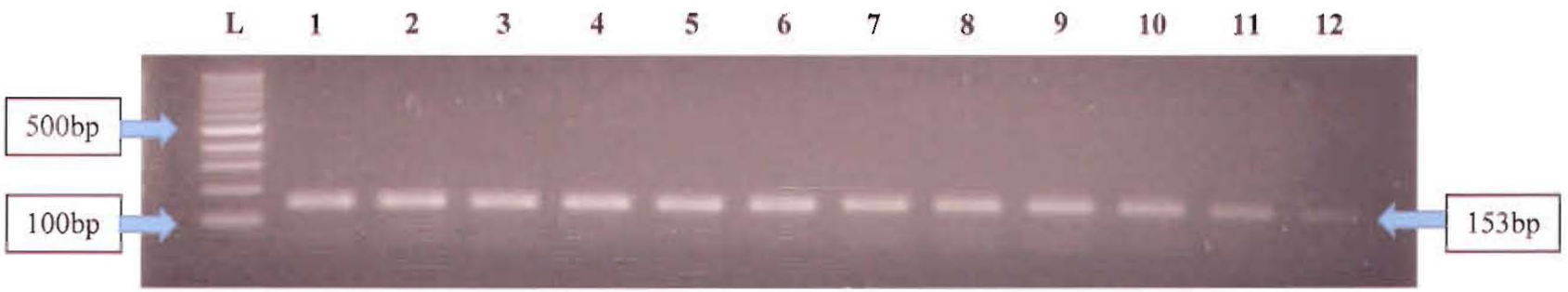

**Appendix 3-2:** The AGE for *β-actin2* before normalization. (L: DNA Ladder 1kb; 1: Control; 2: DPP 4.56 mg/l; 3: DPP 5.10 mg/l; 4: DAP 5.16 mg/l; 5: DAP 3.09 mg/l; 6: DAP+DPP 1.53 mg/l; 7: DAP + DPP: 0.72 mg/l)



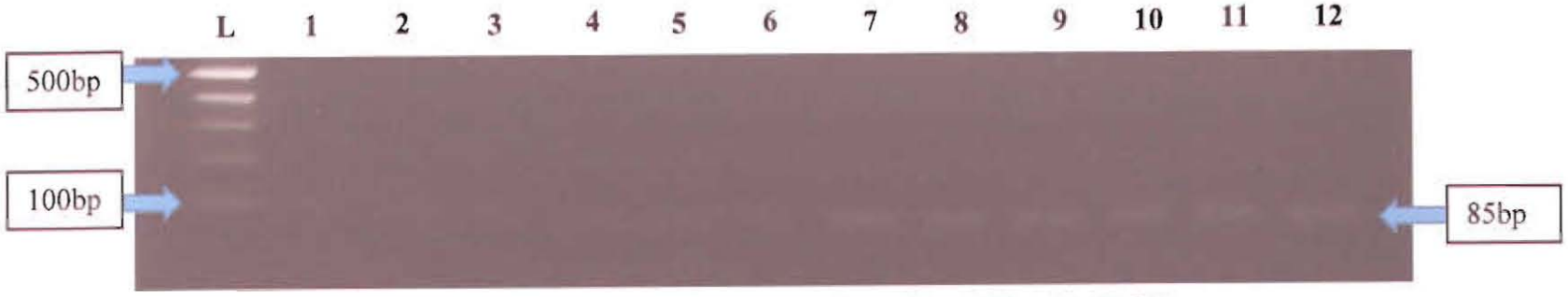

**Appendix 3-3:** The AGE for *β-actin* before normalization. (L: DNA Ladder 1kb; 1: Control; 2: DPP 4.56 mg/l; 3: DPP 5.10 mg/l; 4: DAP 5.16 mg/l; 5: DAP 3.09 mg/l; 6: DAP+DPP 1.53 mg/l; 7: DAP+DPP: 0.72 mg/l)




**Appendix 3-4:** The AGE for *tsh-β* gene in gradient PCR (54 - 65 °C).




**Appendix 3-5:** The AGE for *ttr* gene in gradient PCR (54 - 65 °C).




**Appendix 3-6:** The AGE for *trα* gene in gradient PCR (54 - 65 °C).




**Appendix 3-7:** The AGE for *trβ* gene in gradient PCR (54 - 65 °C).

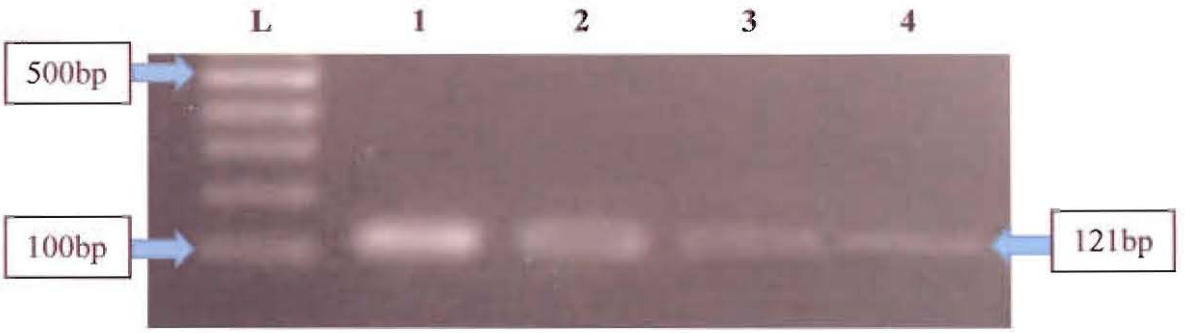



**Appendix 3-8:** The AGE for *dio_1* gene in gradient PCR (54 - 65 °C).

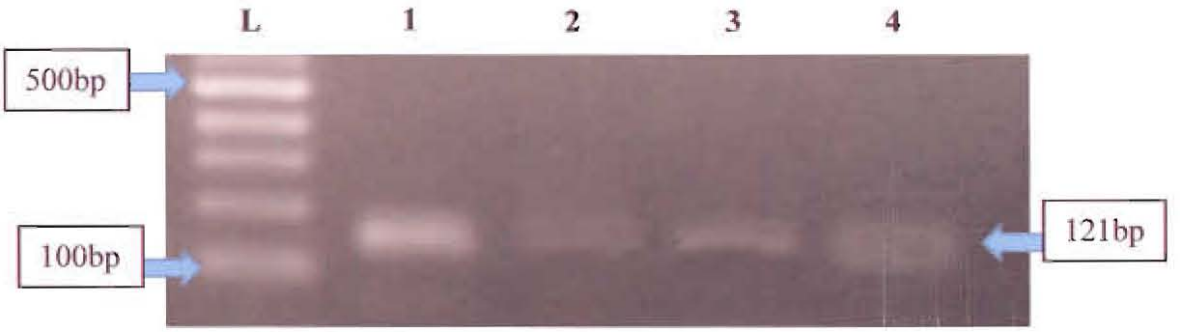



**Appendix 3-9:** The AGE for *dio_2* gene in gradient PCR (54 - 65 °C).

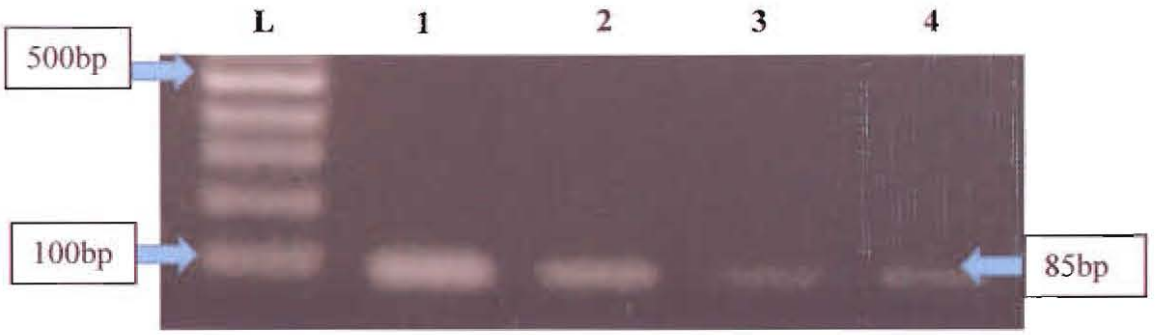
**Appendix 3.1: The Age for PCR of $LC_{50}$ of DPP, DAP, and DPP+DAP of targeted gene.**



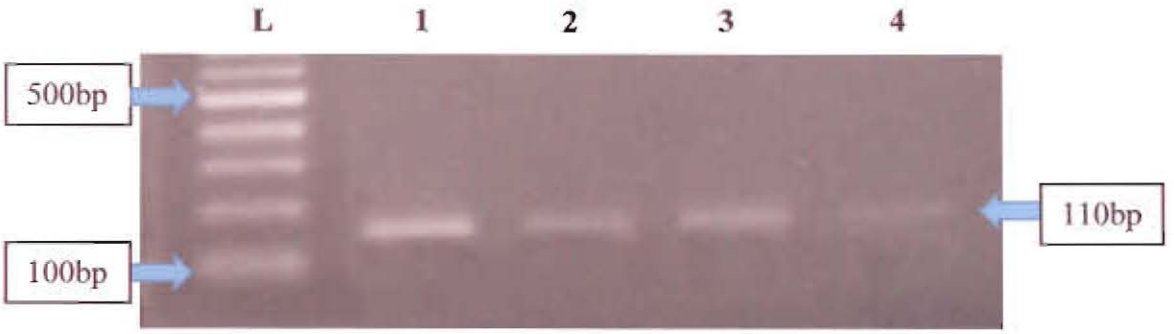

**Appendix 3.1-1:** *tsh_β* treated embryo Control (1), DAP (2), DPP (3) and DAP + DPP (4).



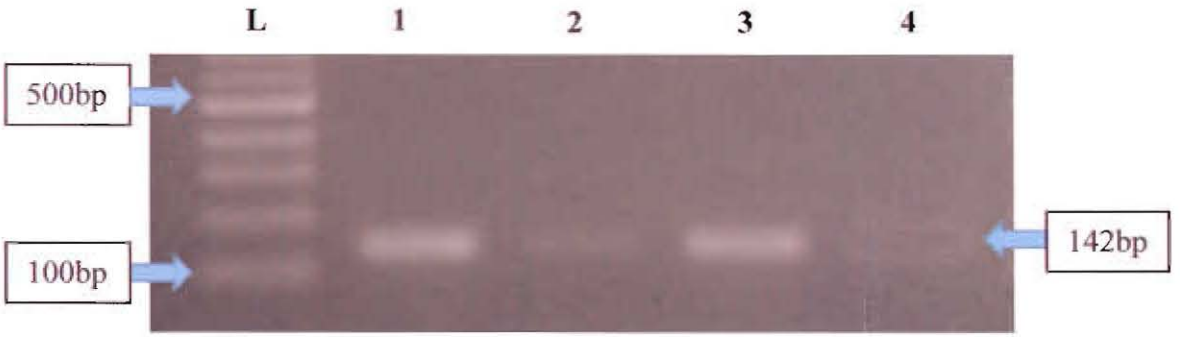

**Appendix 3.1-2:** *ttr* treated embryo Control (1), DAP (2), DPP (3) and DAP + DPP (4).



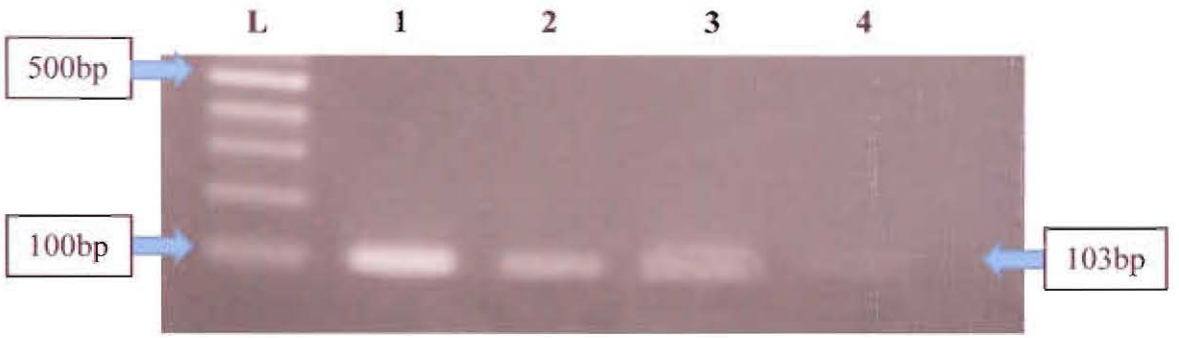

**Appendix 3.1-3:** *trα* treated embryo Control (1), DAP (2), DPP (3) and DAP + DPP (4).




**Appendix 3.1-4:** *tr_β* treated embryo Control (1), DAP (2), DPP (3) and DAP + DPP (4).

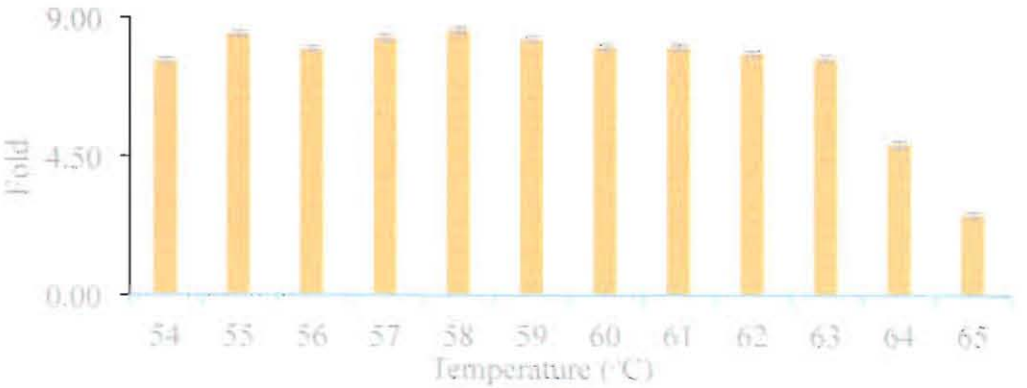



**Appendix 3.1-5:** *dio_1* treated embryo Control (1), DAP (2), DPP (3) and DAP + DPP (4).

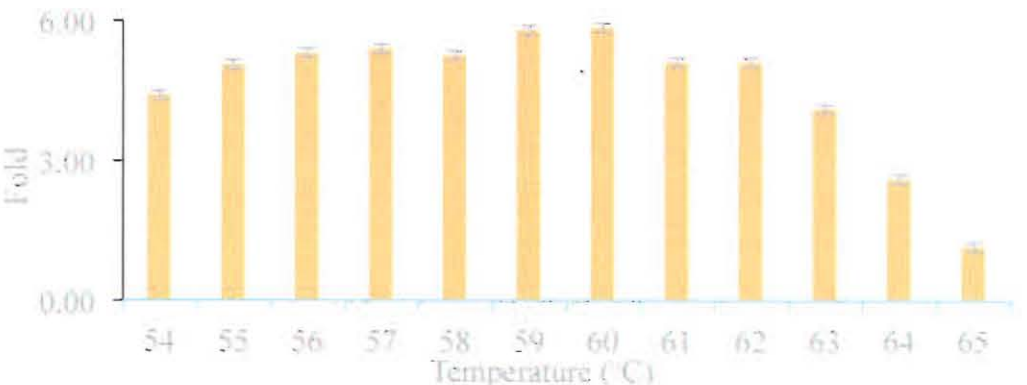



**Appendix 3.1-6:** d*io_2* treated embryo Control (1), DAP (2), DPP (3) and DAP + DPP (4).

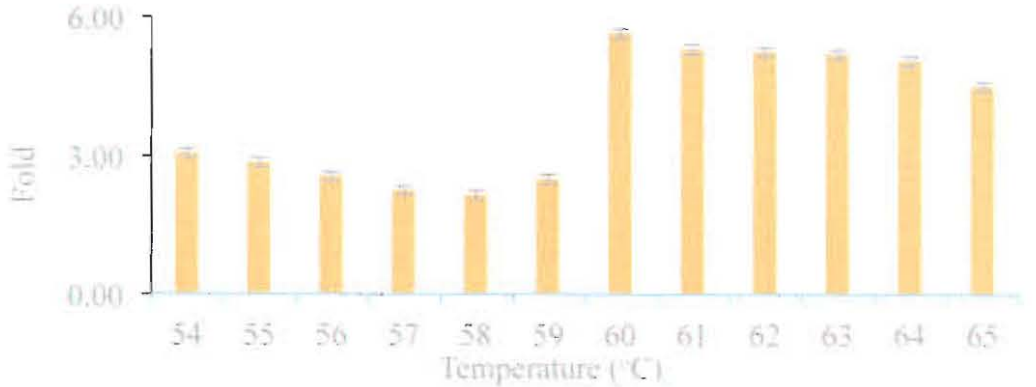
**Appendix 4 : Analysis of AGE gel picture intensity using image J software.**



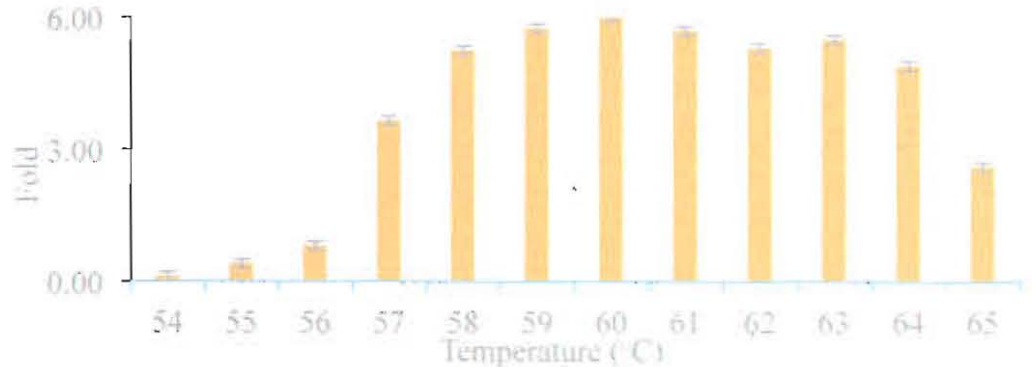

**Appendix 4-1:** The gradient PCR for *ttr* gene by image J.



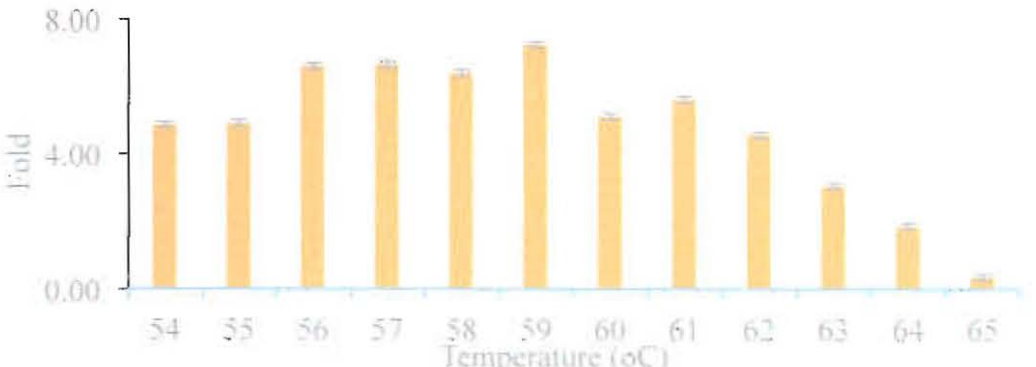

**Appendix 4-2:** The gradient PCR for *tsh_β* gene by image J.



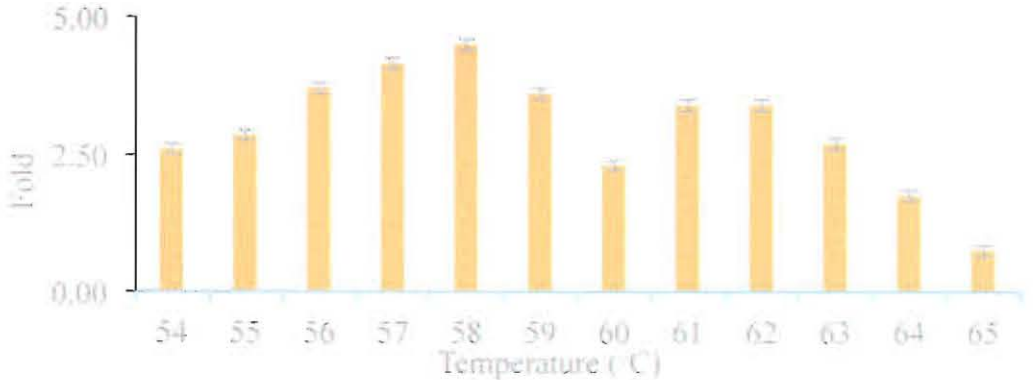

**Appendix 4-3:** The gradient PCR for *trα* gene by image J.




**Appendix 4-4:** The gradient PCR for *trβ* gene by image J.




**Appendix 4-5:** The gradient PCR for *dio_1* gene by image J.




**Appendix 4-6:** The gradient PCR for *dio_2* gene by image J.

## Appendix 5 : Statistical Analysis

DPP
Summary

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.9384 |
| R Square | 0.8806 |
| Adjusted R Square | 0.8567 |
| Standard Error | 0.3999 |
| Observations | 7 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 5.8943 | 5.8943 | 36.8632 | 0.0018 |
| Residual | 5 | 0.7995 | 0.1599 | | |
| Total | 6 | 6.6938 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.0647 | 0.8705 | -0.0744 | 0.9436 | -2.3023 | 2.1729 | -2.3023 | 2.1729 |
| Slope | 5.9906 | 0.9867 | 6.0715 | 0.0018 | 3.4543 | 8.5269 | 3.4543 | 8.5269 |

**Data for Probit Analysis DPP**

| **Concentration mg/L** | **Log10 Dose** | **Empirical Probits** |
|---|---|---|
| 4.31 | 0.63 | 4.16 |
| 5.39 | 0.73 | 4.34 |
| 6.47 | 0.81 | 4.38 |
| 7.01 | 0.85 | 4.48 |
| 8.30 | 0.92 | 5.25 |
| 10.78 | 1.03 | 6.42 |
| 12.94 | 1.11 | 6.59 |

SUMMARY OUTPUT DAP

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.9384 |
| R Square | 0.8806 |
| Adjusted R Square | 0.8567 |
| Standard Error | 0.3999 |
| Observations | 7.0000 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 5.8943 | 5.8943 | 36.8632 | 0.0018 |
| Residual | 5 | 0.7995 | 0.1599 | | |
| Total | 6 | 6.6938 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.0647 | 0.8705 | -0.0744 | 0.9436 | -2.3023 | 2.1729 | -2.3023 | 2.1729 |
| X Variable 1 | 5.9906 | 0.9867 | 6.0715 | 0.0018 | 3.4543 | 8.5269 | 3.4543 | 8.5269 |

| **Data for Probit Analysis DAP** | | |
|---|---|---|
| **Concentration mg/L** | **Log10 Dose** | **Empirical Probits** |
| 3.36 | 0.53 | 3.30 |
| 4.04 | 0.61 | 3.58 |
| 4.84 | 0.68 | 3.78 |
| 4.93 | 0.69 | 4.38 |
| 5.16 | 0.71 | 4.97 |
| 5.61 | 0.75 | 5.97 |
| 6.52 | 0.81 | 6.22 |
| 7.00 | 0.85 | 6.59 |

SUMMARY OUTPUT DAP

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.9384 |
| R Square | 0.8806 |
| Adjusted R Square | 0.8567 |
| Standard Error | 0.3999 |
| Observations | 7.0000 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 5.8943 | 5.8943 | 36.8632 | 0.0018 |
| Residual | 5 | 0.7995 | 0.1599 | | |
| Total | 6 | 6.6938 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.0647 | 0.8705 | 0.0744 | 0.9436 | -2.3023 | 2.1729 | -2.3023 | 2.1729 |
| X Variable 1 | 5.9906 | 0.9867 | 6.0715 | 0.0018 | 3.4543 | 8.5269 | 3.4543 | 8.5269 |

| Group | Log10 Dose | Empirical Probits |
|---|---|---|
| 2 | 0.53 | 3.30 |
| 3 | 0.61 | 3.58 |
| 4 | 0.68 | 3.78 |
| 5 | 0.69 | 4.38 |
| 6 | 0.71 | 4.97 |
| 7 | 0.75 | 5.97 |
| 8 | 0.81 | 6.22 |
| 9 | 0.85 | 6.59 |

SUMMARY OUTPUT DAP+DPP

| *Regression Statistics* | |
|---|---|
| Multiple R | 0.9828 |
| R Square | 0.9659 |
| Adjusted R Square | 0.9574 |
| Standard Error | 0.0418 |
| Observations | 6.0000 |

ANOVA

| | *df* | *SS* | *MS* | *F* | *Significance F* |
|---|---|---|---|---|---|
| Regression | 1 | 0.1982 | 0.1982 | 113.2384 | 0.0004 |
| Residual | 4 | 0.0070 | 0.0018 | | |
| Total | 5 | 0.2052 | | | |

| | *Coefficients* | *Standard Error* | *t Stat* | *P-value* | *Lower 95%* | *Upper 95%* | *Lower 95.0%* | *Upper 95.0%* |
|---|---|---|---|---|---|---|---|---|
| Intercept | -0.7206 | 0.0984 | -7.3218 | 0.0019 | -0.9939 | -0.4474 | -0.9939 | -0.4474 |
| Slope | 0.1976 | 0.0186 | 10.6414 | 0.0004 | 0.1461 | 0.2492 | 0.1461 | 0.2492 |

| **Calculating LD50/LC50 using Probit Analysis** | | |
|---|---|---|
| Group | Log10 Dose | Empirical Probits |
| 2 | 0.04 | 4.10 |
| 3 | 0.18 | 4.36 |
| 4 | 0.27 | 4.78 |
| 5 | 0.30 | 5.26 |
| 6 | 0.48 | 6.22 |
| 7 | 0.60 | 6.59 |