Nature Environment and Pollution Technology
An International Quarterly Scientific Journal

p-ISSN: 0972-6268
e-ISSN: 2395-3454

Vol. 16 | No. 1 | pp. 11-19 | 2017

Original Research Paper | Open Access

Toxicological Analysis of Phthalates from Dust Samples Collected in Selected Philippine Light Rail Transit Stations

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Nat. Env. & Poll. Tech.
Website: www.neptjournal.com

Received: 26-02-2016
Accepted: 16-04-2016

Key Words:
Phthalates
Teratogenicity
Zebrafish
Embryotoxicity

ABSTRACT

Phthalates are the most common form of plasticizers. Due to the ease with which phthalates diffuse from their plastics and the sheer volume of plastics that have been introduced to the environment, they represent one of the most ubiquitous and persistent chemicals known. Phthalates are known to have several toxic effects. The study was carried out to identify the phthalates found in the urban dusts that have accumulated from underneath the LRT1 stations at Monumento, R. Papa, and EDSA in Metro Manila, and to determine their embryotoxic potential. The urban dust samples were subjected to solid phase extraction (SPE) and gas chromatography-mass spectrometry (GC-MS). Six phthalates were identified, and the extract was then used in the zebrafish embryo toxicity (ZFET) test. Lethal and sublethal end points were observed in the zebrafish embryos. Low dilutions were found to be highly embryo toxic and a dose-response relationship was seen in dilutions ranging from 1:4 to 1:40. The LC_{50} of the phthalate mixture was also obtained and found to be 9.188 µg/mL. This study elucidated some of the toxic effects of the phthalates, but further studies must be performed to evaluate the toxicity of the individual phthalates, some of whose effects are relatively unknown.

INTRODUCTION

Phthalates, or phthalic acid esters (PAEs), are a group of compounds composed of alcohol and phthalic anhydride. More commonly known as plasticizers, their use imparts increased flexibility and durability to plastics (Barlow et al. 2007), and the flexibility of the plastic is an indicator of the amount of phthalates present in the material (Crinnion 2010). Very recently, Net et al. (2015) reported that since the 1920s, phthalate plasticizers have experienced widespread use in industry, specifically in the manufacturing and processing of plastics. Phthalates may be found in plastic bags, toys, cosmetics, adhesives, detergents, pharmaceuticals, medical devices, food packaging, nutritional supplements, and building materials (Crinnion 2010).

Despite their prevalent use in plastic, phthalates are not covalently linked to the polymer. This results in the phthalates leaching from the matrix, and they may undergo long-range transport and even enter the food chain (Mankidy et al. 2013). They have become ubiquitous in the environment, and may be found in the atmosphere, sludge, soil, wastewater, solid waste compost, river and marine sediments, and even in drinking water (Net et al. 2015).

The main pathway of phthalates into the body is through ingestion, but secondary exposure may also occur through inhalation and skin absorption. Once within the body, diester phthalates are broken down into monoester phthalates as well as other oxidative metabolites, with the monoester form being more harmful than the diester (Crinnion 2010). In man, phthalates have been observed in various matrices, such as blood, amniotic fluid, saliva, breast milk, and cord blood, and they are primarily excreted through the urine and faeces (Mankidy et al. 2013).

Phthalates are known endocrine-disruptors, and may lead to adverse reproductive and developmental defects, and even cancer (Meeker et al. 2009). *In utero* exposure of phthalates has also produced behavioural and cognitive effects on the fetus. Phthalate exposure is recently being associated with a plethora of health issues, from rhinitis and eczema to asthma to obesity and diabetes type II (Crinnion 2010). Phthalates have been classified as top-priority environmental pollutants, by regulatory agencies such as the China National Environmental Monitoring Center, the European Union, and the Environmental Protection Agency (Chen et al. 2008).

Despite primarily being used in the fields of develop-

mental biology and molecular genetics, the use of the zebrafish model has been recognized for its importance in toxicology and drug discovery (Hill et al. 2005). The benefits of using zebrafish are numerous: they are relatively small, produce a large number of offspring per mating, the embryos and larvae are transparent for easy observation, the route of chemical uptake is direct and straight-forward, and much information has been published about them (Spitsbergen & Kent 2003, Fang & Miller 2012, Hallare et al. 2014). The zebrafish model is also extensively used in various fields of biological research because the results are easily extrapolated to other vertebrate species (Zhai et al. 2014).

The objectives of this study were to (1) identify the phthalates present in the urban street dust underneath selected LRT1 stations and (2) to determine their embryotoxic effects to zebrafish embryos.

MATERIALS AND METHODS

Description of the Study Sites

The three selected stations from the Manila Light Rail Transit System Line (LRT) 1 are Monumento, EDSA and R. Papa. Monumento and EDSA stations are along busy roads and highways and are frequently under moderate to heavy traffic due to the proximity of schools and shopping centres within the area. The vehicles that pass through these areas are buses, jeepneys, motorcycles, and private cars. These stations were chosen due to the presence of heavy human and vehicular traffic. Combining all urban street dust samples to be obtained from these stations would minimize bias.

Urban Street Dust Collection

By using a broom and a dust pan, urban street dust samples were collected from the aforementioned stations. The dust samples obtained were stored in air-tight Reynolds plastic bags and weighed, and 770g, 765g, and 780g of dust were collected from Monumento, EDSA, and R Papa station respectively. The street dust samples were then sieved using a 0.25 µm pore diameter sieve to remove unwanted rocks and debris. The samples were later pooled together and homogenized, and a total of 600g of urban street dust samples was obtained.

Chemical Analysis

Extraction and isolation of phthalates from the urban street dust samples: The street dust samples were initially soaked in petroleum ether for 12 hours, and the extract was filtered in Celite and concentrated under reduced pressure using the Buchirotavapor at 45°C. The resulting residue was transferred to an Erlenmeyer flask and was subjected to a second percolation overnight, using a 1:1 mixture of ethyl acetate and methanol (3 × 200mL). This extract was then collected, and again was filtered with Celite and concentrated *in vacuo* at 42°C. The extract was placed in a vial and stored in the refrigerator for further analysis. The extraction was done three times and a total of 13.75 g of samples were extracted from 600g of urban street dust sample.

Analysis of phthalates: Half of the street dust extract was subjected to phthalate identification and analysis using gas chromatography-mass spectrometry (GCMS). The analysis was conducted via splitless mode on a Perkin Elmer GC-MS. The temperature settings were as followed: 280°C for the injector and the transfer line, 150°C for the ion trap, and 230°C for the ion source. The column temperature program was set, initially, at 55°C, held for 1 minute; ramp of 30°C/min until 140°C; ramp of 5°C/min until 240°C, held for 5 minutes; and ramp of 8°C/min until 300°C, held for 12 minutes. The phthalates present were identified via the NIST database of the mass spectrometer, and were confirmed by the retention times of the standards. The concentrations were determined by analyzing the peaks of the mass spectrometry results manually by dividing the mass of the compound over the total added masses of all the compounds present.

Preparation of the phthalate stock solution: The phthalate stock solution was prepared by mixing 0.59 g of the urban street dust extract with 50mL 1% DMSO under the fume hood. The solution was subjected to ultrasonication for 1 hour to ensure the full dissolution of the extract.

Zebrafish Embryotoxicity (ZFET) Test

Procurement and maintenance of adult zebrafish: Sexually mature zebrafish (*Danio rerio*) aged four to six months old, twenty male and twenty female, were procured from BFAR Fish Farm SitioKabaritan, Barangay Sto. Domingo, Bay, Laguna.

Two glass aquaria, both with dimensions of 16×8×10 inches, were used to house the sexually mature male and female zebrafish separately. Both aquaria were filled up to 3/4 (three-fourths) of its capacity with dechlorinated tap water, and the aquaria were provided with filters for a continuous flow-through condition and aeration systems to provide a continuous supply of oxygen for the fish.

The aquaria were maintained with the following conditions: a 12-hour light/12-hour dark cycle using 18-watt energy saving light bulbs to match the circadian rhythm of the zebrafish; a temperature of 27-28°C, a pH of 7.5 ± 0.5, dissolved oxygen of 10.5+0.5 mg/L O_2, hardness of 400 mg/L $CaCO_3$, and a conductivity of 800 microSiemens/cm.

Another aquarium with the same dimensions was used as the spawning chamber to allow zebrafish to mate and

later spawn its eggs. A rectangular fishnet with dimensions of 11×6.5×8.5 inches was fitted inside the aquarium to house the fish, and a spawning tray was placed directly below the fishnet for easier egg collection. Turning on a fluorescent lamp that was placed on top of the aquarium triggered the mating process. After two hours with the light on, the spawning tray was taken for egg collection while eggs that settled outside the spawning tray were siphoned to a separate container. Randomly selected eggs were then transferred to Petri dishes through the use of micropipettes with widened tips to avoid egg abrasion and for easier suction and release.

Zebrafish exposure to test substance: Glass Petri dish plates were used as the primary exposure chambers of the different dilutions of phthalates from the urban street dust samples as well as for the positive and negative controls for the zebrafish embryos. Each Petri dish plate housed twenty-five zebrafish embryos for the initial exposure.

The degree of dilutions was made by varying the concentrations of the prepared phthalate stock solution from the collection of urban street dust samples mixed with specific amounts of reconstituted water depending on the ratios. Dilutions of 1:0, 1:2, 1:4, 1:8, 1:10, 1:12, 1:16, 1:20, 1:24, 1:28, 1:32, 1:36, and 1:40 were prepared as treatments. 5% acetone was used as the positive control while the reconstituted water served as the negative control. The preparation of the reconstituted water was based on the protocol set by ISO 6341.

After two hours post-fertilization (hpf) within the primary exposure chambers, twenty viable eggs from each dilution were transferred to pre-saturated 96-well plates that served as test chambers. An egg was considered viable if no irregularities were observed under the digital microscope. Viable eggs at this stage would have 64 to 128 blastomeres characterized as a round mass of cells attached to the yolk.

The 96-well plates were pre-saturated with 0.2 mL of the test solutions for 24 hours prior to the addition of viable 2-hour pre-exposed zebrafish eggs into their respective varying dilutions. One viable pre-exposed zebrafish egg taken from the primary exposure chambers along with 0.1 mL of the test solution was placed on each well, using a micropipette. Twenty wells in two rows from the well plate contained a total of 0.3 mL of a certain dilution and one viable 2-hour pre-exposed zebrafish egg. A total of four well plates were used.

Qualitative bioassay and data evaluation: Key selected post-fertilization time points (t= 0 h, 24 h, 48 h, 72 h) were monitored to observe the development of zebrafish embryos from blastula to the early juvenile stages using a digital microscope. Critical end points for the evaluation of embryotoxicity were adapted from (Selderslaghs et al. 2009) based on lethal end points at 24 hpf and 48 hpf and sublethal end points at 72 hpf. Lethal end points at 24 hpf include non-formation of somites, egg coagulation and non-detachment of the tail, while lethal end points at 48 hpf include non-development of the eyes and absence of heart beat. Sublethal end points at 72 hours include lack of pigmentation, yolk sac oedema, pericardial oedema, spinal deformity and delayed hatching.

The overall results of the experiment were recorded and encoded in an Excel template (Microsoft Excel 2007) using a binomial convention where the corresponding effects would have corresponding scores. Zebrafish embryos with normal development were given a score of 0 at each specified time points, whereas zebrafish embryos that exhibited lethal end points and those that were considered dead were given a score of 1 at each specified time points. Percent mortality for a test solution was calculated by dividing the number of dead embryos over the number of surviving and normal embryos at the beginning of exposure (n = 20). Sublethal end points were also monitored at 72 hpf on significant dilutions.

Data Processing and Analysis

The Kruskal-Wallis ANOVA test with $p < 0.05$ was used to analyse the statistical significance of mortality due to lethal end points at 24 and 48 hpf and the occurrence of sublethal end points at 72 hpf due to the effect of the treatment dilutions. Dunett's Post Hoc Test was used to compare the dilutions with the positive and negative controls, respectively, for both lethal and sublethal end points. The LC50 of the sample was calculated using the Toxicity Relationship Analysis Program.

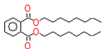
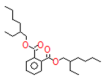
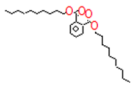
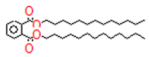
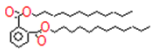
RESULTS AND DISCUSSION

Gas chromatography-mass spectrometry analysis: All in all, six phthalates were identified in the street dust found beneath the LRT1 stations. Table 1 lists down the six compounds, as well as their chemical structures and their relative percentages in the dust extract.

Fig. 1 shows the concentrations of phthalates obtained from GC-MS. The concentration of diisooctyl phthalate (387.0563 μg/g) is much greater than bis(2-ethylhexyl) phthalate (114.5313 μg/g) and di-n-octyl phthalate (134.1376 μg/g). The GC-MS also identified trace amounts of other compounds, such as fatty acids, which are not included in the scope of this study.

Lethal end points: Manifestations of morphological abnormalities such as coagulation, non-formation of somites, and non-detachment of tail at 24 hpf as well as the absence of heartbeat and non-development of the eyes at 48 hpf during

Table 1: Individual phthalates detected from the urban street dust extracts using GC-MS analysis.

Identity	Phthalates in urban street dust samples Chemical structure	Relative Percent
Diisooctyl phthalate		1.689
Di-n-octyl phthalate		0.500
Bis(2-ethylhexyl) phthalate		0.585
Didecyl phthalate		0.026
Bis (tridecyl) phthalate		0.017
Didodecyl phthalate		0.005

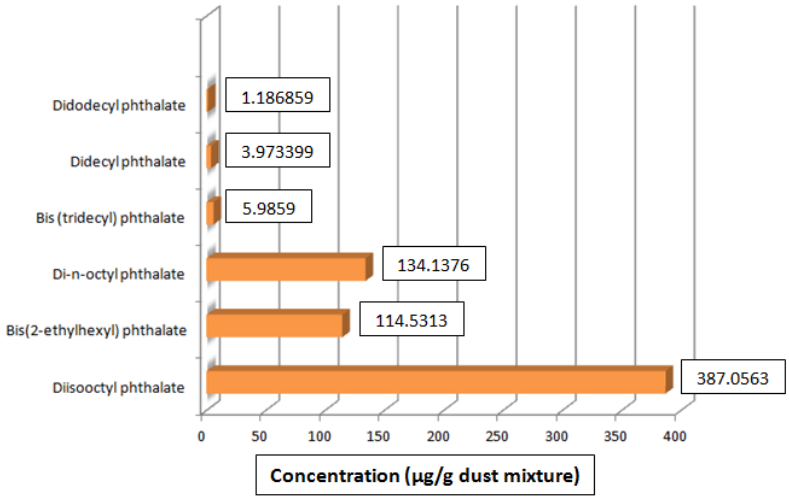


Fig. 1: Concentrations of phthalates in composite urban street dust samples from three LRT stations determined through GC/MS.

zebrafish embryonic development are considered fatal as these deformities prevent the further survival of the embryo until it is ready to hatch from its egg. All lethal end points except for the non-development of the eyes were observed in embryos exposed to the urban street dust phthalate extract as seen in Fig. 2.

At 1:0, 1:2 and 1:4 dilutions, zebrafish embryos exhibited 100% mortality. Embryos subjected to 1:8 dilution showed signs of survival (with a survival rate of 20%) and it is observed that there is a direct relationship between the survival rate of the embryos and the dilutions as the dilutions are increased. Coagulation (B in Fig. 2) is the highest cause of embryo mortality in all dilutions, especially at 1:0 and at 1:2 where embryos exhibited 100% mortality due to coagulation. A dose-response relationship is exhibited with regards to embryo coagulation as this lethal end point decreases as the dilutions are increased. In addition, it was also observed that non-formation of somites (C in Fig. 2) also showed a dose-response relationship since 20% of the embryos exhibited this lethal end point at 1:4 dilution whereas a drop to 5% was seen in 1:8 dilution. The non-formation of somites was no longer observed in the next

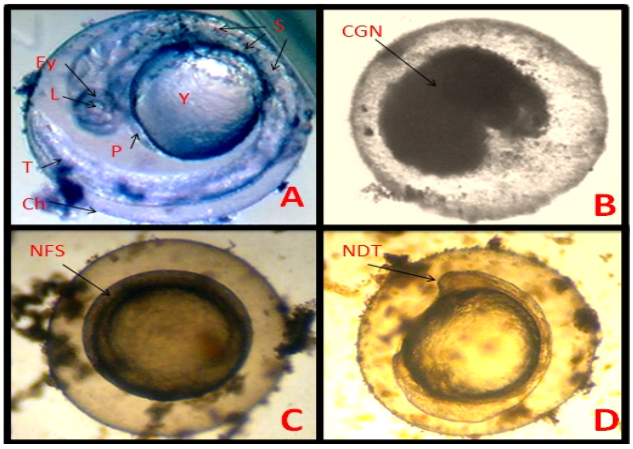


Fig. 2: 24 hours post fertilization (hpf) of zebrafish embryos undergoing the ZFET test normal control embryo (A); yolk (Y); somites (S); pericardial sac (P); chorion (Ch); tail (T); lens (L); and eyes (E); B-D: showing embryos with lethal endpoints: coagulation (B) as seen in an embryo exposed to 1:8 dilution; non-formation of somites (C) as seen in an embryo exposed to 1:4 dilution; and non-detachment of tail (D) as seen in an embryo exposed to 1:4 dilution.

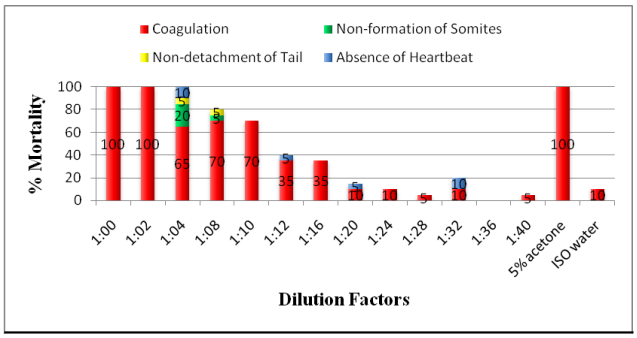


Fig. 3: Response of zebrafish embryos to varying dilutions of phthalate compounds extracted from urban street dust samples from Monumento, EDSA and R. Papa LRT stations showing the contribution of four lethal endpoints to zebrafish mortality.

increasing dilutions. No dose-response relationship can be inferred in both non-detachment of tail (D in Fig. 2) and absence of heartbeat with the data collected.

A decreasing mortality trend as well as a decrease in severity in embryotoxicity is seen in Fig. 3 starting from 1:4 up to 1:28 dilutions. 100% mortality was seen in dilutions 1:0, 1:2 and 1:4, but embryos exposed to 1:4 survived up to 48 hours, long enough to manifest the absence of heartbeat. Other lethal end points such as the non-formation of somites and the non-detachment of tail were also observed in 1:4 dilution, whereas all embryos in both 1:0 and 1:2 dilution exhibited coagulation. A drop in mortality rate to 80% was seen in 1:8 dilution, down to 70% in 1:10, and a sudden

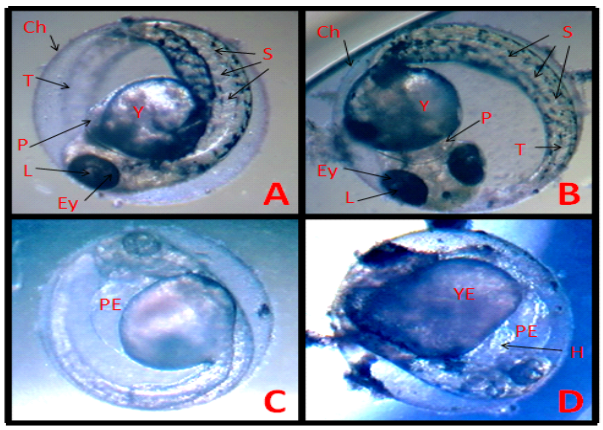


Fig. 4: 48 hours post fertilization of zebrafish embryos undergoing the ZFET test, normal control embryo with pigmentation (A) and (B); yolk (Y); somites (S); pericardial sac (P); chorion (Ch); tail (T); lens (L); and eyes (E); C and D: showing embryos inside eggs with sublethal endpoints: pericardial edema (PE) as seen in an embryo exposed to 1:8 dilution (C); and pericardial edema (PE) and yolk sac edema (YE) as seen in an embryo exposed to 1:4 dilution that exhibited a lethal endpoint: absence of heartbeat; heart (H).

drop in mortality rate down to 40% then 35% was seen in 1:12 and 1:16 dilutions, respectively. All other dilutions (1:20 up to 1:40) had a mortality rate below 20%. A 5% and 0% mortality rate was observed in 1:28 and 1:36 dilutions, respectively but 20% mortality attributed to coagulation at 24 hpf and absence of heartbeat at 48 hpf was observed in 1:32 dilution. This may be due to chance events, as well as the 1:40 dilution having one egg that coagulated at 24 hpf as these results deviate from the observed trend of survivability.

Fig. 4 A and B show two pigmented control embryos exhibiting normal development in two different views. Zebrafish embryos may hatch as early as 48 hours, and 48 hour embryos may already exhibit sublethal end points as shown in Fig. 4 C and D where the embryos are still developing inside their chorion.

Sublethal end points: Manifestations of morphological abnormalities upon hatching or at larval stages at 72 hpf such as yolk sac oedema, pericardial oedema, lack of pigmentation, spinal deformity and delayed hatching are considered as sublethal, where these deformities indicate that the larvae may survive, but the larvae may not live up to its maximum lifespan. In addition, larvae developing such sublethal abnormalities may also fail to develop normally into a healthy adult zebrafish. All sublethal end points except for the lack of pigmentation were observed in embryos exposed to the urban street dust phthalate extract as seen in Fig. 5.

No sublethal end points were observed in 1:0, 1:2 and

1:4 dilutions because all embryos exposed to these dilutions exhibited 100% mortality. In addition, no sublethal end points were also observed in 1:28 and 1:36 since all surviving embryos exhibited normal development. As for the remaining dilutions, (1:8 to 1:24, 1:32 and 1:40) the percent occurrences of each end point were calculated by dividing the number of embryos manifesting the deformities over the number of surviving embryos after 48 hours.

As seen in Fig. 5, majority of the surviving embryos developed pericardial oedema. This sublethal end point showed a dose-response relationship among surviving embryos with a 100% occurrence in 1:8 dilution, a 66.67% occurrence in 1:10 dilution, 58.33% occurrence in 1:12 dilution, 23.1% occurrence in 1:16 dilution, and below 12.5% occurrence in 1:20 and in 1:32 dilutions. Note that in 1:20 and 1:32 dilutions, one embryo exhibited pericardial oedema in both dilutions. A higher percentage occurrence is observed in 1:32 dilution because there were more surviving embryos in the 1:20 dilutions, therefore decreasing the quotient for the 1:20 dilution. Spinal deformity also exhibited a dose-response relationship with a 33.33%, 16.72%, and 7.7% occurrence in 1:10, 1:12, and 1:16 dilutions, respectively. Spinal deformity is no longer observed in increasing dilutions starting from 1:20. In 1:12 dilution, a single embryo both exhibited pericardial oedema and yolk sac oedema as seen in Fig. 6 D. One embryo in 1;16 dilution was observed to develop yolk sac oedema, pericardial oedema and spinal deformity at the same time as seen in Fig. 6 E. The occurrence of yolk sac oedema shows no dose-response relationship but it was found to occur in moderately low dilutions. Lastly, No dose-response relationship can also be inferred with the delayed hatching end point since one embryo from 1:12 and 1:24 dilutions and two embryos from 1:40 dilutions exhibited delayed hatching. The occurrence of delayed hatching was scattered throughout the dilutions.

Positive and negative controls: All zebrafish embryos exposed to 5% acetone, which served as the positive control, exhibited coagulation at 24 hpf, thus a 100% mortality rate was observed. Zebrafish embryos exposed to ISO water in contrast, had a mortality rate of 10% where two out of the twenty embryos exhibited coagulation at 24 hpf. The rest of the surviving embryos underwent normal development at 24 hpf and 48 hpf, and exhibited a 100% hatching rate at 72 hpf. An acceptable survival rate of 90% was met for the negative control as stated by the EURL ECVAM (European Commission 2014).

Statistical analysis: Using the Kruskal-Wallis ANOVA Test with $p<0.5$, it was determined that the occurrence of lethal end points and sublethal end points in zebrafish embryos is

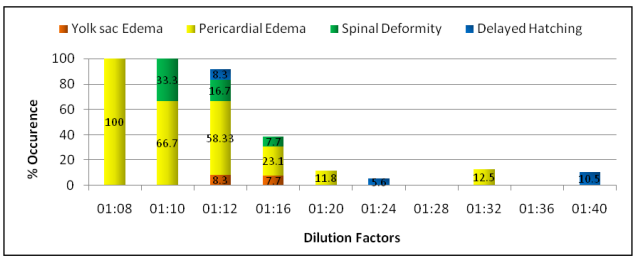


Fig. 5: Occurrence of sublethal end points in surviving zebrafish embryos exposed to phthalates extracted from urban street dust samples from Monumento, EDSA and R. Papa LRT stations after 72 hours post fertilization.

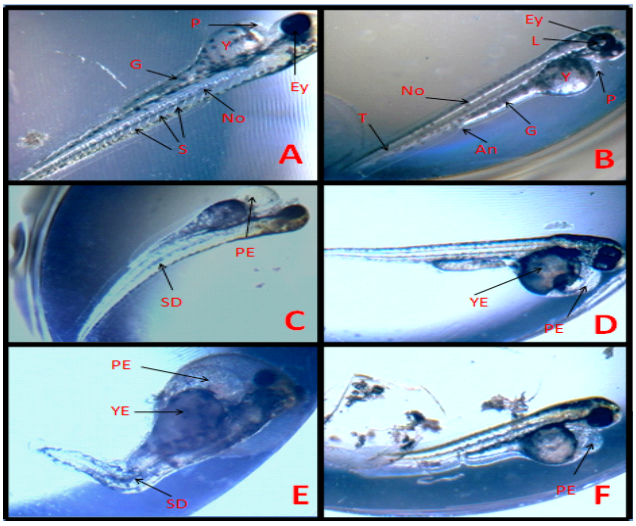


Fig. 6: 72 hour post fertilization of zebrafish embryos undergoing the ZFET test; normal control embryo with pigmentation (A) and (B); yolk (Y); somites (S); pericardial sac (P); tail (T); anus (An); gut (G); notochord (No) lens (L); and eyes (E); C, D, E and F: showing embryos manifesting sublethal endpoints: pericardial edema (PE) as seen in an embryo exposed to 1:12 dilution (C); 1:12 dilution (D); 1:16 dilution (E); and 1:8 dilution (F); spinal deformity as seen in (C) and (E); and yolk sac edema as seen in (D) and (E).

not the same across all dilutions. The results are said to be statistically significant.

The Dunett's Post Hoc Test has revealed that the comparison with the positive control and the dilutions for the lethal end points was closest with 1:0, 1:2 and 1:4 dilutions; that the comparison with the negative control and the dilutions for the lethal end points was closest with 1:20, 1:24, 1:28, 1:32, 1:36 and 1:40 dilutions; and that the comparison with the negative control and the dilutions for the sublethal end points was closest with 1:24, 1:28, 1:32 and 1:36.

The LC50 was obtained through the use of the Toxicity Relationship Analysis Program, which was endorsed by the US EPA. The value obtained was 1.1944mL of the phthalate solution, corresponding to 9.188 µg/mL.

Phthalates as well as other compounds such as fatty acids and hydrocarbons were extracted from the urban street dust samples underneath the LRT stations of Monumento, EDSA, and R Papa but only six significant phthalates were considered in this study as these compounds were found to be relatively higher in concentrations as opposed to the other compounds present only in trace amounts.

Bergh (2011) compiled numerous studies worldwide regarding PAEs in indoor dust samples and found out that bis-(2-ethylhexyl) phthalate or DEHP was the most abundant phthalates in all locations. Three sites from Germany obtained DEHP levels of 740μg/g, 416 μg/g and 659 μg/g; one site from USA obtained 340 μg/g; two sites from Japan obtained 1200 μg/g and 880 μg/g; two sites from Denmark obtained 210 μg/g and 500 μg/g; and lastly, one site from Sweden obtained 770 μg/g of DEHP. Indoor dust samples typically contain more phthalates compared with outdoor dust samples because phthalates outside are more easily subjected to biodegradation, photodegradation, and anaerobic degradation (Liang et al. 2008). In both indoor and outdoor samples however, the concentration of DEHP was found to constitute the main bulk of the phthalates extracted (Sule 2013, Barlow et al. 2007, Wang et al. 2013). Compared to the data gathered in this study, diisooctyl phthalate was found to be the most abundant phthalate in the extract from urban street dust samples with 387.0343 μg/g extracted, while DEHP only came in second with 134.0527 μg/g extracted.

These findings suggest that urban street dust samples obtained from LRT 1 stations in the Philippines contain lesser DEHP levels in contrast with other countries mentioned since diisooctyl phthalate is more than twice the amount of DEHP, which is unusual because DEHP in all other mentioned studies had the highest concentration. These findings, however, cannot determine whether one location is more polluted than the other since the distribution of phthalates in the environment is determined by numerous interacting factors including natural biota, climate, and human activity (Peijnenburg & Struijs 2006). The US Environmental Protection Agency declared both DEHP and DNOP as priority environmental pollutants (Chen et al. 2014) and thus they are compounds of interest. However, the toxicological properties of diisooctyl phthalate, didecyl phthalate, bis (tridecyl) phthalate, and didodecyl phthalate have not been fully investigated. Extracts from dust samples may also contain trace amounts of other phthalates, fatty acids, and hydrocarbons. As such, organisms are not singly exposed to individual compounds in the environment. A combination of these compounds from dust extracts may potentially be more embryotoxic as compared to individual phthalates because of the increase in number of toxic substances present in the dust extracts.

Evidences of lethal and sublethal end points have been observed in lower dilutions of the administered phthalate mixtures, indicating that the extracts were indeed positive in inducing embryotoxicity and teratogenicity on zebrafish embryos in high concentrations. The dose-related response of embryo mortality suggests that the embryotoxicity was related to the exposure of the embryos to different dilutions of the phthalate mixtures as supported by significant statistical results. The results have been consistent with the concern regarding phthalates causing fetal mortality and fetal malformations (Crinnion 2010).

Majority of embryo mortality observed in this study is attributed to embryo coagulation which accounted for 100% mortality dilutions up to 1:2. Coagulation exhibited a dose-dependent response. The same pattern has also been observed in the lethal end point, lack of somite formation in lower dilutions, and in two sublethal end points namely pericardial oedema and spinal deformity in the succeeding higher dilutions. The dose-response relationship observed may also be credited to the increased permeability and the decrease in the barrier function of the egg chorion or vitelline membrane due to the use of 1% DMSO that allowed the zebrafish embryos to be exposed with the phthalates present in the solution (Kais et al. 2013).

A study by Mayer & Sanders (1973) suggests that the low degree of toxicity and high excretion rate of these phthalates, would be relatively safe for aquatic organisms but low chronic concentrations of these phthalates in aquatic ecosystems may be detrimental to the reproductive capacity and endocrine functions of the aquatic organisms as these PAEs have been found to accumulate in aquatic organisms such as in fish and invertebrates. As such, despite the findings of zebrafish embryos developing normally up to 72 hours in higher dilutions using the ZFET Test, the evaluation of the potential toxic effects of PAEs pollution in aquatic organisms is still of particular importance in the field of ecotoxicology and environmental safety.

Mankidy et al. (2013) found that the critical mechanism of toxic action of DEHP and DEP was oxidative stress, and this may be the mechanism involved in the mortality of the zebrafish embryos in this study. The presence of the products of lipid peroxidation on the egg membrane supports this, and DEHP was found to elevate the expression of the mRNA of the enzymes superoxide dismutase and catalase, which are formed as countermeasures to oxidative stress. Apoptosis has been linked to oxidative stress, and so apoptosis may be the end-result of oxidative stress. The concentration of peroxidase and malondialdehyde were directly affected by the amount of phthalates present, indicating that phthalates induced oxidative stress, and may lead

to teratogenesis and developmental deficiencies (Zhou et al. 20011). When *Venerupis philippinarum* was treated with DEHP, glyceraldehydes-3-phosphate dehydrogenase was found to have a 6.4 fold decrease in intensity. This GAPD is necessary in glycolysis, and so this downregulation may instead lead the organism to shift to the pentose phosphate pathway (Li et al. 2014). This shift is advantageous as the NADPH produced is used as reducing equivalents in the thioredoxin and glutaredoxin antioxidant systems (Rubin & Jaeger 1973). The enzymes coded for by CYP3A and GPX, which are monooxygenases and glutathione peroxidises respectively, are key anti-oxidant and detoxification proteins. PAE exposure directly affected the activity of both genes, which activated the detoxification defence of the embryo (Zhou et al. 2011).

Rubin & Jaeger (1973) had found that treatment of embryonic chick cardiomyocytes with 4 µg/ml DEHP for 30 mins resulted in a total cessation of cellular beating, and after 24 hours, total loss of cell viability. Treatment of 100 µg/ml of DEHP on isolated, perfused rat hearts lead to decreased spontaneous rate, isometric systolic tension, and coronary flow. These effects may have been replicated by the developing heart of the zebrafish in our experiment.

Phtalates are also known to have embryotoxic effects on the embryos of *Haliotis diversicolorsupertexta* (Zhou et al. 2011). Examples of the malformations observed were yolk sac leakage and edema, reductions in pigmentation, irregular morulas, and retardation of growth. Increases in the levels of Ca^{2+} and Na^{2+} pumps, which affect osmoregulation and membrane integrity, may be used to explain these defects. PAEs may adversely affect the embryonic membrane, thus altering the osmotic balance and membrane permeability. This may then lead to the entry of phthalates into the embryo proper and lead to contamination. 17β-hsd-12 is known as an essential regulator of embryo development due to its role in steroid biosynthesis. Steroid homoeostasis is necessary for the maintenance of cell-to-cell communication, which is an integral process in embryonic ontogenesis, the development of organs, and the differentiation of cells. Because phthalates downregulate the 17β-hsd-12 gene, endocrine disruption takes place as the mechanisms for steroid synthesis are negatively affected, and this may lead to teratogenicity (Zhou et al. 2011).

CONCLUSION

This study enumerated the various phthalates found in the road dust underneath selected LRT 1 stations. The phthalates identified were diisooctyl phthalate, bis(2-ethylhexyl) phthalate, di-n-octyl phthalate, bis(tridecyl) phthalate, didecyl phthalate, and didodecyl phthalate, with their masses totalling 647 µg/g dust extract. The noteworthy discovery of diisooctyl phthalate, which was the most abundant phthalate in the street dust sample raises questions regarding its potential embryotoxic capabilities.

The results of the ZFET test are statistically significant, meaning that the phthalates in the dust sample obtained triggered a significant embryotoxic response. However, due to the lack of studies regarding the embryotoxic effect of phthalates, the researchers were unable to clearly pinpoint the mechanism of action leading to the deaths of the embryos. However, this study has still proven that the urban street dusts beneath the LRT1 stations have considerable toxic effects. Several mechanisms of action are suggested to explain the embryotoxicity observed in the experiment. Oxidative stress is likely the leading cause, but the effects of PAEs on steroid synthesis, as well as membrane integrity may also contribute to the overall effect. The occurrence of pericardial edema, which is the highest among all other end points besides coagulation is one of the major key points in this study since DEHP was indeed found to have cardiotoxic effects in mice and in humans. However, little is still known regarding the extent of cardiac toxicity and its mechanisms of action to cause such effects.

Further investigation regarding the characteristics of these compounds comes highly recommended. The discovery and analysis of the mechanisms and pathways as to how these phthalates induce embryotoxic and teratogenic effects, singly or in mixtures, will contribute greatly to the scientific community. The other compounds identified in the GC-MS analysis must also be accounted for, since they may have additive and synergistic effects in combination with phthalates.

Lastly, there is currently a lack of research regarding the assessment of phthalate levels across many areas in the Philippines. This includes a comparison between indoor and outdoor dust samples in houses, schools, churches, offices etc. Conducting research regarding this matter would provide insights as to the extent of pollution in these areas as phthalate concentrations are associated with the utilization of various plastic products. Studies focusing on the daily phthalate intake of individuals, through urine analysis, will also provide a window into the current status of phthalate exposure in Filipinos.

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