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Assessment of Embryotoxicity of Urban Highway Runoff in Manila Using the Zebrafish (*Danio rerio*) Embryo Toxicity (ZFET) Test.

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ABSTRACT

Urban highway runoff contains a complex mixture of contaminants which are known to be embryotoxic to the aquatic biota. While there have been a number of studies that focus on the inorganic contaminants found in urban highway runoff (*e.g.*. heavy metals), few have emphasized on the organic contaminants present in urban highway runoff. In this study, an assessment on the embryotoxicity was performed using the zebrafish embryotoxicity test (ZFET). Urban highway runoff samples were collected from five sample sites in Manila and were used as the test samples while 5% acetone and reconstituted water were used as positive and negative controls, respectively. Prior to chemical analysis, water chemistry parameters including pH, dissolved oxygen, total dissolved solids (TDS), conductivity, and salinity were measured. An identification of the possible organic toxicants was conducted using GC-MS analysis. One-way ANOVA with Dunnett's post-hoc analysis was conducted to compare mortality and abnormality rates with the positive and negative control. Only the urban highway runoff samples collected from Sites 1 and 5 were found to be highly embryotoxic and showed no significant difference ($p>0.05$) with that of the positive control (5% acetone). GC-MS results showed that Sites 1 and 5 were sources of chromone derivatives, dioxolane, and phthalates. Site 5 had the highest concentration of organic contaminants and consequently registered the highest mortality among the exposed zebrafish embryos. Pearson correlation analysis for toxicological indices and water chemistry parameters revealed no correlation while the presence of increasing concentrations of organic toxicants showed significant association with increased incidence of 24hpf coagulation and delayed hatching. Given these results, we support the claim that the presence of these organic toxicants impact embryo development in aquatic biota.

Keywords: runoff, embryotoxicity, ZFET, chromones, dioxolane, phthalates, zebrafish.

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INTRODUCTION

Urban highway runoff is found to contain salts, metals, polyaromatic hydrocarbons and other organic compounds, and xenobiotic compounds [1]. These compounds have been known to pose a threat to aquatic organisms and alter freshwater ecosystems, leading to the disruption and possible destruction of these ecosystems [2,3]. Studies have shown that urban highway runoff toxicity can be attributed to heavy metals and polycyclic aromatic hydrocarbons (PAHs) from tires and gas exhausts from vehicles [4,5]. Other studies have also related toxicity of urban highway runoff to organic pollutants such as phthalates [6,7].

The zebrafish embryo is useful in the assessment of toxicity of various substances including, but not limited to urban highway runoff. Various studies using zebrafish embryos as biomarkers have proved them to be easy to use, practical, rapid, and sensitive in assessing water quality [8,9]. The heavy metals, polyaromatic hydrocarbons, and phthalates present in the urban highway runoff have been shown to have a toxic effect on the zebrafish embryos as well as on its early life stages [10,11].

Urban highway runoff as a significant source of impairment in rivers and lakes leads not only to degradation and loss of aquatic habitats but to the reduction in the numbers and diversity of fish and macroinvertebrates. Destruction of aquatic habitats can impede economic development as well since contaminated fishing beds negatively affect the fishing industry and local economies. In line with this, the toxicity of urban highway runoff becomes a major concern to health and ecology. The City of Manila, as the country's capital and a highly urbanized area with majority, if not all of its lands converted into highways, is thus a crucial site for study regarding urban highway runoff. Manila also has access and close proximity to a number of water bodies that act as immediate basins for urban highway runoff, including but not limited to Manila Bay, of which countless studies have indicated its toxicity to various aquatic and terrestrial biota.

Urban highway runoff is a major pathway for the spreading of non-point source pollutants from urban environments. Previous urban highway runoff research has focused mainly on nutrients, heavy metals, organic and particulate matter, and PAHs. Despite the potential environmental risks, only limited studies have addressed the toxicological status of urban highway runoff in Manila. Therefore, this study underscores the identification and determination of the concentrations of organic pollutants in urban highway runoff on five (5) selected areas across Manila and assessment of their embryotoxic potential using the Zebrafish (*Danio rerio*) Embryo Toxicity (ZFET) test.

MATERIALS AND METHODS

Population and Sampling

The cohort study design was used in the study to observe the embryotoxicity over time after exposure to crude highway runoff samples from five different sites. The Zebrafish Embryo Toxicity (ZFET) Test involves the observation of embryos exposed to runoff samples and determination of potential disruption on the developmental process. The exposed embryos were randomly selected without replacement from a pool of pre-exposed embryos.

"First flush" samples of urban highway runoff were collected in 1L glass bottles with polypropylene caps in the following sites: (1) Shell; UN Ave. cor. Roxas Blvd., (2) Shell; J. Abad Santos Ave., Tondo, (3) Liwasang Bonifacio, Lawton, (4) Recto Ave. cor. Rizal Ave., and (5)P. Faura St. cor. Taft Ave.

Data Collection and Procedure

Half liter of the collected urban runoff water samples was subjected to organic solvent extraction and chemical analysis using gas chromatography-mass spectrometry (GC-MS). A spitless mode on a Varian 400 GC-MS device was utilized, with the injector and transfer line temperature set at 280°C, ion trap at 150°C, and the ion source at 230°C. the column temperature program will be set as follows: initial temperature at 55°C, hold at 1 minute; ramp of 30°C/min to 140°C; ramp of 5°C/min to 240°C, hold at 5 minutes; and ramp of 8°C/ min to 300°C, hold at 12 minutes. With the use of NIST software, the presence and concentration of specific organic compounds was identified. The quantity of the identified toxic organic compounds was obtained using the external calibration method.

Water chemistry parameters (conductivity, TDS, salinity and dissolved oxygen) of urban highway runoff samples and reconstituted water were measured using CyberScan Series 600 Waterproof Portable Meter. Eco Tester was used to measure pH. The urban highway runoff samples were maintained at room temperature.

Two hundred (200) embryos were collected for exposure to the test samples obtained from five (5) urban highway runoff samples. The embryos were rinsed with reconstituted water (ISO 7346/3: 294 mg/L $CaCl_2 \cdot H_20$, 123 mg/L $MgSO_4 \cdot 7H_20$, 123mg/L $NaHCO_3$, 5.5 mg/L KCl) before 40 randomly selected eggs were transferred to petri plates containing their respective test solutions for pre-exposure.

After two (2) hours of pre-exposure, twenty (20) viable embryos were selected and transferred to 96-well cell culture plates—pre-saturated with the test solutions 24 hours prior to testing—using a micropipette and a dissecting microscope. Twenty (20) fertilized eggs were placed individually in 2 ml of the respective test solutions to exclude mutual influences. The negative control involved twenty (20) zebrafish embryos exposed to reconstituted water diluted to a ratio of 1:5 with deionized water and twenty (20) zebrafish embryos exposed to 5% acetone [12] served as the positive control. The 96-well-plates were incubated at 26°C ± 1°C.

Embryo development from blastula to early juvenile stages was monitored and observed at specific time points post fertilization (t = 0h, 24h, 48h, 72h). The lethal endpoints that were observed at 24 hpf include: non-formation of somites, egg coagulation, and non-detachment of tail; while the lethal endpoints that were observed at 48 hpf include absence of heartbeat. Sublethal endpoints that were observed at 72 hpf include: yolk sac edema, pericardial edema, spinal deformity and delayed hatching. The control treatment was used as the basis for normal development of zebrafish embryos. A binomial convention was utilized to determine the overall effect of the treatments on the embryos; collected data was encoded in a Microsoft Excel file. A score of 0 will be given to normally-developed embryos, while a score of 1 will be given to embryos exhibiting the aforementioned lethal and sublethal endpoints at specific times. The percent mortality per test solution was computed by dividing the number of dead embryos with the total number of embryos at the beginning of exposure (n = 20).

Data Processing and Analysis

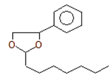
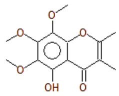
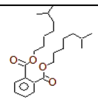
The statistical significance of mortality due to the effect of test solutions was analyzed using One-way ANOVA among groups of five test samples for analysis. One-way ANOVA was used to determine if there is a significant difference between the mortality rates of the five sites and was followed by Dunnett's test to compare the five treatments with the positive and negative control. Pearson correlation analysis was also done to determine if there is a relationship between toxicological indices (lethal and sublethal endpoints) and water chemistry parameters; and between toxicological indices and concentration of organic contaminants. IBM® SPSS® ver.20 statistical software was used to analyze the data. Statistical significance was accepted at 95% confidence interval ($p<0.05$) for all tests.

RESULTS AND DISCUSSION

Gas Chromatography-Mass Spectrometry Analysis

Three toxic organic compounds were detected in the test samples collected from both Site 1 (Shell; UN Ave. cor. Roxas Blvd.) and Site 5 (P. Faura St. cor. Taft Ave.). A high molecular weight phthalate (diisooctyl phthalate), however, was only detected in Site 5, and was the highest in concentration among the organic contaminants found in urban highway runoff.

Table 1. Toxic organic compounds detected in urban highway runoff from five sample sites in Manila

Site	Toxic organic compounds in Urban Highway Runoff		
	Identity	Chemical Structure	Relative Percent
1	1,3-Dioxolane, 2 heptyl-4-phenyl		2.87%
	Chromone,5-hydroxy-6,7,8-trimethoxy-2,3-dimethyl-		1.97%
2	No identity		
3	No identity		
4	No identity		
5	Diisooctyl phthalate		21.45%

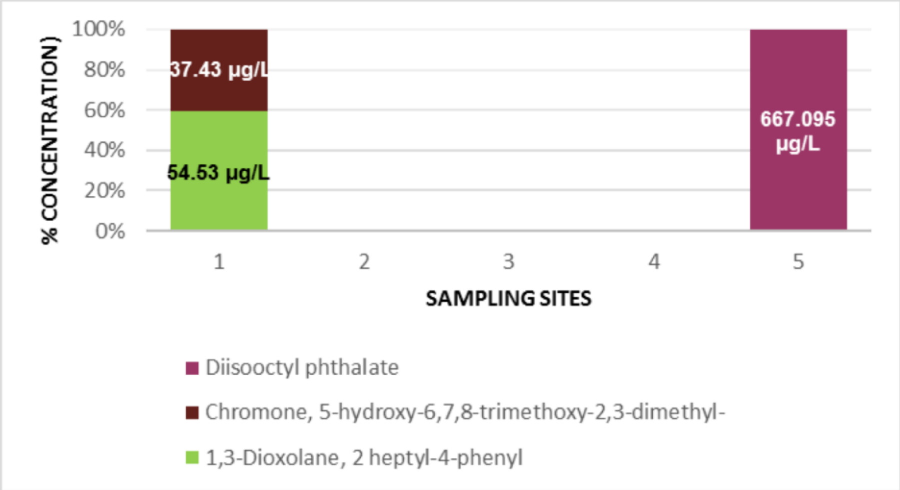


Figure 1: Concentrations of individual toxic organic compounds in five sites determined through GC/MS.

Water Chemistry Parameters at Different Sites

Throughout the zebrafish embryotoxicity test (ZFET), pH levels of reconstituted water ranged from 7-7.2, whereas temperature levels were measured to be fairly constant at 26 ± 1°C. These levels are within the accepted parameters set by the ZFET Standard Operation Procedure. Conductivity of the reconstituted water solution was consistent at 545.3 µS (Table 2).

Table 2: Water chemistry parameters (pH, TDS, DO and conductivity) of the reconstituted water and test samples from five sites

	pH (Std units)	Total Dissolved Solids/ TDS (ppm)	Salinity (ppm)	Dissolved oxygen (mg/L)	Conductivity (µS)
Site 1	7.5	225.6	218.2	6.50	221.2
Site 2	6.9	47.56	48.92	6.00	47.28
Site 3	7.1	384.3	375.4	6.20	375.8
Site 4	7.2	156.2	151.4	6.50	153.2
Site 5	6.8	248.0	242.7	6.30	242.8
Negative Control (Reconstituted Water)	7.0	556.3	558.1	6.50	545.3

Lethal Endpoints

General morphological abnormalities exhibited at 24h and 48hpf larval stages are said to be lethal endpoints. These lethal endpoints indicate that the developing embryo will eventually die as these deformities would not allow its survival. Three lethal endpoints (coagulation, non-formation of somites, and non-detachment of tail) were seen at 24hpf while the absence of heart beat was observed 48hpf in zebrafish embryos exposed to urban highway runoff samples.

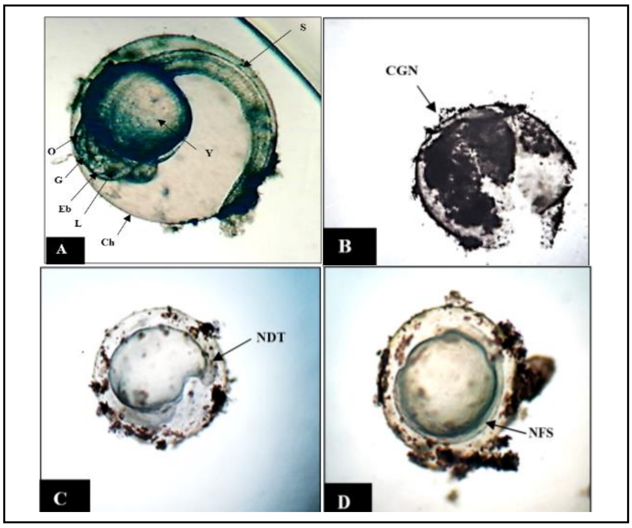


Figure 2: Twenty-four (24) hours post fertilization (hpf) developing control embryo (A); chorion (Ch); ear bud (O; brain (GE); lens (L); eye buds (Eb); yolk (Y); somites (S); tail (Sh); B-D: 24 hpf test embryos showing lethal endpoints: coagulation (B) as seen in the embryo exposed to test sample (specify); non-formation of somites (C) as seen in an embryo exposed to test sample (Sites 1, 4 and 5); and non-detachment of tail (D) as seen in an embryo exposed to test sample (Sites 3, 4 and 5).

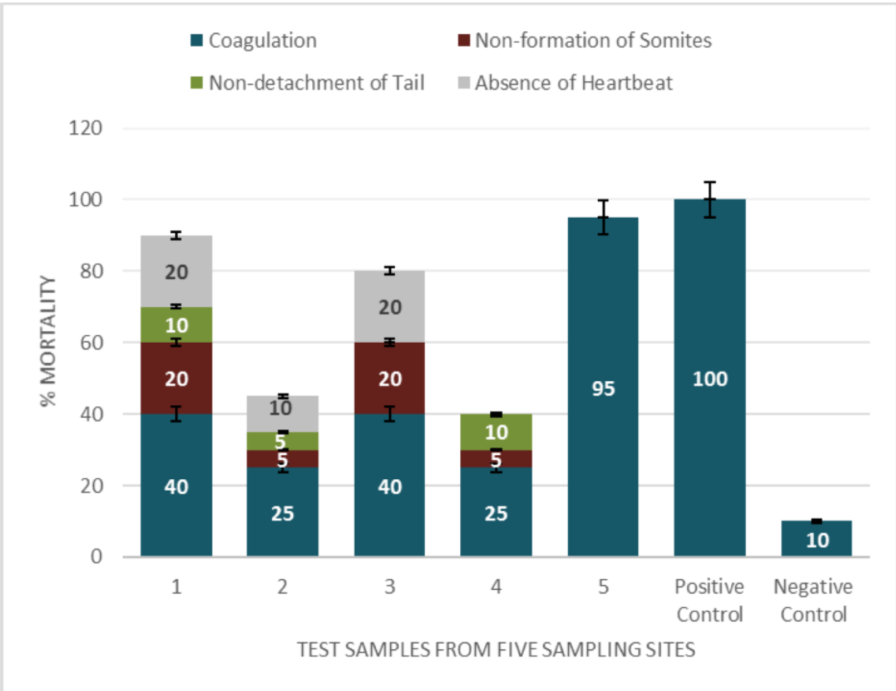


Figure 3: Response of zebrafish embryos to the test samples obtained from urban highway runoff showing the contribution of the four lethal endpoints, namely, coagulation (blue), non-formation of somites (red), non-detachment of tail (green) and absence of heartbeat (gray). Values in red are the overall percent mortality per test sample; mortality rates with significant difference at p<0.05

Sublethal Endpoints

General morphological abnormalities during the hatching and larval stages at 72hpf are considered as sublethal endpoints. Sublethal endpoints indicate that embryos/larvae may survive but does not guarantee a long lifespan and a normal development into a healthy adult zebrafish. These are the four sublethal endpoints observed in zebrafish embryos yolk sac edema, pericardial edema, spinal deformity and delayed hatching.

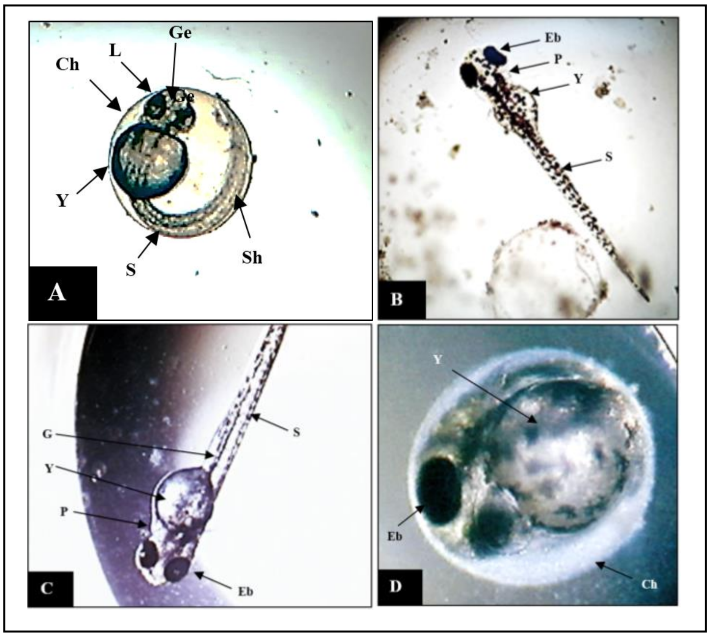


Figure 4: 48 hpf developing control embryo (A); chorion (Ch); lens (L); pericardium (P); yolk (Y); somites (S); gut (G); 72hpf developing control embryo (B and C); Delayed hatching, a sublethal abnormality seen in D (wells containing test samples obtained from Sites 4 and 5).

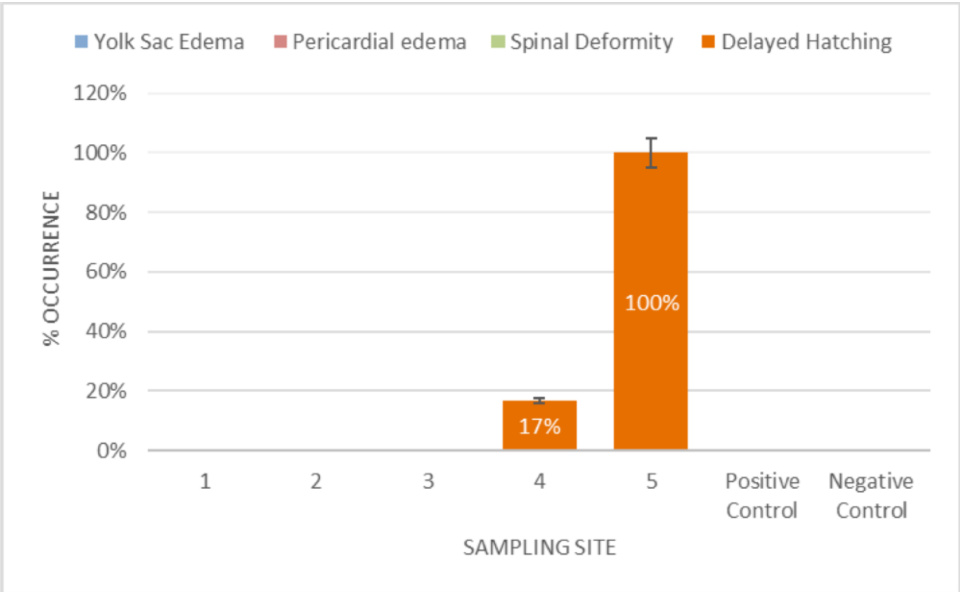


Figure 5: Percent occurrence of sublethal endpoints developed by zebrafish embryos exposed to urban highway runoff test samples; no value for positive control since all embryos died due to coagulation at 24hpf; no value for negative control since 100% of the embryos lived without abnormalities; abnormality rates due to sublethal endpoints with significant difference at $p<0.05$

Positive and Negative Controls

In zebrafish embryos used as negative control, 10% mortality was observed. Only 2 embryos out of 20 embryos exhibited coagulation while normal development at 24 hpf and 48hpf was observed in the rest of the zebrafish embryos in the negative control. At 72 hpf, the surviving embryos exhibited 100% hatching rate. For the positive control, where zebrafish embryos were exposed to 5% acetone (diluted with ISO water), 100% mortality occurred. All zebrafish embryos exhibited coagulation at 24hpf.

Relationship of Lethal and Sublethal endpoints with Water Chemistry

Parameters and concentration of toxicants

Table 3: Pearson correlation values between concentration of organic compounds and water chemistry parameters and toxicological indices

	Pearson R *(p* value)					
	Overall Mortality	Coagulation (24hpf)	NFS (24hpf)	NDT (24hpf)	Absence of Heartbeat (48hpf)	Delayed Hatching (72hpf)
pH	-0.104	-0.658	0.181	0.000	0.452	-0.585
TDS	0.182	0.031	0.639	-0.625	0.848	0.108
DO	0.187	0.156	-0.662	0.102	-0.201	0.515
Salinity	0.184	0.037	0.643	-0.628	0.847	0.110
Conductivity	0.183	0.030	0.639	-0.626	0.849	0.108
Toxicants	0.665	**0.914***	-0.550	-0.438	-0.128	**0.956***

*Correlation is significant at the 0.05 level

**Correlation is significant at the 0.01 level

Pearson correlation analysis was performed to identify if there is a relationship between the toxicological indices (overall mortality, coagulation at 24hpf, non-formation of somites at 24hpf, non-detachment of tail at 24hpf, absence of heartbeat at 48hpf and delayed hatching at 72hpf) and water chemistry parameters including concentration of toxicants determined in urban highway runoff samples. From the statistical analysis, a significant correlation (p=0.914) was found between concentration of chemical toxicants and coagulation at 24hpf; a significant correlation (p=0.956) was also found between concentration of toxicants and delayed hatching at 72hpf.

DISCUSSION

As a tropical country with regular occurrences of rainfall and flooding, the Philippines experiences a large amount of urban highway runoff and consequently, acquires a large amount of pollutants known to be present in urban highway runoff. With increasing urbanization, the deterioration of receiving water quality becomes a primary concern, and as urban highway runoff is a main pathway of non-point source pollutants, it becomes imperative to assess its quality and possible toxicity. This study has found that urban highway runoff samples obtained from Manila have embryotoxic properties to zebrafish embryos and harbor toxic pollutants.

Organic Contaminants in the runoff samples

Chromone, 5-hydroxy-6,7,8-trimethoxy-2,3-dimethyl- identified as an organic toxicant in urban highway runoff sample collected from Site 1 is considered a chromone derivative. Site 1 namely, UN avenue cor. Roxas blvd. is lined by establishments such as hotels and restaurants which can be the point source of this chromone derivatives. Chromones are known components of biological pesticides [13]. Pesticides, although not directly related to vehicles may also be present in urban highway runoff [14]. Pesticides also belong to the most common nonpoint source pollutants from residential areas and improper household management.

1, 3- Dioxolane is commonly used as a process solvent and stabilizer for halogenated solvents. It is also present in paints and rubber products and can thus enter the environment via degradation of such materials. This is also present in Site 1, namely UN Ave. cor. Roxas Blvd. which is lined by different business establishments i.e., hotels, restaurants and stores. The construction, renovation and maintenance of these establishments could have involved usage of paints, thinners and cleaning products. This organic compound is not readily biodegradable and is highly soluble in water.

A group of phthalate esters referred to as the dioctyl phthalates include di-ethylhexyl, diisooctyl phthalate (DIOP), and di-n-octyl phthalate (ATSDR, 2015). The commercial mixture of DIOP in the plasticizers is not pure and may be comprised of ≤2% dioctyl phthalate esters including di-ethylhexyl phthalate (DEHP). Dioctyl phthalates are considered to be the most toxic phthalates [15].

Diiosooctyl phthalate (DIOP) was found to be the highest in concentration among the organic chemical pollutants (21.45%) discovered in Site 5, namely Padre Faura St. cor. Taft Avenue. This site is predominantly comprised of school, government establishments, and hospital (Philippine General Hospital). This site is also the nearest to streets which are residential areas. Improper waste management most especially of plastics could have resulted to its leaching to the environment when washed by highway runoff. As phthalates are not chemically bound to the polymeric matrix in soft PVC they can enter the environment by losses during manufacturing processes and by leaching or evaporating from final products. Once released to the environment phthalates tend to adsorb on particles. Pthalates have been detected in rainwater, surface water, treated and untreated wastewater and in stormwater. Dioctyl phthalate contributes 0.6 ± 0.5% of overall phthalate emission [7].

Embryotoxicity of Organic Contaminants

Chromone derivatives, which have the same IC_{50} (half maximal inhibitory concentration) as Emodin and coleon AL was discovered as novel angiogenesis inhibitors [16]. Angiogenesis inhibitors can cause defective cardiac development defects and heartbeat and bloodflow abnormalities [17]. Coumarin, which is an isomer of chromone was found to produce teratogenic and lethal effects in zebrafish embryos. A similar study [18] which made use of a series of compounds having similar structures showed coherent trends in their toxicological effects. For coumarin, it induced malformation of head, tail and growth retardation [19].

Dioxolane is of low concern to aquatic environmental species because it has a low order of toxicity. However, acute toxicity is well characterized among mammalian species through dermal and inhalation route which is dependent on time of exposure and concentration of dioxolane [20].

Pearson correlation also revealed (with a value of p=0.914) a strong relationship between phthalates concentration and 24hpf mortality due to the occurrence of coagulation. In general, phthalates can produce developmental disturbance such as reduced hatching success [21], which explains the 100% occurrence of delayed hatching for the remaining alive embryo at 72hpf. This has been shown in studies [11.22] wherein embryotoxicity was attributed to phthalates such as di-n-butyl phthalate (DBP) and bis-2(ethylhexyl) phthalate (DEHP). Phthalic acid esters including dioctyl phthalates significantly reduced embryo hatchability, and increased developmental malformations. The typical toxicity symptoms caused by phthalates among 72h zebrafish embryos were death, tail curvature, necrosis, cardio edema and no touch response [23].

CONCLUSION

This study has identified the various types of toxic organic compounds which can be found in urban highway runoff in Manila relevant to the aquatic ecosystems. Organic contaminants discovered from two of the five sample sites were extracted through GC-MS spectrometry and were identified as chromone derivatives and dioxoloane in Site 1 and diisooctyl phthalate in Site 5. The embryo mortality rates of Site 1 and 5 showed no significant difference with the embryo mortality rate exposed to 5% acetone (positive control). As what the Pear-son correlation analysis revealed, although no significant correlation was found between the water chemistry parameters and toxicological indices, a significant relationship was discovered between concentrations of organic contaminants and mortality rate due to coagulation at 24hpf and abnormality rate due to delayed hatching at 72hpf. Although these correlation values represent relationship, it should be noted however, that these do no not signify causation.

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REFERENCES

[1] Waara S, Färm C. Environ. Sci. Pollution Res Intl 2008;15(3): 205–210.
[2] Clement B, Raevel V., Renard O. J. Soils Sediments 2010;10:1255–1266.
[3] Brown J, Peake B. Sci Total Environ 2006;359(1-3), 145-155.
[4] Karlsson K, Viklander M, Scholes L, Revitt M. J Hazard Mater 2010;178(1-3), 612-618.
[5] Kayhanian M, Fruchtman BD, Gulliver JS, Montanaro C, Ranieri E, Wuertz S, Bari P. Water Res 2012; 46(20): 6609–6624.
[6] Bjorklund K, Cousins AP, Stromvall AM, Malmqvist PA. Sci. Total Environ 2009;407: 4665-4672.
[7] Clara M, Windhofer G, Hartl W, Braun K, Simon M, Gans O. Chovanec A. Chemosphere 2010;78(9): 1078–1084.
[8] Oberemm A. Lab Anim 2000;29 (7): 32-40.
[9] Hallare A, Kosmehl T, Schulze T, Hollert H, Kőhler H, Triebskorn R. Sci Total Environ 2005;347: 254-271.
[10] Wassenberg DM, Di Giulio RT. Environ. Health Persp 2004;112(17): 1658–1664.
[11] Lin Y, Wei J, Chen J, Zhou Z. Am J Physiol Endocrinol Metab 2011;301(3):527-538.
[12] Henn K, Braunbeck T. Comp. Biochem. Physiol.- C Toxicol Pharmacol 2011;53(1): 91–98.
[13] Nollet LML, Rathore HS. (2009). Handbook of Pesticides: Methods of Pesticide Residues Analysis. CRC Press. Retrieved from https://books.google.com.ph/ books?id=pSsz45_yAP4C&dq= herbicides+pesticides+chromone&hl=fil&source=gbs_navlinks_s
[14] Herrera Environmental Consultants. (2007). Untreated Highway Runoff in Western Washington, (May).
[15] Crinnion WJ. Toxic effects of the easily avoidable phthalates and parabens, 2010;15(3): 190–196.
[16] Crawford AD, Liekens S, Kamuhabwa AR, Maes J, Munck S, Busson R, de Witte P. PLoS ONE 2011;6(2): 1–9.
[17] Gerety SS, Anderson DJ. Development 2002;129(6): 1397–1410.
[18] Hermsen SB, van den Brandhof EJ, van der Ven LTM, Piersma AH. Toxicology in Vitro 2011;25(3): 745–753.
[19] Weigt S, Huebler N, Strecker R, Braunbeck T, Broschard TH. Reprod Toxicol 2012;33(2): 133–141.
[20] Czajkowska T, Krysiak B Popiânska E. Acute toxic effect 1987;38: 84–90.
[21] Oehlmann J, Schulte-Oehlmann U, Kloas W, Jagnytsch O, Lutz I, Kusk KO, Tyler CR. Series B Biol Sci 2009;364(1526): 2047–2062.
[22] Zhou J, Cai ZH, Xing KZ. Environ Pollution 2011;159(5): 1114–1122.
[23] Chen X, Xu S, Tan T, Lee ST, Cheng SH, Lee FWF., Ho KC. Intl J Environ Res Public Health 2014;11(3): 3156–3168.