RESEARCH ARTICLE

Embryotoxic and teratogenic effects of polyethylene microbeads using the zebrafish *Danio rerio* (Hamilton, 1822)

Margaret L.C. de Guzman*, Patricia Anne P. Chua, Franceska S. Sedano, Arnold V. Hallare†, Jay T. Dalet

*Corresponding author's email address: mcdeguzman4@up.edu.ph

Department of Biology, College of Arts and Sciences, University of the Philippines Manila, Manila, Philippines

Deposited as pre-print in BioRxiv (doi:https://doi.org/10.1101/2020.09.16.299438)

ABSTRACT

Background: The escape of polyethylene microbeads from waste-water treatment facilities to aquatic habitats has been a major concern by scientific communities due to the adverse effects on aquatic organisms as well as the well-being of the marine and terrestrial ecosystems.

Objective: This study was conducted to evaluate the embryotoxic and teratogenic effects of polyethylene microbeads on the early development of the zebrafish *Danio rerio* using the Fish Embryo Acute Toxicity Test (FET).

Methodology: Sixty (60) zebrafish embryos were exposed to polyethylene microbead suspensions (PE-MBS) of 20 µg/L, 200 µg/L, and 2000 µg/L concentrations. Using FET, the toxicological endpoints (i.e., egg coagulation, lack of somite formation, non-detachment of tail, and lack of heartbeat) were observed every 24 hours until the 96th-hour exposure. Hatching of the embryo from the chorion was observed from 48-96 hpf (hours-post fertilization), and at least four parameters of teratogenicity (i.e., edema of the pericardium and yolk sac, bent axis, tail curvature, and collapsed swim bladder) was observed at 144 hpf.

Results: Significant differences between means and variances in the embryotoxic and teratogenic effects were observed for all treatment groups in relation to the negative control (reconstituted water). The emulsifier control (0.01% Tween 80, p-value=0.9), the solvent control (1% DMSO, p-value = 0.9), and the 20 µg/L PE-MBS (p-value = 0.92) did not significantly differ with the negative control group. However, the positive control (5% ethanol, p-value = 7.8) and 200 µg/L (p-value = 1.1), and 2000 µg/L (p-value = 1.48) of PE-MBS were significantly embryotoxic and teratogenic to the developing organism.

Conclusion: The high concentrations of PE-MBS (200 µg/L and 2000 µg/L) may induce early hatching, mortality, and malformations. Tukey Kramer post hoc test substantiated that PE-MBS toxicity is dose-dependent since embryotoxicity and teratogenicity increase at higher concentrations. Further studies should be conducted to know more about the adverse effects of polyethylene microbeads on the development, physiology, and genomics of freshwater fishes.

Keywords: *Danio rerio, embryotoxicity and teratogenicity, polyethylene microbeads*

Introduction

Microplastics (MPs) such as polyethylene microbeads (PE-MB) are polysynthetic resin products that serve as abrasives or bulking agents in cleaning products and exfoliants in numerous beauty products [1]. Due to their microscopic sizes, most waste-water treatment plants are unable to effectively filter these microbeads. As a result, these microplastics infiltrate the aquatic ecosystem and exposure to these microbeads may result in developmental toxicity in aquatic organisms [2]. These substances may also cause physical or functional defects in a developing embryo and are, thus, classified as teratogenic substances [3].

Microplastics have two forms: primary and secondary microplastics. Primary MPs are particles that were originally

manufactured to be of that size such as pellets, granules, or microbeads while secondary MPs, on the other hand, are particles that have resulted from the gradual breakdown, degradation, or fragmentation of larger plastic items during use or by the actions of the environment (*e.g.*, exposure to UV radiation, mechanical transformation, or by biological degradation by microorganism) [4]. The polyethylene microbeads incorporated in facial washes are classified as primary microplastics.

The ubiquity of microplastics in aquatic habitats has alerted scientists and environmentalists worldwide because of its potential risk to the biota. In the last decade or so, several investigations have been conducted to determine the presence of these polymers in aquatic systems including sediments and coastal areas [5,6].

The prevalence of microplastic pollution is not well studied yet in the Philippines even if it is ranked third in the world for the highest plastic waste inputs into the ocean [7]. But in the past four years, there is a rise in documented studies on microplastics in the Philippines after a call was made by Abreo in 2018 [8] and by the National Scientist Alcala in 2019 [9]. Recent studies have investigated the occurrences of microplastic litter in the estuary and coastal waters of San Juan, Batangas and an urban coast of Cagayan de Oro, which revealed that a substantial percentage of plastic litter were brightly colored spherules and were speculated to have originated from facial washes and other cosmetic products that contain microbeads [10,11].

There are several facial washes sold in the Philippines that contain polyethylene microbeads and some of these are Céleteque® DERMOSCIENCE + ™, Oil-free Acne Wash Daily Scrub, Clear Pore Daily Scrub, and Deep Action Exfoliating Scrub [12]. The popular use of these facial scrubs for personal care and cosmetic purposes unfortunately adds up to the worsening plastic pollution of the country's aquatic ecosystems. The EcoWaste Coalition, along with other private groups such as the Coastal Conservation, Marine Conservation Philippines, and many other groups, wrote a letter to the Department of Health (DOH) and Food and Drug Administration (FDA) pleading for an expedited implementation of a microbead ban. These concerned groups stated that since plastic microbeads in drainage systems leach their way into the bodies of water, quick action must be done before they negatively affect the food chain, especially seafood consumed by man [13].

The impact of these persistent pollutants is great since it might affect the health and overall physiology of the animal biota because most of these spherules are mistaken as food by the animals. Intentional or accidental ingestion of these microbeads has been proven by studies wherein the gastrointestinal tracts (GITs) of fishes have microplastics in them. A few studies here in the country have confirmed the presence of MPs in the GITs of aquatic animals of the Philippines that are commercially sold and consumed by Filipinos. An important fishery in Negros Oriental, the rabbitfish (Siganus fuscescens) were also found to have MPs in their gastrointestinal tracts [14]. Three fishes commercially sold in the markets of Cebu Island, namely, *Auxis rochei*, *Rastrelliger kanagurta*, and *Chanos chanos* were also found to have MPs in their GITs [15]. The presence of MPs in the GITs of these food animals can pose a risk, especially to humans since there can be potential bioaccumulation and biomagnification of these emerging/persistent pollutants along the food chain [10].

Danio rerio is a tropical freshwater fish that is readily available, inexpensive, and is a prolific breeder exhibiting high fecundity and rapid development [16]. Their lifespan can reach up to five years and are omnivorous in nature. Furthermore, the transparency of *Danio rerio* embryos allows researchers to observe normal embryonic development as well as teratogenesis or malformations in the developing organs of the fish just by using a compound light microscope or a stereomicroscope. The zebrafish also has a high degree of genomic conservation and is likened to humans in terms of cellular, molecular, and physiological processes of almost all organ systems [17]. It is also documented that 70% of human disease genes also have similar homologs in the genes of *Danio rerio* [18]. This fish has also been used in many toxicity studies because it is one of the best-known models of vertebrate development.

The Fish Embryo Acute Toxicity Test (FET) is a method used to study chemical toxicity in aquatic ecosystems in vivo wherein the fish is observed when exposed to varying concentrations of the test solutions [19]. Fishes are primarily used in this toxicity testing because they can be primary targets of water pollution and can damage their metabolic activities [20]. There are four embryo-toxicological endpoints in this test observed during the 24–96 hpf (hours post-fertilization): (a) coagulation of fertilized egg; (b) lack of somite formation; (c) non-detachment of tail-bud from the yolk-sac, and; (d) lack of heartbeat; while at 144 hpf, the teratogenicity of a substance can be assessed and evaluated by observing for at least four parameters such as pericardial or yolk sac edema, bent body axes, tail curvature, and collapsed swim bladder [21].

This study is so far the first in the Philippines that assessed and evaluated the embryotoxicity and teratogenicity of polyethylene microbeads which are added to facial washes commonly bought and used by Filipino consumers. Due to the increasing evidence of microplastics being detected in the aquatic habitats of the Philippines, it is just timely to fill in the wide gap of knowledge on the potential risks and hazards of microplastics to the aquatic biota of the country. Also, the results of this study might hasten the passing into law the bill submitted by former Senator Loren Legarda in 2018 that will ban the use of microbeads in our country [22]. The results of this study can also encourage local and international skincare manufacturers and companies to produce and import more environmentally friendly exfoliant alternatives to polyethylene microbeads.

Methodology

Husbandry of Danio rerio

Thirty (30) female and twenty-five (25) male *Danio rerio* (about 5 months-old) void of any pharmaceutical treatment were purchased from the Freshwater Aquaculture Center, College of Fisheries in Central Luzon State University Science City of Munoz, Nueva Ecija. The females were separated from the males and placed in two 15-gallon glass tanks 3/4 filled with dechlorinated water that was maintained at 26 ± 1 °C, well-aerated with dissolved oxygen at a concentration of 6.6 mg/L, with electrical conductivity of 0.256 mS/cm, water hardness of 185 mg/L CaCO3, and at a constant pH of 7.2 ± 1. These conditions were measured and maintained using the API® Freshwater Master Test kit. The fish were fed with fish flakes twice a day at 8:00 am and 4:00 pm daily. The adult zebrafish were subjected to 12-hour light and dark cycle and were acclimated for 2 weeks prior to the experiment. The fish were fed with egg yolk the night before mating to increase the likelihood of breeding [23].

Preparation of Polyethylene Microbead Suspensions

Clear polyethylene microbeads (PE-MB) 300-355 µm in diameter and 1.10 g/cc in density were purchased from Cospheric. These measurements were based on a study by Chang who characterized microbeads from various commercial facial exfoliating cleansers [24]. The PE-MB were treated with 0.01% Tween 80 for 24 hours, or until equal dispersion was achieved, and were then filtered from the 0.01% Tween 80 using Whatman® Grade 1 filter paper (pore size of 11 µm). The polyethylene microbead suspensions (PE-MBS) were then prepared by adding the polyethylene microbeads to a solution consisting of reconstituted water (RW) and 1% DMSO, an organic solvent which can dissolve PE-MB and produce a suitably concentrated stock solution [25]. The composition of RW consisted of 294.0 mg/L CaCl2·2H2O, 123.3 mg/L MgSO4·7H2O, 63.0 mg/L NaHCO3, and 5.5 mg/L KCl [25]. The RW solution was aerated for a minimum of 24 hours before it was used in the experiment. The three concentrations of microbead test suspensions used in this study were 20 µg/L, 200 µg/L, and 2000 µg/L which were based on previous studies on microplastic toxicity that used the same concentrations [26].

Egg Production and Collection of Fertilized Eggs

Groups of *Danio rerio* with a sex ratio of 1 female to 3 males were placed in spawning tanks and were exposed to a 14-hour-light 10-hour dark cycle the day before the eggs were collected [25]. A trap was placed inside the spawning tank as a means of collecting *Danio rerio* eggs. Spawn traps were covered with an inert wire mesh with a size of approximately 2 ± 0.5 mm to prevent predation of the eggs by the adult *Danio rerio*. Mating, spawning, and fertilization took 30 minutes after the onset of light on the day of testing and eggs were collected.

After collecting the eggs from the spawning tank, they were rinsed with RW. The transparent fertilized eggs were sorted from the opaque unfertilized eggs. The number of unfertilized and fertilized eggs was counted to check the validity of the results obtained from FET. The overall fertilization rate of all eggs collected must be ≥ 70% in the batch tested.

Fish Embryo Acute Toxicity Test (FET)

Sixty (60) viable fertilized eggs per treatment group (20 eggs for each replicate, with three replicates) were placed in the Petri plates containing their respective test concentrations for initial exposure. A dropper was used to transfer the viable fertilized eggs into the 96 well plates containing 0.5 ml of the microbead test suspensions and the controls (RW for the negative control and internal plate control, 5% ethanol for the positive control, 1% DMSO as the solvent control, and 0.01% Tween 80 as the emulsifier control).

Observations for the Fish Embryo Acute Toxicity Test

The following toxicological endpoints were observed using a Leica ES2 Stereoscope with a magnification of 100x:

(a) coagulation of embryos, wherein the embryos appeared milky white with the naked eyes but appeared dark under the microscope; (b) lack of somite formation, wherein the embryos did not develop 20 somites in 24 hpf and no side-to-side contractions of the embryos; (c) non-detachment of the tail, wherein the tails of the embryos did not separate from the posterior end of the body, and; (d) lack of heartbeat, wherein the embryos 48 hpf did not have a visible pulsating heart tube at the midventral region of the body. Hatching was also noted wherein the embryos were freed from the enveloping chorion starting at 48 hpf until 96 hpf. Any positive results observed in any of the toxicological endpoints rendered the *Danio rerio* embryo dead. The toxicological endpoints were recorded every 24 hours until the end of the 96-hour exposure.

At 144 hpf, the *Danio rerio* larvae were euthanized by quickly immersing the larvae in an ice bath consisting of five parts ice and one part distilled water for 40 minutes or until cessation of gill and heart movement was observed. Once movement was no longer visible, the *Danio rerio* were preserved in vials with 10% glycerol. The preserved embryos were then observed under Leica ES2 Stereoscope with a magnification of 100x to assess some of the different teratogenic effects induced by PE-MBS such as yolk sac edema, pericardial edema, bent body axes, tail curvature, and collapsed swim bladder.

Statistical Analysis for the Fish Embryo Acute Toxicity Test

The cumulative mortality, cumulative hatching, number of malformations, and the number of embryos that represent coagulation, lack of somite formation, non-detachment of tail, lack of heartbeat, and hatching, respectively, for all treatments after the 24-, 48-, 72-, and 96-hour exposure were recorded. Probit analysis for the estimation of LC50 values at 96 hours of exposure for mortality with a 95% confidence limit was recorded for graphing and interpretation [25].

The treatment effects of the different concentrations of microbead suspensions on the developmental parameters and mortality of *Danio rerio* embryos were determined using one-way analysis of variance (ANOVA). The Kruskal-Wallis test was performed if data did not pass Shapiro Wilk's test of normality. The Dunnett's test was used to compare the treatment means with their corresponding controls if parameter assumptions of normality and homogeneity of variances were met whereas the Dunn's test was used to analyze obtained data if assumptions were not met. Multiple comparisons among the three treatments were performed through Tukey-Kramer post hoc test. Statistical analyses were executed using the Microsoft Excel Real Statistics Software. Data is significant for $p \leq 0.05$.

Animal Care and Use Guidelines

The Institutional Animal Care and Use Committee (IACUC) of the University of the Philippines Manila reviewed the methods used in this study (Protocol No. 2019030) and was granted approval for implementation. The researchers ensured the proper handling of the live specimens and ethical euthanasia and disposal of the dead embryos and larvae.

Results

Embryotoxicity

A total of 702 eggs were collected in February 2020, and 84% (587) of these eggs were fertilized and used for the experiment. A normal embryo and the embryos that exhibited three of the four toxicological endpoints after the 96 hpf period are shown in Figure 1. Of the four toxicological endpoints, coagulation of the eggs accounted for the most occurring lethal endpoint in all control solutions and PE-MBS concentrations (38% to 80%). The second most toxicological endpoint observed was lack of heartbeat (0% to 33%), followed by non-detachment of tail (0% to 29%), and lack of somite formation that accounted for the least occurring endpoint (0% to 12%).

The concentration-mortality graph and curve of Danio rerio embryos exposed to varying concentrations of PE-MBS used in this study are shown in Figure 2a and 2b, respectively. It can be seen in Figure 2A that as the concentration of the polyethylene microbead suspension increases, the percentage of mortality significantly increased. It can also be seen in the graph that the mortality of the embryos also increased at every observation hour. The results of the Tukey Kramer's post hoc test showed that there is a significant difference between the means of the dead embryos treated with different concentrations of PE-MBS at 96 hpf. There was also a low mortality rate of the zebrafish exposed to the RW, the internal plate control, 0.01. Tween 80, and 1% DMSO. The Dunnet's test showed that there is no significant difference in the means and variances among these groups, including the group of embryos exposed to 20 µg/L PE-MBS.

Hatching

The hatching of the zebrafish embryo from its chorion is also a critical stage of development. Looking at Figure 3,

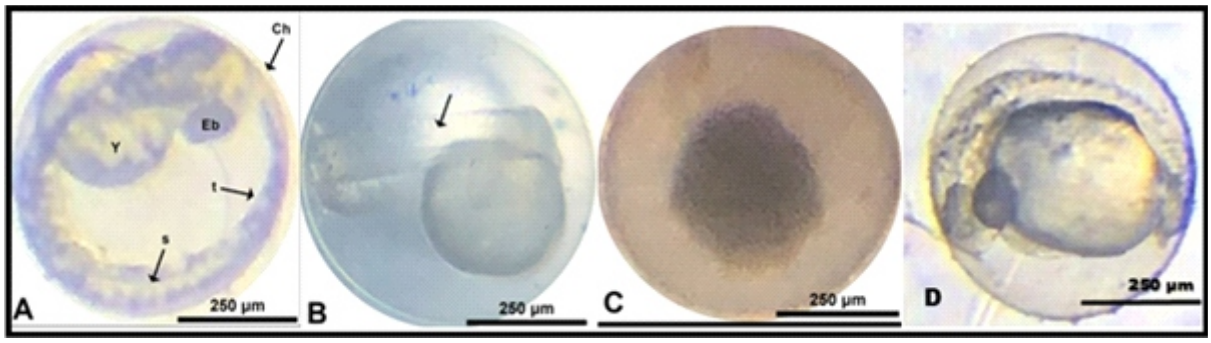

Figure 1. T*oxicological endpoints. (A) Normal embryo at 48 hpf exhibits a distinct chorion (Ch), eye bud (Eb), yolk sac (Y), (S), and tail (t). These structures were observed in embryos exposed to the negative control (RW), 0.01% Tween 80, 1% DMSO, and 20 µg/L PE-MBS. The embryos that exhibited three of the four toxicological endpoints are shown in B-D: (B) embryo with no somite formation; (C) coagulation of the egg; and (D) non-detachment of the tail. These appearances were observed in the positive control (5% ethanol), 200 and 2000 µg/L PE-MBS.*

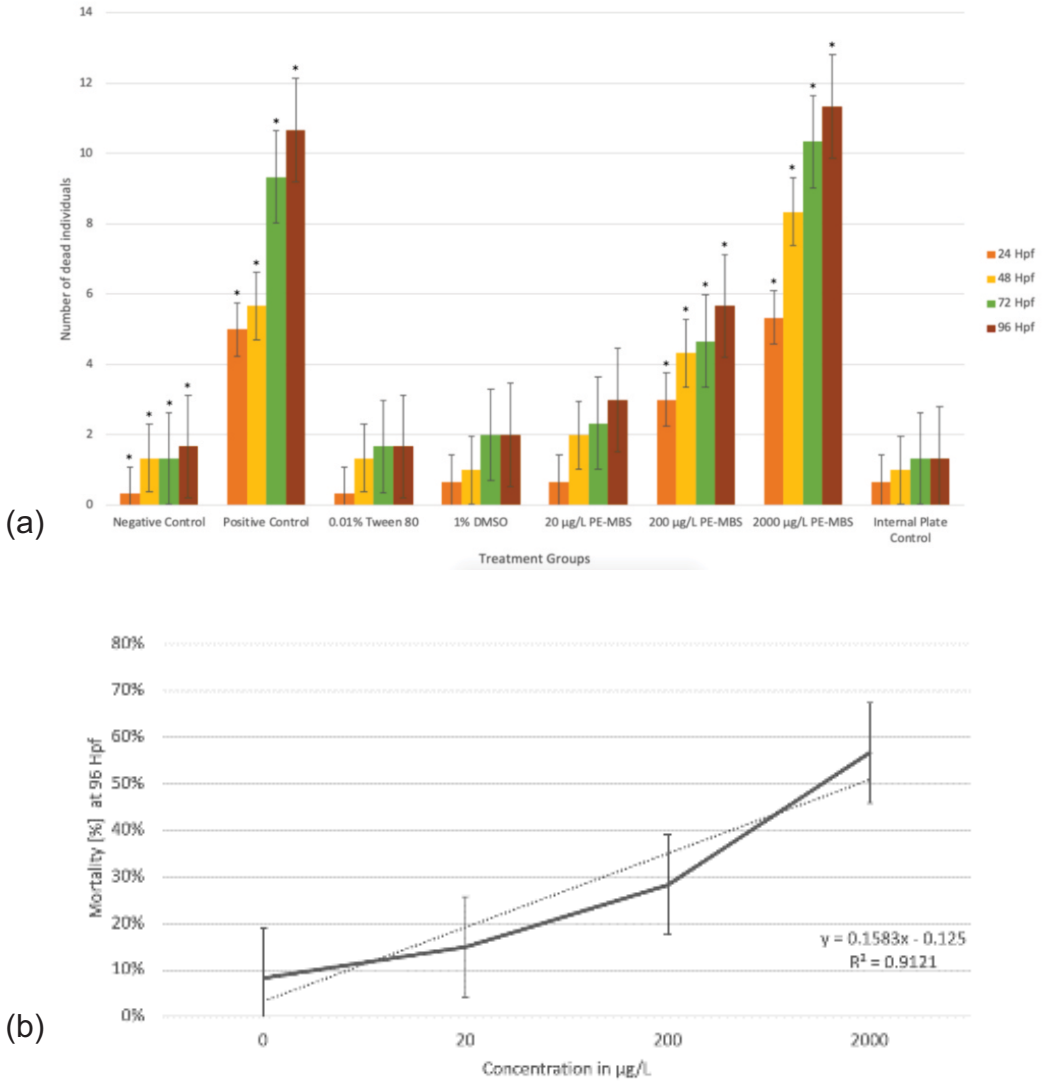

Figure 2. *Concentration-Mortality (a) bar graph from 24 to 96 hpf and; (b) curve at 96 hpf of Danio rerio embryos exposed to varying concentrations of polyethylene microbead suspensions (PE-MBS) using the Fish Embryo Acute Toxicity Test (FET). Data is based on the average of three replicates Error bars indicate standard error. The asterisk indicates a statistically significant difference in cumulative mortality among the embryos (p < 0.05).*

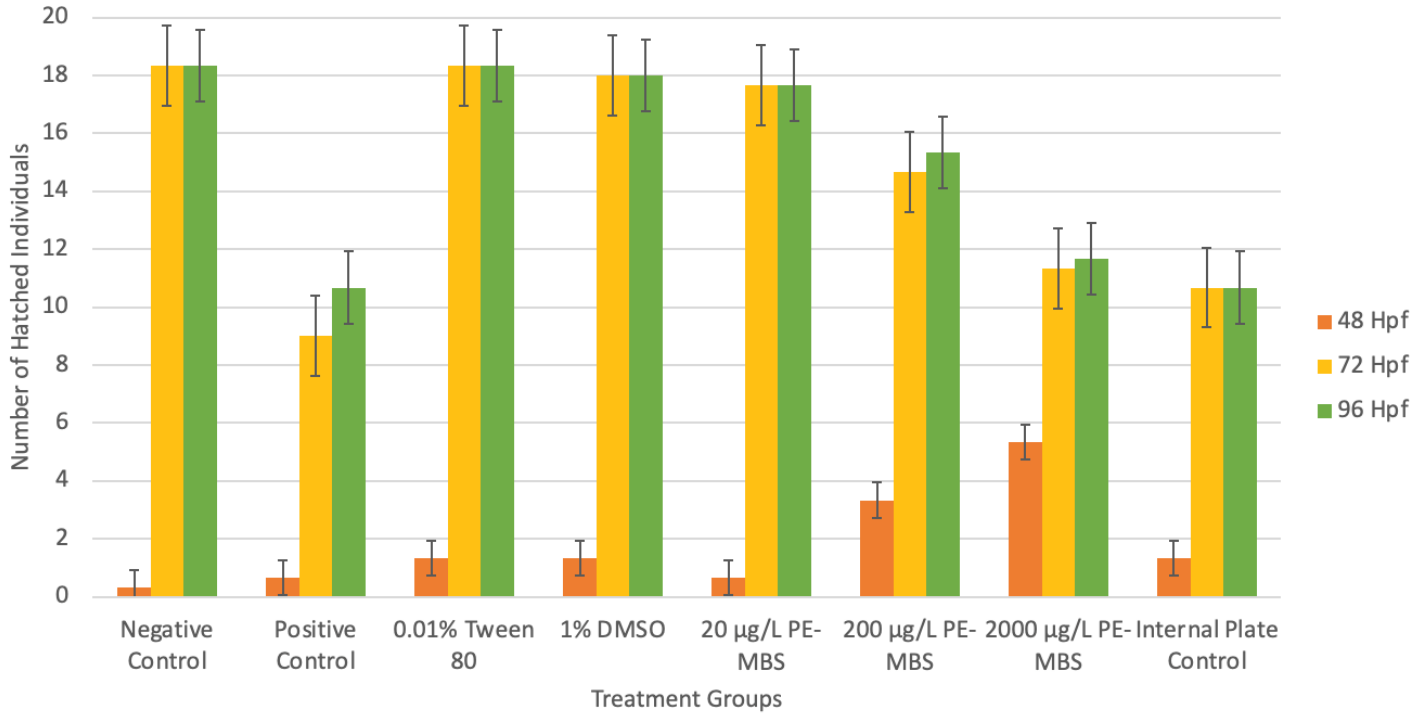

Figure 3. *Hatching of the embryos. A cumulative number of hatched embryos (n=20) within the 96 hpf observation period for the ecotoxicological assay of polyethylene microbeads. Data shown is based on the average of three replicates. Error bars indicate standard error. The asterisk indicates a statistically significant difference in cumulative hatching among the embryos (p<0.05).*

most of the embryos (18/20) hatched at the 72-96 hpf period while 1-2 embryos hatched at the 48hpf for eggs that were exposed to negative control (RW), 0.01% Tween 80, 1% DMSO, and the treatment group at 20 µg/L PE-MBS. For the embryos that were exposed to the test concentrations of 200 and 2000 µg/L PE-MBS, it can be seen in the graph that significantly fewer embryos hatched at the 48-96 hpf period and were statistically comparable with the 9-10 embryos that hatched exposed to 5% ethanol, a known positive control for the FET. Results from the Tukey's-Kramer's test for the cumulative number of hatched individuals within the 96 hours exposure period showed that all three concentrations of PE-MBS are significantly different from each other.

Teratogenicity

The teratogenic potential of a chemical at varying concentrations is evaluated by looking at the teratogenic endpoints using the FET [28]. The graph seen in Figure 4 shows that no malformations were observed in the larvae that developed at the negative control (RW), 20 µg/L PE-MBS, internal plate control, surfactant used (Tween 80), and solvent (DMSO) set-ups. However, analysis of the data using the Kruskal-Wallis Test, indicated that the means and variances of the malformations seen in larvae that developed from the treatment groups of 200 and 2000 µg/L PE-MBS, as well as the 5% ethanol (positive control) group, were significantly different from the groups with no malformation. The highest number of incidents of malformations for each group was edema of both yolk sac and pericardium (62% - 100%), followed by bent body axis (8% - 33%), then bent tail (21% - 24%), and the least observed teratogenic endpoint for all the groups was the collapsed swim bladder (3% - 13%).

Four of the teratogenic features observed in the larvae are shown in Figures 5B-F. Edema is the accumulation of pellucid fluid in the pericardium or in the yolk sac (Figure 5B-C); bent body axis is an abnormal flexion of the primary axis (Figure 5D); bent tail is an abnormal, dorsoventral, or lateral flexion of the tail at the axial level of the caudal fin (Figure 5E); and collapsed swim bladder (Figure 5F) is the unexpanded swim bladder compared to the normal phenotype (Figure 5A) of *Danio rerio*.

Discussion

Embryotoxicity of Polyethylene Microbeads

Polyethylene microbeads are added to several personal care and cosmetic products (PCCPs) such as facial wash or

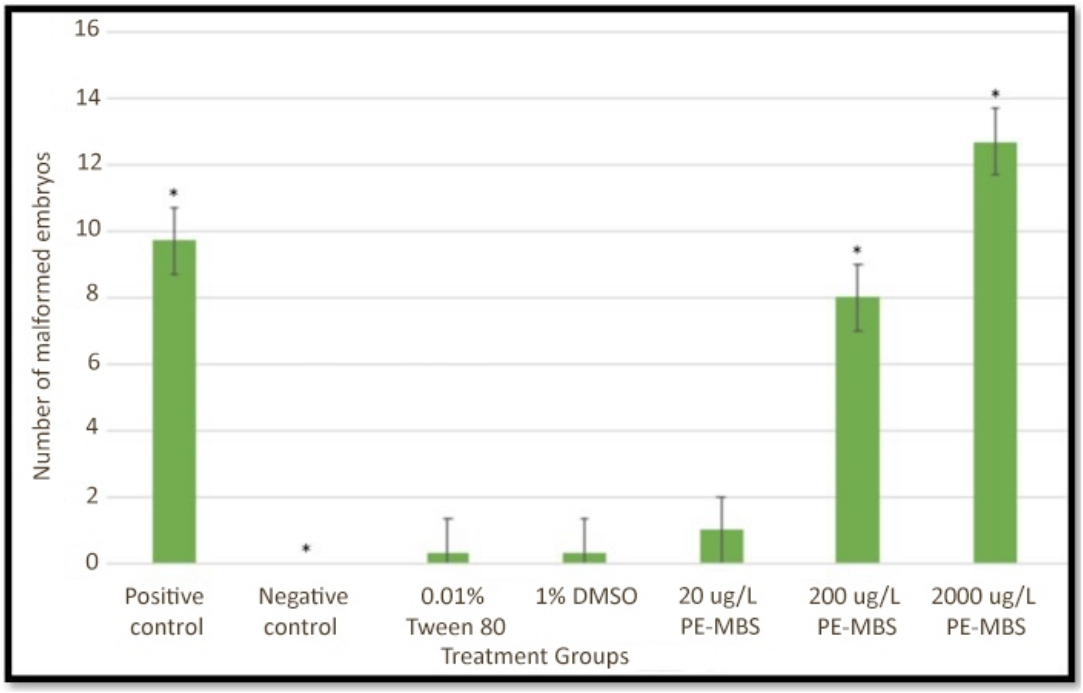

Figure 4. *Teratogenicity of polyethylene microbeads. The number of malformed larvae of D. rerio larvae at 144 hpf exposed to the positive control (5% ethanol), negative control (RW), 0.01% Tween 80, 1% DMSO (surfactant and solvent, respectively), and the three concentrations of the polyethylene-microbead suspensions used in this study. Error bars indicate standard error. The asterisk indicated a statistically significant difference in the number of malformations observed across the group ($p < 0.05$).*

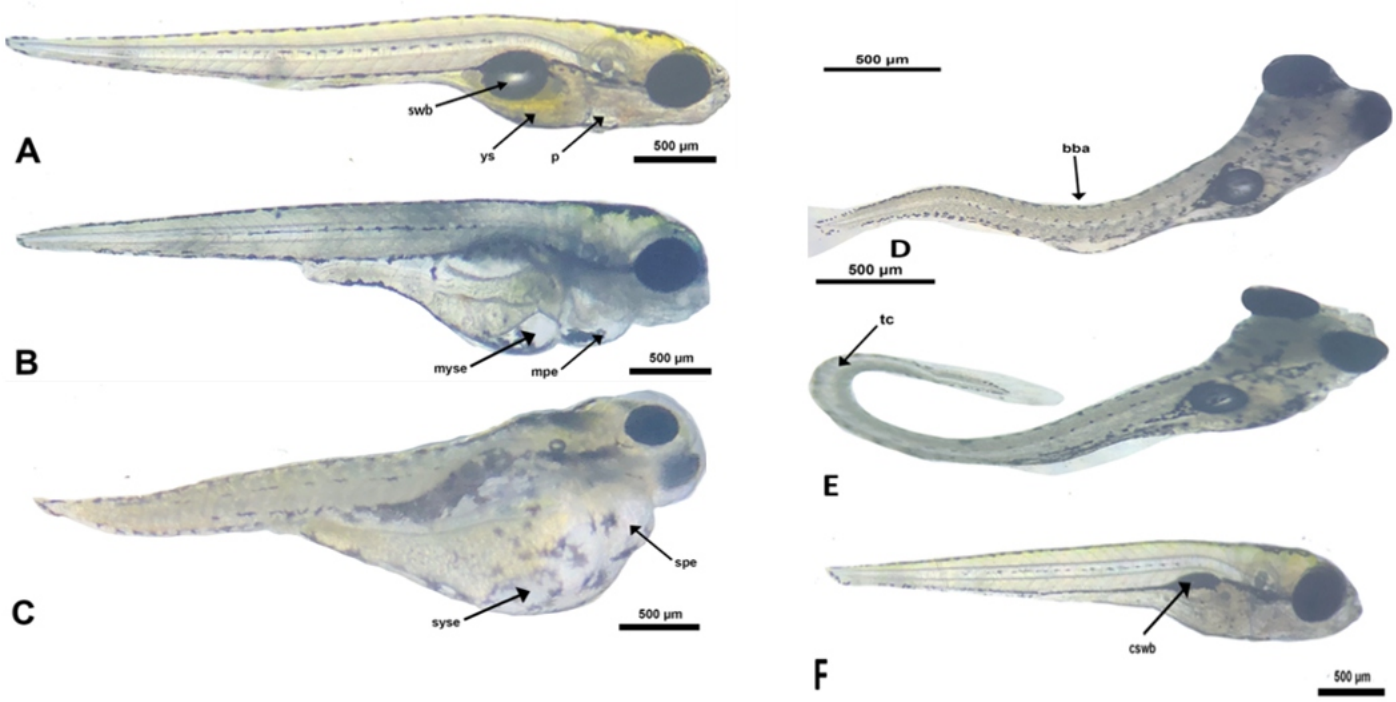

Figure 5. *Malformations in Danio rerio larvae at 144 hpf. (A) Normal larva observed in the negative control (RW) and 20 µg/L PE-MBS with a normal inflated swim bladder (swb), yolk sac (ys) and pericardium (p), no bent body axis and no tail curvature. (B) Larva with mild yolk sac edema (myse) and mild pericardial edema (mpe) were observed in the 0.01% Tween and 1% DMSO, respectively. (C) Severe yolk sac edema (syse) and severe pericardial edema (spe); (D) bent body axis (bba); (E) tail curvature (tc) and (F) collapsed swim bladder (cswb). Figures C-F were all observed in the 5% ethanol group, 200 and 2000 µg/L PE-MBS groups.*

cleanser, shower gel, and toothpaste that act as scrubbing agents [27]. These microscopic plastic beads, when disposed of improperly or unintentionally escape the waste-water filtration system, can find their way into the freshwater ecosystems and pose a risk to the development and health of their biota. In this study, the effects of white, polyethylene microbeads (300–355 µm in diameter and 1.10 g/cc density) found in facial wash products on the embryos and larvae of *Danio rerio* were assessed using the Fish Embryo Acute Toxicity Test (FET).

The injury of an embryo resulting in its abnormal development or death due to exposure to toxic substances is referred to as embryotoxicity. Using the zebrafish embryos as models for the embryotoxicity of the polyethylene microbeads, the results of this study have shown that these microbeads are embryotoxic to the embryos as the concentration of the test substance increases. The results on the embryotoxicity of PE-MB to zebrafish embryos used in this study coincided with one toxicological study of the zebrafish [28]. Since there were no significant toxic effects nor mortality in the negative control, emulsifier, solvent, and the 20 µg/L PE-MBS groups, it is possible that these treatments had no substantial effects on the early life stages of development of the zebrafish fertilized eggs from the 24-96 hpf period. It is also likely that the 300-355 µm diameter microbeads at that concentration was not able to get through the chorion, which is said to be < 1 µm [29].

All four toxicological endpoints in assessing the embryotoxicity of substances were observed in embryos exposed to 200 and 2000 µg/L of PE-MBS which were comparable with the results observed in embryos exposed to 5% ethanol. This would also mean that since all four of the toxicological endpoints were observed in the 200 and 2000 µg/L of PE-MBS, it can be said that these concentrations are lethal to the embryos, comparable with the results obtained from the positive control (5% ethanol).

The first sign of somite differentiation in zebrafish occurs after gastrulation usually at 12 hpf. Somites are necessary to differentiate starting at this stage since most of the muscle and connective tissues of the vertebrate will be derived from this group of cells. It is also during somitogenesis when the tail begins to extend and separate itself from the yolk. So, it is most likely that the non-detachment of the tail observed in the embryos might also be due to the absence of somite formation (Figure 1B) in those embryos. The toxicity of a substance may affect the normal development of the somites as well as the non-detachment of the tails. In this study, 12% of the embryos in all test groups exhibited a lack of somite formation with the greatest number of affected embryos in the 200 and 2000 µg/L PE-MBS, and 5% ethanol groups. It can be implied also that aside from the polyethylene composition of the microbeads, there could be some chemical additives and endocrine disrupting chemicals added to the beads that are toxic to the embryos and could have altered gene integrity of the cells affecting the normal development of the embryos [30].

Of the four toxicological endpoints, coagulation of the egg (Figure 1C) accounted for most of the frequently occurring endpoints (38%-80%). Although the mechanism behind egg coagulation remains unclear due to lack of related literature, the exposure to high concentrations of PE-MBS (*i.e.*, 200 and 2000 µg/L) may have induced coagulation of the eggs. There might be a temporal expression or lack of specific metabolic enzymes that may allow the eggs to detoxify the harmful substances from the 24 to 48 hpf period of observation [31]. The coagulation of the eggs observed in the two highest concentrations of PE-MBS is comparable with the 5% ethanol (positive control) used in this study. It is likely that the coagulation of the eggs at 200 and 2000 µg/L PE-MBS may be due to the desiccating and denaturing properties of ethanol and may be due to the toxic properties of the alcohol [32].

The lack of observable heartbeat was the second most recorded toxic endpoint, with 33% of the embryos in all treatment groups affected. The development of the heart begins at 16 hpf wherein the cardiac precursor cells start to differentiate and travel towards the central midline of *Danio rerio* embryos [33]. The lack of heartbeat was seen in embryos in the 200 and 2000 µg/L PE-MBS groups which were significantly not different from the positive control groups. This lack of heartbeat may be attributed to a possible hypoxia caused by the high concentrations of PE-MBS before the 24 hpf observation period. Evidence has shown that polyethylene microbeads cause hypoxia in *Danio rerio* embryos as these microplastics may adhere to the chorionic membrane [34]. The large diameter of the PE-MB used in this study (i.e., 300-355 µm) and their high concentrations could have become a barrier on the surface of the chorion (pore size of < 1 µm) and hindered the passage of oxygen or interfered with the process of gas exchange in the chorion. Once gas exchange is disrupted, this can result in critically low amounts of available oxygen to the embryos and could have caused the developing heart to stop beating [35].

The non-detachment of tail from the yolk (Figure 1D) was observed in 29% of the embryos of all the treatment groups

used in this study after 24, 48, 72, and 96 hpf, with the highest incidence observed at the 200 and 2000 µg/L PE-MBS, and 5% ethanol groups. These observations were similar to a study wherein the test substance in high concentration caused the embryonic tail not to detach at the posterior end of the yolk sac [36]. The tail of a developing zebrafish embryo must detach from the yolk since the fish in its early stage of development will need its tail to swim for its survival.

Since all four toxicological endpoints were observed in the 200 and 2000 µg/L PE-MBS, it can be said that polyethylene microbeads with 300-355 µm diameter can be lethal at these concentrations. The lethal effects of these treatments after 96 hpf are significantly not different from the results obtained from the 5% ethanol group. Although there were mortalities of embryos exposed to the 20 µg/L of PE-MBS, these were not significantly different from the negative control group (RW). The low mortality at this concentration of PE-MBS is still within the requirement of the OECD standards wherein at least 90% of the embryos are still alive at 96 hpf [25]. The coagulation of the eggs, as well as the absence of heartbeat from 24 to 96 hpf observation period, provides enough reasons to consider the test substances to be lethal (Figure 2a).

The concentration-mortality curve of *Danio rerio* at 96 hpf (Fig 2b) indicates that the mortality of the embryos is concentration dependent, such that as the concentration of the polyethylene microbeads increases, mortality increases. The LC50 was computed using probit analysis taking into consideration the results obtained from the negative control group. The lethal concentration of the microbeads to cause 50% mortality of the population under study is 2455.096 µg/L (graph not shown) which is higher than the highest treatment concentrations used in this study at 2000 µg/L.

Hatching

A critical stage in the embryogenesis of aquatic vertebrates is hatching from the chorion or egg sac/shell. A delay or failure of hatching in the prescribed period of development is a sign that the substance tested or evaluated is toxic to the early development of the organism, in this case, *Danio rerio*. The typical hatching of *Danio rerio* is between 48 to 72 hpf. Any embryo that hatches at 48 hpf is considered an early hatcher while an embryo that hatched at 96 hpf is considered a late hatcher. In this study (Figure 3), only one embryo hatched at the 48 hpf in these treatment groups (*i.e.*, negative control, 0.01% Tween 80, 1% DMSO, and 20 µg/L PE-MBS). Statistical analysis of the results was not significantly different from each other. The number of embryos (3 to 5) that hatched on the 48 hpf at treatment groups of 200 and 2000 µg/L PE-MBS is significantly higher compared to those in the negative control. These results may indicate that high concentrations of PE-MBS can induce early hatching of *Danio rerio* embryos.

It is likely that hypoxia due to the presence of high concentrations of PE-MBS in the medium made the embryos become early hatchers for them to survive at 48hpf. The possible adherence of the microbeads on the chorionic membrane may have resulted in clogged pores, thus hindering gas exchange, and consequently, insufficient gas supply. Hypoxia in *Danio rerio* embryos increases truncal muscle movement to agitate water contained inside the chorion and accelerates certain metabolic and respiratory processes to compensate for the lack of oxygen [37]. These stressed-induced responses due to a hypoxic environment can induce early hatching since removing the chorionic membrane around the embryo can increase oxygen uptake by the embryos. Although the embryos exposed to 200 and 2000 µg/L PE-MBS hatched early, there were lower survival rates after hatching. This can reinforce the hypothesis of this study that high concentrations of PE-MB are both embryotoxic and lethal.

Teratogenicity

The extension of the observation period to 144 hpf allowed the visualizations of the teratogenic effects of polyethylene microbeads on the larvae of the zebrafish. A substance can be considered teratogenic when some malformations such as edema, bent body axis, bent tail, and collapsed swim bladder can be observed at 144 hpf. In this study, 200 and 2000 µg/L PE-MBS caused the indicated malformations which were also significantly comparable to the 5% ethanol group. These results imply that PE-MB at these concentrations can be teratogenic to zebrafish embryos. The low concentration of PE-MBS used in this study (20 µg/L), was not significant enough to cause malformations in the embryos which were seen also in the negative control and other control set-ups used in this study.

The edema of the yolk and the pericardium (Figs. 5B-C) had the highest incidence of malformation in all PE-MBS treatment groups (62% to 100%). These results are comparable with the edema caused by ethanol, the positive control of this study, which is known to be teratogenic in animals and humans [36]. The polyethylene microbeads may have caused disturbance to regulate barriers in internal water

diffusion, possibly substantiating increasing incidents of edema in increasing concentrations. The presence of edema, particularly pericardial edema, may also be regarded as a symptom of hypoxia in the *Danio rerio* embryos that led to the loss of heartbeat at 48 hpf. Yolk sac edema is the swelling of the yolk sac due to the accumulation of fluid that was observed in 144 hpf zebrafishes at 200 and 2000 µg/L PE-MBS which were also observed in the positive control group. The yolk sac of larvae at 20 µg/L PE-MBS did not deviate from the normal external features seen in the negative control group. These observations on the yolk sac were like the results of a salmon study wherein the edema of the yolk sac was an indication of toxicity and teratogenicity in salmon fry exposed to 1000 µg/L of bisphenol-A [37]. A malformation of the yolk sac may cause the yolky nutrients to be damaged also, thus causing poor nutrition for the growing zebrafish larvae.

The bent body axis (Figure 5D), often referred to as scoliosis and bent tail (Figure 5E) was also observed in embryos exposed to 200 and 2000 µg/L of PE-MBS at the 144 hpf period. These results are not significantly different from the malformations of these two body parts seen in the 5% ethanol group, which is a known teratogen. The bent appearances of the tails may possibly be associated with "knockdown" of the cysteine-rich motor neuron 1 gene (crim1) which is involved in the development of the central nervous system or missense mutation in polycystin2 (pkd2), a gene that encodes for the activation of the Ca^{2+} cation channel which is important in the skeletal muscle excitation-contraction process [38] that may be depicted in the tail movement of the zebrafish.

The collapsed swim bladders were seen at the larvae exposed to 200 and 2000 µg/L of PE-MBS. The swim bladder is an important organ in bony fishes such as the zebrafish since it aids in making upward hydrodynamic forces to prevent the sinking of the fish [39]. The collapse of this structure may be attributed to hypoxia and edema in the ventral region of the body as observed in embryos with pericardial edema [38].

Conclusions and Recommendations

The inclusion of polyethylene microbeads in personal care and cosmetic products such as facial washes has captured the attention of the scientific community due to the risks it poses to aquatic organisms. In this study, the *Danio rerio* was chosen to be the animal model for toxicity and teratogenicity assessment of polyethylene microbeads because of its availability, easy care, high fecundity, and great similarity with the human genome. Polyethylene microbeads used in this study were based on measurements of microbeads found in commercial facial washes. Results from the Fish Embryo Acute Toxicity test revealed that 20 µg/L did not have a significant difference in the observed parameters seen in the negative control group (*i.e.*, embryotoxicity, hatching, and teratogenicity). However, at 200 and 2000 µg/L of PE-MBs, these high concentrations were significant to cause embryotoxic and teratogenic effects on the developing zebrafish embryos. These negative effects were comparable with the positive control used in this study which is 5% ethanol, a known toxic and teratogenic substance.

The results of this study require urgent actions against the production and selling of facial washes with polyethylene microbeads here in the Philippines. The unintentional release of these wastes from water treatment facilities into the aquatic bodies of the ecosystem can add to their pollution and can harm the aquatic biota. It is also suggested that investments and policy reforms on improving plastic waste management must be enacted to minimize the leaching of microplastics such as microbeads. Through these efforts, problems concerning bioaccumulation and toxicity of these pollutants may be prevented and consequently save the lives of both aquatic organisms and humans as well.

It is also recommended to investigate the embryotoxic and teratogenic effects of other types of microbeads such as polypropylene and polyamide of varied sizes other than the sizes used in this study. It is also recommended to investigate the effects of microbeads at the gene level, by looking into mutations of genes such as hsp70, crim1, and pkd2 that might have contributed to the embryotoxicity and teratogenicity of the zebrafish embryos observed in this study. The discovery of new biomarkers due to the influence of polyethylene microbeads can also be done in the future which can be used to monitor the health of the aquatic habitats and their biota.

Acknowledgments

This study was funded by the National Institute of Health – University of the Philippines Manila Student Researcher Grant, with Registration No. RGAO-2019-1096. The authors are grateful for the assistance of Mr. Edgar Acantilado and Mr. Maxcitar Amar, the laboratory technicians of the Department of Biology, College of Arts and Sciences, University of the Philippines Manila.

References

1. Australian Government Department of the Environment and Energy (n.d.) Plastic Microbeads. http://www.environment.gov.au/protection/waste-resourcerecovery/plastics-andpackaging/plastic-microbeads
2. Malafaia G, de Souza AM, Pereira AC, *et al.* (2020) Developmental toxicity in zebrafish exposed to polyethylene microplastics under static and semi-static aquatic systems. Science of The Total Environment 700: 134867.
3. Tantibanchachai C. (2014) Teratogens. The embryo project encyclopedia.
4. Triebskorn R, Braunbeck T, Grummt T, *et al.* (2019) Relevance of nano- and microplastics for freshwater ecosystems: A critical review. Trends in Analytical Chemistry. 110: 375-392. https//doi.org/10.1016/j.trac.2018.11.023
5. Graca B, Szewe K, Zakrewska D, Dolega A. (2017) Source and fate of microplastics in marine and beach sediments of the Southern Baltic Sea. Environ Sci Pollut Res.doi:10.1007/211356-017-8419-5
6. Genc AM, Vural N, Balas L. (2020) Marine Pollution Bulletin. Modelling transport of microplastics in enclosed coastal waters: A case study in the Fethiye Inner Bay. Marine Pollution Bulletin 150: 110747. https://doi.org/1016/j.marpolbul.2019.110747
7. Prokíc MD, Radovanovic TB, Gavric JP & Faggio C. (2019) Ecotoxicological effects of microplastics: examination of biomarkers, current state, and future perspectives. Trends in Analytical Chemistry 111: 37-46. https:/doi.org/10.1016/j.trac.2018.12.001
8. Abreo, Neil Angelo S. (2018) Marine plastics in the Philippines: a call for research. Philippine Science Letters 11(1): 1-2.
9. Alcala AC. (2019) Collaborative research on microplastics. Environment Connection. MetroPost. http://dumaguetemetropost.com/july-pdf-version-p11277-192.htm
10. Espiritu EQ, Dayrit SASN, Coronel ASO, *et al.* (2019) Assessment of quantity and quality of microplastics in the sediments, waters, oysters, and selected fish species in key sites along the Bombong estuary and the coastal waters of Ticalan in San Juan, Batangas. Philippine Journal of Science 148(4): 789-801.
11. Esquinas GGMS, Mantala AP, Atilano MG, Apugan RP, Galarpe VRKR. (2020) Physical characterization of litter and microplastic along the urban coast of Cagayan de Oro in Macalajar Bay, Philippines. Marine Pollution Bulletin 154: 111083. https://doi.org/10.1016/j.marpolbul.2020.111083
12. Salaverria LB. (2018) Legarda to file bills to ban microplastics, single-use plastics. Inquirer. https://newsinfo.inquirer.net/997882/legarda-to-filebills-to-ban-microplastics-single-use-plastics
13. Philippine Information Agency. (2017) Groups press DOH, FDA to ban plastic microbeads in personal care and cosmetic products. Science.ph: http://www.science.ph/full_story.php?key=124053: groups-press-doh-fda-to-ban-plasticmicrobeads-in-personal-care-and-cosmetic-products&type=latest
14. Alcala AC, Bucol AA, Bucol LA, *et al.* (2020) Microplastics in the mottled rabbitfish (Siganus fuscescens) in Negros Oriental, Philippines with notes on the Siganid Fishery. Siliman Journal 61(1):19-36. https://www.researchgate.net/publication/351744882_Microplastics_in_the_Mottled_RabbitfishSiganus_fuscescens_in_Negros_Oriental_Philippines_with_Notes_on_the_Siganid_Fishery/comments
15. Abiñon BSF, Camporedondo BS, Mercadal EMB, *et al.* (2020) Abundance and characteristics of microplastics in commercially sold fishes from Cebu Island, Philippines Int. J. Aquat. Biol. 8(6): 424-433. doi: https://doi.org/10.22034/ijab.v8i4.874
16. JoVE Science Education Database. (2020) Biology II: Mouse, Zebrafish, and Chick. Zebrafish Breeding and Embryo Handling. JoVE, Cambridge, MA.
17. Gerlach G, Morales E, Wingert R. (2015) Microbead implantation in the zebrafish embryo. Journal of Visualized Experiments. doi: 10.3791/52943
18. Rahman KF, Sulaiman AS. (2019) Zebrafish (*Danio rerio*) as a Model Organism. Current Trends in Cancer Management. doi: 10.5772/intechopen.81517
19. Arellano-Aguilar O. *et al.* (2015) Use of zebrafish embryo toxicity test for risk assessment purpose: case study. Journal of Fisheries Sciences doi: 10.1371/Journal.pone.0187590
20. Braunbeck T, Kais B, Lammer E, *et al.* (2015). The fish embryo test (FET): origin, applications, and future. Environmental Science and Pollution Research 22(21): 16247-16261.
21. Selderslaghs IWT, Hooyberghs J, Blust R, Witters HE. (2013) Assessment of the developmental neurotoxicity of compounds by measuring locomotor activity in zebrafish embryos and larvae. Neurotoxicology and Teratology 37:44-56. http://dx.doi.org/10.1016/j.ntt.2013.01.003
22. Philstar Global. (2018) Legarda bill seeks to ban use of microbeads. https://www.philstar.com/business/

science-and environment/2018/06/14/1824289/nation
23. Ochokwu IJ, Apollos TG, Oshoke JO. (2015) Effect of egg and sperm quality in successful fish breeding. IOSR Journal of Agriculture and Veterinary Science 2: 48-57.
24. Chang M. (2015) Reducing microplastics from facial exfoliating cleansers in wastewater through treatment versus consumer product decisions. Marine Pollution Bulletin 101(1): 330-333.
25. OECD (2013) Test No. 236: Fish Embryo Acute Toxicity (FET) Test, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris. doi: 10.1787/9789264203709-en
26. Lu , Zhang Y, Deng Y, *et al.* (2016) Uptake and accumulation of polystyrene microplastics in zebrafish (*Danio rerio*) and toxic effects in liver. Environmental Science & Technology 50(7):4054-4060.
27. Leslie H. (2014) Review of microplastics in cosmetics. Institute for Environmental Studies (IVM).
28. Mendis JC, Tennakoon TK, Jayasinghe CD. (2018) Zebrafish embryo toxicity of a binary mixture of pyrethroid insecticides: d-tetramethrin and cyphenothrin. Journal of Toxicology, 2018.
29. LeMoine CMR, Kelleher BM, Lagarde R, Northam C, Elebute OO, Cassone BJ. (2018) Transcriptional effects of polyethylene microplastics ingestion in developing zebrafish (*Danio rerio*). Environmental Pollution 243:591-600. https://doi.org/10.1016/j.envpol.2018.08.084
30. Rochman CM, Kurobe T, Flores I, Teh SJ. (2014) Early warning signs of 993 endocrine disruption in adult fish from the ingestion of polyethylene with and without 994 sorbed chemical pollutants from the marine environment. Science of the Total 995 Environment, 493, 656-661.
31. Reimers MJ, Flockton AR, Tanguay RL. (2004) Ethanol-and acetaldehyde-mediated developmental toxicity in zebrafish. Neurotoxicology and Teratology 26(6):769781.
32. Bakalinsky AT, Penner MH. (2003) ALCOHOL Properties and Determination.
33. Torres-Arraut E, Singh S, Pickoff AS. (1984) Electrophysiologic effects of Tween 80 in the myocardium and specialized conduction system of the canine heart. Journal of Electrocardiology 17(2): 145-151
34. Kim KT, Tanguay RL. (2014) The role of chorion on toxicity of silver nanoparticles in the embryonic zebrafish assay. Environmental Health and Toxicology 29.
35. Burggren WW, Pinder AW. (1991). Ontogeny of cardiovascular and respiratory 1240 physiology in lower vertebrates. Annual Review of Physiology, 53(1), 107-135.
36. Basnet RM, Guarienti M, Memo M. (2017) Zebrafish embryo as an in vivo model for behavioral and pharmacological characterization of methylxanthine drugs. International Journal of Molecular Sciences 18: 596. doi:10.3390/ijms18030596
37. Honkanen JO, Holopainen IJ, Kukkonen JVK. (2004) Bisphenol A induces yolk-sac oedema and other adverse effects in landlocked salmon (Salmo salar m. sebago) yolk-sac fry. Chemosphere 55:187-196. doi:10.1016/j.chemosphere.2003.10.028
38. Bertola L, Schoonheere E, Wilms I, Kabli S, Alia A, Groot H, Bagowski C. (2011) Effects of hypoxia on buoyancy control and the development of lordosis in physostomous and physoclistous fish species. Biosciences 1-7.
39. Xiyuan Z, Fink RHA, Mosqueira M. (2017) NO-sGC pathway modulates Ca^{2+} release and muscle contraction in zebrafish skeletal muscle. Front. Physiol. 8(607):1. doi: 10.3389/fphys.2017.00607