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2 Embryotoxic and teratogenic effects of polyethylene microbeads found in facial wash products
3 in Zebrafish (*Danio rerio*) using the Fish Embryo Acute Toxicity Test

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24 Abstract

25 Use of polyethylene beads in facial cleansers has been continuously questioned by
 26 scientific communities for they adversely affect aquatic organisms once these beads find their way
 27 into their habitats. This study specifically aims to determine *Danio rerio* mortality rate using lethal
 28 endpoints and to evaluate sublethal teratogenic effects in *Danio rerio* due to polyethylene
 29 microbead exposure. *Danio rerio*, a model organism for ecotoxicology, was subjected to the Fish
 30 Embryo Acute Toxicity Test. Embryos were exposed to polyethylene microbead suspensions (PE-
 31 MBS) of varying concentrations (i.e., 20 µg/L, 200 µg/L, 2000 µg/L). They were also exposed to
 32 5% ethanol (positive control), reconstituted water (negative control), 0.01% Tween 80 (emulsifier
 33 control), and 1% DMSO (solvent control). Toxicological endpoints (i.e., egg coagulation, lack of
 34 somite formation, non-detachment of tail, and lack of heartbeat) were observed every 24 hours
 35 until the 96th hour exposure. Hatching was observed from 48 hpf while teratogenicity was
 36 observed at 144 hpf. Significant differences between means and variances were observed for all
 37 treatment groups in relation to the negative control. For all groups, 0.01% Tween 80, 1% DMSO
 38 and 20 µg/L PE-MBS did not significantly differ with the negative control due to negligible
 39 concentration but 5% ethanol and higher concentrations of PE-MBS did. This indicated that high
 40 concentrations of PE-MBS exposure may induce early hatching, mortality, increased
 41 malformation, and increased heart rate. Tukey Kramer *post hoc* Test substantiated that PE-MBS
 42 toxicity is dose dependent since embryotoxicity and teratogenicity increases at higher
 43 concentrations. LC₅₀ obtained using probit analysis based on experimental data was 2455.096
 44 µg/L, and was higher than the concentrations used in this study. Further studies should be
 45 conducted to know more about the adverse effects of polyethylene microbeads to the biota.
 46 Keywords: embryotoxicity, teratogenicity, polyethylene microbeads, zebrafish

47 **Author Summary**

48 Margaret De Guzman, MSc, Patricia Chua, and Franceska Sedano have all
 49 equally contributed to this work in conceptualization, formal analysis, funding
 50 acquisition, and investigation. All authors have also equally headed project
 51 administration, procurement of resources and writing.

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66 **Introduction**

67 **Background of the Study**

68 Marine pollution caused by plastic microbeads has been an emerging concern for the
69 embryonic development and cellular health of many organisms [1]. Polyethylene microbeads (PE-
70 MB) are polysynthetic resins found in beauty products and generally serve as abrasives or bulking
71 agents in cleaning products and exfoliants in numerous beauty products [2]. Due to their minuscule
72 size, most sewage treatment plants are unable to effectively filter these microbeads. As a result,
73 these microplastics infiltrate the aquatic ecosystem and pose adverse effects to its constituents.
74 Since microbeads are usually treated with additives and plasticizers during the production process
75 [3], and have the ability to adsorb chemical pollutants [4], exposure to these microbeads may result
76 in developmental toxicity in aquatic organisms [5].

77 Substances that may cause physical or functional defects in a developing embryo are
78 considered to be teratogenic [6]. Polyethylene, the most common type of plastic used for
79 microbeads [7], is a polymer of repeating CH₂ units [8]; however, this polymer degrades for a long
80 period of time [9], rendering them to be highly persistent and toxic to the environment. The
81 chemical composition and ability of polyethylene to be carriers of toxins from industrial
82 manufacturers makes it a potential teratogen to living organisms [10]. Once these microbeads
83 come in contact with low trophic organisms such as fish larvae, exposure to toxic additives
84 contained in polyethylene microbeads may interfere with metabolic pathways, alter gene integrity,
85 and consequently lead to embryotoxicity and formation of teratogenic abnormalities [5,11].

86 Currently, there are no known studies conducted regarding the quantity of polyethylene
87 microbeads in Philippine waters as well as the harmful effects that they pose. This study differs
88 from others as this study is limited to polyethylene among other microplastics such as

polypropylene, polyester, polyethylene terephthalate, and nylon. This study will also use polyethylene microbeads with sizes based on the ones contained in facial cleansers being commercially sold in the Philippines. This study will also be done *in vitro* instead of obtaining polyethylene bead samples from the marine or freshwater environment.

Danio rerio is the chosen test organism of the study. *Danio rerio* is a tropical freshwater fish and is readily available, inexpensive, exhibits high fecundity [12] and rapid development [13]. Its genes are also likened to 70% of genes in humans [14]. Furthermore, transparency of *Danio rerio* embryos allows researchers to observe teratogenesis in the embryonic development of the zebrafish [13]. In this study, *Danio rerio* were subjected to the Fish Embryo Acute Toxicity Test [15]. The Fish Embryo Acute Toxicity Test is used to evaluate the toxicity of certain chemicals on the embryonic development of vertebrates [16] and exposes fertilized eggs to varying concentrations of the toxin for 96 hours [17].

101

102 **Statement of the Problem**

Do polyethylene microbeads induce embryotoxic and teratogenic effects on *Danio rerio* embryos?

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106 **Research Objectives**

The main objective of this study is to assess if polyethylene microbeads can induce teratogenic and embryotoxic effects in *Danio rerio*. The study specifically aims to:

- 1.) to determine the mortality rate of *Danio rerio* using lethal endpoints such as lack of heartbeat, coagulation of fertilized eggs, lack of somite formation, and lack of detachment of tail-bud from yolk sac

- 112 2.) to determine the lethal concentration (LC₅₀) or the minimum concentration that is lethal
113 to 50% of the exposed population, and;
114 3.) to evaluate the sublethal teratogenic effects of polyethylene microbead exposure such
115 as yolk sac and pericardial edema, bent tail and spine axes, and deflated swim bladder.

116

117 **Significance of the Study**

118 This study is deemed significant as it provides information on the deleterious
119 effects of polyethylene microbeads found in facial cleansers on the developing embryo of
120 freshwater organisms such as *Danio rerio*. In addition, this study is timely and relevant since there
121 has been an observed increase of microplastics in marine and freshwater environments [18],
122 resulting in biomagnification and bioaccumulation [19]. Due to this occurrence, the Microbead-
123 Free Waters Act has been observed in Canada, America, and the United Kingdom. According to
124 Romero [20], this act may also be passed by Senator Loren Legarda in the Philippines soon, a
125 country known to be third in the list of countries with the most ocean plastic pollution in a 2015
126 study conducted by the University of Georgia. Additionally, data gathered from this study may
127 prompt institutions to take action in protecting bodies of water from plastic pollution and
128 encourage local as well as international skincare manufacturers to produce a more environmentally
129 friendly exfoliant alternative to polyethylene microbeads.

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132 **Scope and Limitations**

133 This study is primarily focused on the assessment of teratogenic and embryotoxic effects
134 of virgin polyethylene microbeads in *Danio rerio*. The size of polyethylene microbeads used in

the study is based on the size of commercially sold facial cleansers that contain polyethylene microbeads in the Philippines. Embryotoxic and teratogenic effects induced by polyethylene microbeads on zebrafish embryos will be assessed in accordance with the Fish Embryo Acute Toxicity Test.

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140 **Review of Related Literature**

141 **Polyethylene**

Polyethylene is one of the most widely manufactured polymers in the industry [21]. Its structure consists of a long chain of carbon atoms, with two hydrogen atoms attached to each carbon atom. It is a highly versatile material that can be used to make plastic bags, plastic films, bottles, and microbeads. The plastic's melting point ranges from 110-130°C, making it highly malleable. Despite its malleability for producing a wide variety of products, it makes it a poor candidate for recycling. Despite its universal use, improper disposal of polyethylene microplastics into bodies of water makes it a vector for heavy chemical adsorption. Heavy metals such as cadmium and lead are adsorbed by these plastics and can be detrimental for both wildlife and humans [22]. It has also received criticism for containing pro-oxidants and disintegrating into smaller fragments upon exposure to light, heat, and oxygen [23].

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153 **Microbeads**

Since its introduction to the industry in 1972, microbeads have been a popular ingredient in facial washes and facial scrubs as they serve to exfoliate and scrape away dry cells from the surface of the skin [24]. They are also incorporated in soaps and function as abrasives that remove dirt and debris found in the epidermis. The presence of plastic microbeads has been increasing in

158 aquatic systems and yet its presence has only received attention a few years ago. Recently,
 159 microbeads have been given much attention such that countries such as Canada, New Zealand,
 160 United Kingdom, and the United States of America have banned the use of microbeads in
 161 commercial products [25]. A study conducted by Jingyi et al. [10] found that due to the continuous
 162 increase of synthetic plastic production in beauty product companies and poor management of
 163 plastic waste, water pollution by microbeads has exponentially escalated and has been a great issue
 164 of concern from public authorities. In 2018, a study found many urban areas with a maximum
 165 microplastic concentration of about 517,000 particles m⁻² [26]. Additionally, evidence of plastic
 166 microbeads from beauty products has been reported to bypass sewage treatments and found afloat
 167 in Hong Kong bays [27] while microplastic fragments and polyethylene microbeads mistaken for
 168 fish food were found in the gastrointestinal tract of commercial Japanese Anchovy [28]. Ingested
 169 microbeads have also affected other deep-sea organisms such as mussels and oysters. These
 170 bivalves were found to contain 0.36 to 0.47 particles of microplastic per gram [29]. Recent
 171 evidence has also shown that microplastics such as microbeads have the capacity to adsorb toxic
 172 chemicals, carry harmful bacteria and release them in digestive systems once ingested [30]. The
 173 production process of polyethylene microbeads usually include intentional treatment of additives
 174 such as flame retardants, plasticizers, pigments, and UV stabilizers as these additives prevent fire
 175 hazards and maintain product integrity [31] (Gallo et al., 2018). Polyethylene may also contain
 176 some monomers such as vinyl chloride and Bisphenol A (BPA) that contain endocrine disrupting
 177 components and induce adverse effects upon ingestion or exposure [31]. In a similar study, when
 178 mice were fed with microbeads, microplastics were seen to accumulate in the liver, kidney, and
 179 intestines. The increase of this foreign substance in bodily tissues have also heightened the levels
 180 of oxidative stress in mice [32]. In lieu of microbeads easily adsorbing pollutants, another

pollutant associated with microbeads is polybrominated diphenyl ethers (PBDEs) known to accumulate in shellfish consumed by humans. A study by Wardrop et al. [33] showed PBDE accumulation of up to 12.5% sourced from polyethylene microbeads of Nivea Exfoliating Face Scrub in the rainbow fish. PBDE pollutant is associated with neurological, fertility, and immune system problems, biomagnifying the aquatic food chain [4]. In effect, these studies have been a rising concern for humans and animals alike.

Facial washes with polyethylene microbeads in the Philippines

The prevalence of microplastic pollution is not uncommon in the Philippines as it ranked third in the world for the highest plastic waste inputs into the ocean [34]. Statistically, the Philippines generates about 0.28 to 0.75 million metric tons of plastic litter, yearly [34]. Studies by Kalnasa et al. [35] and Palar et al. [36] investigated the occurrences of microplastic litter in Macajalar Bay and Southwestern Luzon, respectively and revealed that a large percentage of plastic litter were brightly colored spherules. Another study by Bucol et al. [37] quantified microplastics ingested by rabbitfish (*Siganus fuscescens*) from coastal areas of Negros Oriental and found an average of 0.6 particles/fish. These microplastic spherules were speculated to have originated from facial cleansers and other cosmetic products that contain microbeads.

In the Philippines, there are a number of facial cleansers being sold in the market that contain polyethylene microbeads such as Oil-free Acne Wash Daily Scrub, Clear Pore Daily Scrub, and Deep Action Exfoliating Scrub [38]. The rise of microbead consumption and worsening of marine litter over the years have prompted government officials like Senator Loren Legarda to draft a bill that seeks to ban microbead production in the Philippines last 2018 [20] to mitigate microbead production just as New Zealand, Austria, Belgium, and the Netherlands have. In

204 addition, Senator Loren Legarda also proposed to file a bill that will ban microplastic consumer
205 products and single-use plastics that would otherwise bring harm to the environment [39]. In 2017,
206 EcoWaste Coalition, along with other private groups such as Coastal Conservation, Marine
207 Conservation Philippines, and many others endorsed a letter to the Department of Health (DOH)
208 and Food and Drug Administration (FDA) pleading for an expedited implementation of the
209 microbead ban. These groups stated that since plastic microbeads in drainage systems leach their
210 way into the bodies of water, quick action must take place before they negatively affect the food
211 chain, especially those who consume seafood [40]. Currently, DENR issued a resolution of
212 Republic Act No. 9003 that implements the ban of single use plastics in the Philippines [41].

213

214 ***Danio rerio***

215 In this study, *Danio rerio* was chosen as the test organism. The zebrafish is a valuable
216 genetic model system for the study of developmental biology and disease [42]. They are prolific
217 breeders that can lay up to hundreds of eggs per week [43], exhibiting high fecundity and rapid
218 development. Their lifespan can reach up to 5 years and are omnivorous in nature [44]. For the
219 past several years, the use for *Danio rerio* for scientific studies has been popular as it provides
220 optical clarity when observing embryos with developing pathologies [45] as well as the
221 developmental stages of a typical organism. They also have a high degree of genomic conservation
222 and are likened to humans in terms of cellular, molecular, and physiological processes [42].
223 Furthermore, these genetic and physiological similarities with humans include the brain, digestive
224 tract, musculature, vasculature, and innate immune system. 70% of human disease genes also have
225 similar homologs found in the genes of *Danio rerio* [46]. *Danio rerio* are preferentially used for
226 embryonic studies as they allow clear visualization of the dynamics of organogenesis using a

227 simple stereomicroscope [42]. *Danio rerio* has been used in many toxicity studies for the reason
228 that it is one of the best-known models of vertebrate development. The use of *Danio rerio* in
229 studying the toxicity of microplastics is not uncommon. Despite toxicity of microplastics being
230 common, this study differs from other studies because it focuses on polyethylene microbeads
231 found in commercial products such as facial washes commonly used in the Philippines.

232

233 **Embryotoxic and teratogenic effects of microplastics**

234 Teratogens are agents or substances that induce abnormality following fetal exposure.
235 Likewise, teratology is the study of abnormal development in embryos and the causes of congenital
236 malformations or birth defects. These teratogens may be present on either the body surface or
237 internal to the viscera [47]. Embryotoxicity, on the other hand, refers to injury to the embryo
238 resulting in death or abnormal development due to exposure to toxic substances [48]. A study by
239 Oehlmann et al. [49] conducted showed that ingestion of microplastics can affect reproduction and
240 hormone function of marine animals like annelids, mollusks, crustaceans, insects and fish. When
241 retained in the internal viscera for an extended amount of time, ingested microplastics may cause
242 reproduction malfunction, increased risk of death, bioaccumulation, and even eggshell thinning
243 [50].

244 However, a similar study conducted by Batel et al. [51] found that exposure to
245 microplastics did not induce morphological effects on *Danio rerio* embryos nor did microplastics
246 permanently accumulate in adult *Danio rerio* gills under 6 or 24 hours of incubation.

247 Another study stated that exposure to 1000 µg/L of microplastics significantly lessened
248 swimming competence and speed in larval zebrafish. At gene level, this exposure resulted in
249 upregulated expression of genes concerning “inflammation (il1b) and oxidative stress (cat)” [52].

250 In relation to exposure of zebrafish to microplastics, a study by Cormier et al. [53] stated
251 that microplastics may be vectors for organic pollutants such as oxybenzone (BP3),
252 benzo[a]pyrene (BaP), and perfluoro octane sulfonate (PFOS). This exposure effected alteration
253 in cyp1a gene transcription, larval swimming behavior, and hatching rate at 72 hours. For other
254 organisms such as *H. azteca*, exposure to specifically polyethylene microplastics was found to
255 cause lessened organism growth and significant decrease of reproduction for 5000 and 10,000
256 microplastics/mL [54].

257 A study by Gallo et al. [31] stated that exposure of marine organisms to micro and nano
258 plastics results in bioaccumulation and adverse toxic endpoints as these microplastics contain
259 endocrine disrupting properties such as alkylphenols, bisphenol A (BPA), and phthalate esters
260 (DEHP) in concentrations as high as 500,000 mg/kg (ppm). The presence of microplastics
261 increases BPA uptake in *Danio rerio* and in turn causes gene-upregulation in the central nervous
262 system and inhibition of acetylcholinesterase (AChE), which entail that microplastics are
263 neurotoxic [55]. A similar study by Nobre et al. [56] studied the effects of polyethylene pellets on
264 the embryonic development of *Lytechinus variegatus* (sea urchin) and found that exposure to these
265 microplastics induced toxic effects and increased anomalous embryonic development by 58.1%
266 and 66.5% respectively. These findings substantiate that plastic pellets have the ability to act as
267 vectors of pollutants that include additives contained in the surface of virgin pre-processed
268 particles.

269 Other studies show the detrimental effects of microbeads to aquatic organisms, particularly
270 in *Danio rerio*. A study by Träber et al. [57] implanted polyacrylamide beads into developing
271 *Danio rerio* embryos to quantify cell-scale stress in its morphogenesis and organ formation.
272 Stresses induced by microbead implantation had a detrimental effect on neural rod formation.

273 Lei et al. [58] observed that microplastics such as polyamides, polyethylene,
274 polypropylene, polyvinyl chloride, and polystyrene cause intestinal damage and other adverse
275 effects in zebrafish and in the nematode, *Caenorhabditis elegans* in freshwater pelagic and benthic
276 environments. Absence or insignificant levels of lethality were observed in the zebrafish upon
277 exposure at 0.001-10 mg L⁻¹ microplastics for 10 days. Meanwhile, concentrations of microplastics
278 at approximately 70 µm resulted in intestinal damage, cracking of villi, and splitting of enterocytes
279 in zebrafish. On the other hand, exposure of 5.0 mg m⁻² microplastics for 2 days notably impeded
280 survival rates, reproduction and body length of the nematodes. For both organisms, exposure to
281 microplastics at specific sizes contributed to decrease in calcium levels and increased expression
282 of the glutathione S-transferase 4 enzyme in the intestine. This increase confirmed intestinal
283 damage and increase of oxidative stress as effects of exposure to specific concentrations of
284 microplastics. From these results, researchers suggested that toxicity of microplastics were based
285 on size instead of their composition [58]. Although past toxicity studies related to polyethylene
286 microplastics already exist in literature, this study differs since it investigated embryotoxicity and
287 teratogenicity of polyethylene microbeads based on sizes found in commercially sold facial
288 cleaners in the Philippines.

289

290 **Fish Embryo Acute Toxicity Test**

291 The Fish Embryo Acute Toxicity Test (FET) is a method used to study chemical
292 toxicity in aquatic ecosystems in vivo [59]. This test (FET) is deemed advantageous for studies
293 that need to observe the fish under varying concentrations of the test solution. Fish is primarily
294 used in toxicity testing because of their metabolic capacities and they are, more often than not, the
295 primary targets of water pollution and heavy metal effluents [12]. A study by Gül den et al. [60]

296 compared cytotoxicity data from fish and mammalian cell lines and found that both are equally
297 sensitive. This study produced evidence that the Fish Embryo Acute Toxicity Test, while it is
298 performed on fish, is extremely relevant in humans as they both show high sensitivity to FET tests.
299 It uses 4 toxicological endpoints for the determination of toxicity in zebrafish eggs: coagulation of
300 fertilized egg, lack of somite formation, detachment of the tail-bud from yolk sac, and lack of
301 heartbeat [16].

302

303 **Methodology**

304 ***Danio rerio* maintenance**

305 Thirty (30) female and twenty-five (25) male *Danio rerio* approximately 5-months-
306 old and void of any pharmaceutical treatment were purchased from Freshwater Aquaculture
307 Center, College of Fisheries in Central Luzon State University Science City of Munoz, Nueva
308 Ecija. Female and male *Danio rerio* were separated and placed in two 15-gallon glass tanks three
309 fourths ($\frac{3}{4}$) filled with dechlorinated water that was maintained at 26 ± 1 °C, well-aerated with
310 dissolved oxygen at a concentration of 6.6 mg/L, electrical conductivity of 0.256 mS/cm, water
311 hardness of 185 mg/L CaCO_3 and at a constant pH of 7.2 ± 1 [17]. These conditions were
312 maintained with the use of API Freshwater Master Test kit. The feeding regime consisted of *Danio*
313 *rerio* being fed with 300 μm of Tetra®Min Tropical Flakes twice a day at 8:00 am and 4:00 pm
314 daily. This slightly deviated from the original OECD protocol of requiring to feed *Danio rerio*
315 with dry flake food and brine shrimp 3-5 times daily. *Danio rerio* were subjected to a 12-hour-
316 light cycle and were acclimated for two weeks prior to the experiment. The fish were fed with egg
317 yolk the night before mating to increase the likelihood of breeding [61].

318

319 **Preparation of polyethylene microbead suspensions**

320 Clear polyethylene microbeads (PE-MB) 300-355 μm in diameter and 1.10 g/cc in density
321 were purchased from Copsheric LLC (Santa Barbara, CA). These measurements were chosen
322 based on a study of Chang [38] who characterized microbeads from various commercial facial
323 exfoliating cleansers. Since PE-MB are hydrophobic in nature, they were treated with 0.01%
324 Tween 80, a surfactant used to disperse hydrophobic particles in aqueous solutions. To prepare
325 0.01% Tween 80 solution, a beaker was filled with distilled water and was brought to a boil for 5
326 minutes. 0.1 g of Tween 80 per 100 ml was slowly dispensed in boiled water. After cooling, the
327 desired amount of PE-MB was added to a test tube and was placed in a vortex mixer for at least
328 five minutes. PE-MB was left to soak in 0.01% Tween 80 for 24 hours or until equal dispersion
329 was achieved [62]. Polyethylene microbeads were then filtered from 0.01% Tween 80 using
330 Whatman® Grade 1 filter paper with a pore size of 11 μm . Afterwards, polyethylene microbead
331 suspensions (PE-MBS) were prepared by adding polyethylene microbeads to a solution consisting
332 of reconstituted water and 1% DMSO, an organic solvent capable of softly dissolving PE-MB and
333 producing a suitably concentrated stock solution [17]. Sterile and aerated reconstituted water was
334 used in the preparation of PE-MBS [63]. Three concentrations of PE-MBS were prepared based
335 on previous studies on microplastic toxicity by [63] that used the same concentrations [1]. The
336 three concentrations of microbead test suspensions used in this study are 20 $\mu\text{g/L}$, 200 $\mu\text{g/L}$ and
337 2000 $\mu\text{g/L}$.

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341 Egg production and collection of fertilized eggs

342 *Danio rerio* eggs were collected using mass spawning. The number of *Danio rerio*
343 used for mass spawning from the original OECD protocol was modified to acquire the desired
344 number of *Danio rerio* eggs for the study. Groups of *Danio rerio* with a sex ratio of 1 female: 3
345 males were placed in spawning tanks [64] and were exposed to a 14-hour-light cycle the day
346 before the eggs were collected [17].

347 A spawn trap was placed inside the spawning tank as a means of collecting *Danio rerio*
348 eggs. Spawn traps were covered with an inert wire mesh with a size approximately 2 ± 0.5 mm to
349 prevent predation by adult *Danio rerio*. Mating, spawning and fertilization took 30 minutes after
350 the onset of light on the day of testing and eggs collected through spawn traps [17]. After collecting
351 the eggs from the spawning tank, they were rinsed with reconstituted water. Reconstituted water
352 consisted of 294.0 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 123.3 mg/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 63.0 mg/L NaHCO_3 , 5.5 mg/L
353 KCl [65]. A volume of 0.05 ml of Methylene blue was also added to reconstituted water to prevent
354 fungal and parasitic infection that may occur in *Danio rerio* eggs [66]. The reconstituted water
355 solution was aerated for a minimum of 24 hours before being used in the experiment. Fertilized
356 eggs were sorted from unfertilized eggs. The fertilized eggs were transferred to multi-well plates
357 with the reconstituted water. The number of unfertilized and fertilized eggs were counted to check
358 the validity of the results obtained from the Fish Embryo Acute Toxicity test as overall fertilization
359 rate of all eggs collected must be $\geq 70\%$ in the batch tested [17].

360

361

362 **Fish Embryo Acute Toxicity Test**

363 The fertilized eggs were immersed in the test solution immediately after egg collection.
 364 The viable fertilized eggs and the unfertilized eggs were separated and counted for raw data. After
 365 separation, 60 viable fertilized eggs per treatment group were placed in a chamber containing their
 366 respective test concentrations (Table 1) for initial exposure [17]. A dropper was used to transfer
 367 viable fertilized eggs from their respective chambers to 96-well plates containing microbead test
 368 suspensions. For the experimental set-up of the Fish Embryo Acute Toxicity Test, 20 *Danio rerio*
 369 embryos per test concentration with 3 replicates each will be placed in 96-well plates containing
 370 the test concentrations.

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380 **Table 1. Experimental setup showing the composition and volume of each treatment used in**
381 **the Fish Embryo Acute Toxicity test.**

Treatment	Volume in mL	Composition
Negative control	0.5	Reconstituted Water
Internal Plate Control	0.5	Reconstituted Water
Positive Control	0.5	5% ethanol
Solvent Control	0.5	1% DMSO
Emulsifier Control	0.5	0.01% Tween 80
Treatment 1	0.5	20 µg/L PE-MBS
Treatment 2	0.5	200 µg/L PE-MBS
Treatment 3	0.5	2000 µg/L PE-MBS

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383

384 Shown in Table 1 is the experimental setup done for the Fish Embryo Acute Toxicity Test.

385 Five internal plate controls containing sterile reconstituted water will be added to each 96-well

386 plate to identify any potential contamination of the plates by the manufacturer that may be

387 suspected to affect the outcome of the results [17]. If more than one embryo dies per plate in the

388 internal plate control, the test is considered invalid and must be performed again. Reconstituted

389 water was specifically used as a reference solution for negative and internal plate control as *Danio*

390 *rerio* embryos have stricter requirements than adult fish and may be more susceptible to disease if

incubated in regular distilled water [67]. Five percent (5%) ethanol served as the reference substance for positive control as it is known to be a neurotoxicant that induces deformations and mortality in *Danio rerio* [68]. 1% DMSO and 0.01% Tween 80 served as solvent and emulsifier controls, respectively, as they were used in the preparation of PE-MBS and to ensure that these substances do not cause embryotoxicity and teratogenic abnormalities to the organisms under investigation.

Water temperature was made sure to be maintained at 26 ± 1 °C in the test chambers at any time during the test. Certain parameters of newly fertilized *Danio rerio* eggs were checked to ensure that it is valid for the Fish Embryo Acute Toxicity Test.

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The following factors were observed in the collected eggs for the test results to be valid:

1. Overall fertilization rate of all eggs collected must be $\geq 70\%$ in the batch tested.
2. Overall survival of embryos in the negative and solvent control must be $\geq 90\%$ at the end of the 96 hour exposure.
3. Exposure to the positive control must result in mortality not less than 30% at the end of the 96 hour exposure.
4. Hatching rate in the negative and solvent control must be $\geq 80\%$ at the end of the 96 hour exposure.

410

411 **Observations for the Fish Embryo Acute Toxicity Test**

The following toxicological endpoints were observed using a Leica ES2 Stereoscope with a magnification of 100x: (1) coagulation of embryos, (2) lack of somite formation, (3) non-

414 detachment of the tail, (4) lack of heartbeat, and (5) hatching rate. For embryo coagulation, it was
 415 observed as milky white, yet it appeared dark under the microscope. For lack of somite formation,
 416 it should be noted that a zebrafish embryo undergoing normal development at 26 ± 1 °C, will form
 417 approximately 20 somites after a day. In addition, side-to-side contractions of the embryo
 418 signifying somite formation were observed. Lack of somite formation was also recorded after 24,
 419 48, 72, and 96 hours. Non-detachment of the tail means absence of a posterior extension of the
 420 body of the embryo. Absence of this was recorded after 24, 48, 72, and 96 hours. Lack of heartbeat
 421 was recorded after 48, 72, and 96 hours since visibility of heartbeat occurs after 48 hours of a
 422 normally developing zebrafish embryo at 26 ± 1 °C. It should be noted that erratic heartbeat and
 423 visible heartbeat in the absence of circulation in aorta abdominalis are non-lethal. Hatching, despite
 424 not being a teratogenic endpoint involved in the calculation for LC₅₀, was observed and recorded
 425 after 47, 72 and 96 hours for it ensures exposure of the embryo in the absence of a potential barrier
 426 function of the chorion.

427

428 Any positive results observed for any of the toxicological endpoints rendered the *Danio*
 429 *rerio* embryo dead. Moreover, hatching and heartbeat were observed in control and treatment
 430 groups from 48 up to 96 hpf were recorded as well. The remaining toxicological endpoints were
 431 recorded every 24 hours until the end of the 96 hour exposure.

432

433 At 144 hpf, *Danio rerio* larvae were euthanized using hypothermic shock. The fish were
 434 quickly immersed in an ice bath consisting of 5 parts ice and 1 part distilled water for 40 minutes
 435 or until cessation of gill and heart movement was observed [69]. Once movement was no longer
 436 visible, *Danio rerio* were mounted in glass slides with 10% glycerol. Prepared microscope slides

were then observed under Leica ES2 Stereoscope with a magnification of 100x to assess the different teratogenic effects induced by PE-MBS such as yolk sac edema, pericardial edema, bent body axes, tail curvature and collapsed swim bladder.

440

441 **Statistical Analysis for Fish Embryo Acute Toxicity Test**

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, 96 hour exposure were recorded. Probit analysis for the estimation of LC₅₀ values at 96 hour exposure for mortality with a 95% confidence limit was recorded for graphing and interpretation as well [17]. It should be noted that LC₅₀

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). Kruskal-Wallis test was performed if data did not pass Shapiro-Wilk's test of normality. 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 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 Microsoft Excel Real Statistics Software. Data is significant for $p \leq 0.05$.

457

458

459 Institutional Animal Care and Use Committee (IACUC)

460 Under guidelines of the Institutional Animal Care and Use Committee, *Danio rerio* was
461 used in this study for the interest of relevance to human and animal health, to improvement of
462 knowledge, and to the good of society [70]. Researchers involved in the experiment ensured proper
463 handling of the specimens.

464 Factors affecting the housing and feeding of the *Danio rerio* such as UV-sterilization,
465 ventilation, aeration (6.6 mg/L O₂) temperature (26 ± 1 °C), water cleaning, water salinity (185
466 mg/L CaCO₃), electrical conductivity (0.256 mS/cm), and pH (7.2 ± 1) were adjusted in
467 accordance to the proper care and breeding of *Danio rerio* as earlier mentioned in the methodology.

468 *Danio rerio* were placed and maintained in 2 15-gallon glass tanks. Dimensions of each
469 glass tank were 20" x 10" x 12". 15% of the water in glass tanks was replaced every week. Before
470 replacement, the tap water acquired in the sanitized gallon-sized bucket was pre-treated first with
471 a water conditioner to adjust the pH level and to remove toxins and metal residue in the tap water.
472 Water was UV-sterilized with a portable UV water sterilizer submerged and stirred in the bucket
473 until light of the sterilizer turned off.

474 After UV sterilization, the water in the tank was removed with a siphon tip placed into the
475 tank's substrate at the bottom. The siphon removed debris and the tank water. Temperature of the
476 remaining water in the tank and that of the new water in the buckets were measured with a
477 thermometer to know if temperatures were near to one another. Pretreated water was poured slowly
478 into the tanks [71].

479 They were fed properly and regularly. The fish were fed twice everyday as earlier stated in
480 the methodology.

481 After conducting the study, adult *Danio rerio* used for breeding were returned to their
482 original tanks. *Danio rerio* embryos and larvae used in the Fish Embryo Acute Toxicity Test were
483 placed in sealed plastic bags for garbage collection.

484

485 **Results and Discussion**

486 The use of the Fish Embryo Acute Toxicity Test in this study has shown that polyethylene
487 microbeads found in facial wash products are embryotoxic and teratogenic to *Danio rerio*
488 embryos. Three concentrations of white PE-MBS 300-355 µm in diameter and 1.10 g/cc density
489 were used in this study (i.e., 20, 200 & 2000 µg/L) as toxicants.

490

491 **Embryotoxicity**

492 Polyethylene microbead embryotoxicity was evaluated using the four toxicological
493 endpoints namely coagulation of eggs, lack of somite formation, non-detachment of tail, and lack
494 of heartbeat [17]. Coagulated embryos are described as milky white eggs void of any structure.
495 Lack of somite formation is characterized by absence of somites and side to side contractions.
496 Non-detachment of tail is the inability of the embryo to extend its posterior extension while lack
497 of heartbeat is absence of a visible heartbeat in a normally developing embryo starting at 48 hpf.
498 Once a single toxicological endpoint is observed within the 96 hour exposure, the embryo was
499 considered dead [17].

500 Cumulative mortality was observed until the 96th hour of the final static exposure. Ninety-
501 six (96) hours were allotted for observing cumulative mortality since there are some chemicals
502 (i.e. cationic polymers) that may not manifest their toxic potential until the embryo has been

completely liberated from the protective outer shell, the chorion. In extending the static exposure to 96 hours, zebrafish development may encompass hatching [72] and cumulative mortality may be recorded as well.

Based on the results shown in Fig 1, the number of observed deceased *Danio rerio* for all control treatments and PE-MBS concentrations were in the following decreasing order: 2000 µg/L, 5% ethanol, 200 µg/L, 20 µg/L, 1% DMSO, 0.01% Tween 80, and reconstituted water for the negative control and internal plate control (S4 Appendix). The trend in Fig 1 shows that exposure to increasing concentrations of PE-MBS increased incidences of mortality as well. Upon statistical analysis, ANOVA (S9 Appendix) indicated that there is a significant difference between the means and variances of cumulative mortality of *Danio rerio* within the 96 hour exposure for all treatments.

Fig 1. Lethal effects of PE-MBS on *Danio rerio* embryos within 96 hour exposure to different concentrations.

Data shown is based on the average of three replicates performed in the study. Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of cumulative mortality between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

Based on the results found in Fig 2, coagulation accounted for the most frequently occurring lethal endpoint in all control solutions and PE-MBS concentrations, with mean percentages ranging from 38% to 80%. Lack of observable heartbeat was the second most recorded

526 toxicological, garnering values from 0% to 33% followed by non-detachment of tail with results
527 ranging from 0% to 29%. Lack of somite formation accounted for the least occurring endpoint in
528 all control solutions and PE-MBS concentrations, with percent values from 0% to 12%.

529

530 **Fig 2. Relative percentages of toxicological endpoints observed in deceased *Danio rerio* at 96**
531 **hpf.**

532 Percentage shown is based on the average of three replicates performed in the study.

533

534 Negative controls are important since they are used to detect confounding variables [73]
535 and serve as a basis of comparison for different test groups. Internal plate controls, on the other
536 hand, are used to identify any potential contamination found in 96-well plates that may affect the
537 outcome of the results [17]. Small discrepancies such as toxic endpoints observed in the negative
538 and internal plate control may be due to extraneous variables (i.e. varying oxygen levels in well-
539 plate, change of pressure of pipette tip into well, position of embryo in well). While extraneous
540 variables have a wide scope that include situational variables, participant variables, investigator
541 effects and demand characteristics; environmental factors, on the other hand, are more specific but
542 may still fall under extraneous variables [74]. Examples of environmental variables are noise,
543 temperature and lighting conditions of the experimental set-up.

544 In lieu of these toxic endpoints seen in the negative and internal plate controls, these results
545 also coincided with toxicological studies of the zebrafish [75, 76]. These studies observed low
546 zebrafish embryo mortality in their negative controls such as dilution water [75] and buffer and
547 egg water [76]. The latter stated that “spontaneous mortality” in the first 24 hpf may have been the
548 reason for the mortality observed in their controls, coinciding with other literature. This may also

549 explain the mortality of the control group in this study. Despite the mortality observed in these
550 controls, these results had no significant difference. Additionally, requirements by OECD
551 standards [17] stating that these controls should observe survival of at least 90% until the 96th
552 hour were still met in this study.

553

554 Upon analysis of data gathered in the study, mortality of *Danio rerio* treated with 5%
555 ethanol garnered results significantly different from the negative control all throughout the 96 hour
556 exposure (S10 Appendix). Coagulation, lack of somite formation, non-detachment of tail, and
557 lack of heartbeat were observed in deceased *Danio rerio*, with coagulation accounting for the most
558 frequently occurring toxic endpoint. Although the mechanism behind egg coagulation remains
559 unclear due to lack of related literature, coagulation induced by toxicant exposure is suspected to
560 be a result of *Danio rerio* having temporal expression or lack of specific metabolic enzymes that
561 may not allow it to metabolize harmful products during the entirety of the first 48 hours of
562 development [77]. Exposure to toxic products such as ethanol may lead to complete cell and
563 biomolecule disintegration as well as disruption to cell fate determination during organogenesis
564 [78], which is manifested by milky white egg coagulation in *Danio rerio* embryos (Fig. 3C).
565 Coagulation induced by 5% ethanol may be due to its toxic properties and ability to act as a
566 desiccant and protein denaturant at high concentrations [79]. Lack of visible heartbeat also
567 occurred and may possibly have been a result of its disruption of the central nervous system and
568 inhibition of acetylcholinesterase [80] that may have caused complications related to heart failure.
569 Data obtained from this study coincide with the study conducted by Hallare et al. [81].

570

571

572 **Fig 3. Toxicological endpoints observed in *Danio rerio*.**

573 (A) normal development of *Danio rerio* at 48 hpf observed in the negative control (RW), 0.01%
 574 Tween 80, 80% DMSO, and 20 µg/L PE-MBS. A. Embryo demonstrates eye bud (Eb), chorion
 575 (Ch), yolk (y), somites (s), and tail (t). 3 of the 4 toxicological endpoints denoting mortality: (B)
 576 lack of somite formation (*arrow*), (C) coagulation of eggs, and (D) non-detachment of tail (*arrow*)
 577 observed primarily in the positive control (5% ethanol), 200 µg/L PE-MBS, and 2000 µg/L PE-
 578 MBS.

579

580 Dunnet's test results revealed that there is no significant difference between the means and
 581 variances of mortality obtained for 0.01% Tween 80 and 1% DMSO with the negative control all
 582 throughout the 96 hour exposure (S10 Appendix). Manifestations of cardiac failure in embryos
 583 treated with 0.01% Tween 80 may be due to its low order toxicity [82] and capability to cause
 584 electrophysiologic changes to the cardiac conduction system [83] whereas visible heartbeat
 585 observed in *Danio rerio* treated with 1% DMSO may be due to its disruption of the central nervous
 586 system and inhibition of acetylcholinesterase [80]. Although both substances are embryotoxic and
 587 inducers of various developmental effects at high concentrations as evidenced in previous studies
 588 [82, 81], Tween 80 and DMSO were diluted to concentrations 0.01% and 1% respectively. Dilution
 589 of these substances were effective in making them appropriate surfactants and solvents for PE-MB
 590 [17] without causing embryotoxicity of remarkable difference with the negative control.

591 The results obtained for *Danio rerio* embryos treated with 20 µg/L PE-MBS did not show
 592 a significant difference with the negative control all throughout the 96 hour exposure (S10
 593 Appendix) which suggests that 20 µg/L PE-MBS is a concentration not sufficient enough to induce
 594 embryotoxic effects to *Danio rerio* embryos. However, Dunnet's test revealed significant

595 differences between the means of the cumulative number of deceased *Danio rerio* treated with 5%
596 ethanol, 200 µg/L PE-MBS, and 2000 µg/L PE-MBS with the negative control at all exposure
597 times within the 96 hour period (S10 Appendix).

598 Occurrences of egg coagulation (Fig. 3C) were most frequently observed during the first
599 48 hours of exposure for embryos treated with PE-MBS; thus, it is speculated that coagulation is
600 associated with a defect in the early embryonic stages of development (e.g., blastulation and
601 gastrulation). These developmental processes are highly conserved as few alterations may cause
602 lethal effects to the embryo [84]. Coagulation induced by PE-MBS is due to the toxic chemical
603 components of polyethylene. According to a study by Gallo et al. [31], polymers of microplastics,
604 even in extremely low concentrations, contain toxic chemical additives such as flame retardants,
605 plasticizers, UV stabilizers and pigments that are intentionally treated to the surfaces of virgin
606 polyethylene microplastics during the production process to reduce fire hazards and maintain
607 product integrity. Another study by Rochman et al. [3] stated that virgin pre-production polyethene
608 microplastics contain Endocrine-Disrupting Chemicals (EDCs) such as bisphenol A (BPA). Aside
609 from BPA being an exogenous compound that interferes with metabolic pathways and proper
610 functioning of the endocrine system [5, 85], accumulated evidence from past studies have
611 ascertained that BPA is cytotoxic, have the ability to alter gene integrity [85, 86] and induce cell
612 apoptosis and organ necrosis to developing vertebrates [87]. In this study, 1% DMSO was used as
613 a solubilizing agent to produce a suitable suspension for polyethylene microbeads [17]. Soft
614 extraction of polyethylene microbeads by DMSO may have caused leaching of toxic additives and
615 other EDCs that permeated through the chorion pores, caused cell disintegration and ultimately
616 led to incidences of milky white embryo coagulation (S2 Appendix).

617 Development of the heart begins at 16 hpf in which cardiac precursor cells start to
618 differentiate and travel towards the central midline of *Danio rerio* embryos [83]. Occurrences of
619 lack of observable heartbeat were observed in *Danio rerio* treated with PE-MBS during the
620 embryonic and larval stage (S4 Appendix). The number of deceased *Danio rerio* due to lack of
621 heartbeat increased upon increase in PE-MBS concentration (S2 Appendix). This may be attributed
622 to hypoxia caused by PE-MBS exposure during the earlier developmental stages of *Danio rerio*.
623 Accumulated evidence supports a study by Malafaia et al. [5] that polyethylene microbeads cause
624 hypoxia in *Danio rerio* embryos as these microplastics may adhere to the chorionic membrane
625 [88]. Since chorionic pores measure approximately less than 1 μm in diameter, polyethylene
626 microbeads that measure 300-355 μm most likely became a barrier that hindered the passage of
627 diffusing oxygen, and consequently interfered with gas exchange. Disruption in gas exchange
628 results in critically low oxygen availability that induces a reactive response in which certain
629 respiratory processes are accelerated [89] and cases of premature hatching may occur [5]. A
630 significant number of early hatching (i.e., hatching at the 48 hpf mark) in *Danio rerio* treated with
631 200 and 2000 $\mu\text{g/L}$ PE-MBS were observed (S5 Appendix) and may be suspected to be due to the
632 breakdown of the chorion as a means to increase oxygen uptake in *Danio rerio* [5]. Despite greater
633 oxygen uptake, premature hatching produces underdeveloped larvae with teratogenic
634 abnormalities that synergistically contribute to post-hatching mortality [5]. In a study by Kuiper et
635 al. [90], they also found that exposure of *Danio rerio* to plastic additives such as flame retardants
636 found in microplastics contain toxic chemicals that cause high post-hatching mortality and
637 pericardial fluid accumulation in juvenile larvae evidenced by manifestations of pericardial edema
638 (S7 Appendix), both in which coincide with the results obtained in the conducted study.

639 The first sign of somite differentiation occurs after gastrulation [84]. It is from these
640 somites that muscle cells are derived from. It is also during somitogenesis when the tail begins to
641 extend and separate itself from the yolk. Alterations in somitogenesis due to substance toxicity
642 affect normal development and may cause a defect in somite formation and tail detachment [84].
643 Incidences of embryos exhibiting lack of somite formation and non-detachment of tail were
644 observed in *Danio rerio* treated with PE-MBS (S2 Appendix) and may be attributed to chemical
645 additives and EDCs added to pre-production polyethene microplastics [85]. Related literature
646 outside of this study suggests that observed toxic endpoints related to somite defect may be
647 associated with ectodermal implications during somitogenesis [91]. Somite formation is initiated
648 by the motion waves of gene expression that originate from the head [92], and since EDCs alter
649 gene integrity, they may have affected normal development as well. However, further studies must
650 be conducted to investigate to know the specific genes and the level of gene expression EDCs pose
651 an effect on.

652

653

654 The concentration-mortality curve of *Danio rerio* at 96 hpf as shown in Fig 4 indicates that
655 there is an increasing trend in mortality rate as the concentration of PE-MBS increases. Results
656 from Tukey Kramer's *post hoc* test (S11 Appendix) revealed that there is a significant difference
657 between the means of the cumulative number of deceased *Danio rerio* treated with different
658 concentrations of PE-MBS at 96 hpf which substantiates early speculations that PE-MB toxicity
659 is dose dependent and causes concentration-dependent reduction in *Danio rerio* survival.

660

661

662 **Fig 4. Concentration-Mortality curve in FET of *Danio rerio* treated with PE-MBS at 96**
663 **hpf.**

664 Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of
665 cumulative mortality between *Danio rerio* at 96 hpf ($p < 0.05$). (*: $p < 0.05$).

666

667

668 In Fig 5, the computation of LC_{50} using probit analysis, while taking into consideration
669 results obtained from the negative control, revealed that the lethal concentration of polyethylene
670 microbeads causing mortality to 50% of the population under study is 2455.096 $\mu\text{g/L}$ (S12
671 Appendix). The value garnered for the LC_{50} of PE-MB is higher than treatment concentrations
672 used in the study (i.e., 20, 200, and 2000 $\mu\text{g/L}$ PE-MBS).

673

674 **Fig 5. Probit analysis for the estimation of LC_{50} values of *Danio rerio* exposed to PE-MBS.**

675 Analyzed results showed that the LC_{50} is 2455.096 $\mu\text{g/L}$ with 95% confidence limits. Error bars
676 indicate standard error.

677

678

679 **Hatching**

680 Hatching is a critical stage in the embryogenesis of *Danio rerio* for it aids in the evaluation
681 of developmental delays and toxicity caused by different substances [93]. A normally developing
682 *Danio rerio* typically hatches between 48 to 72 hpf [94]. *Danio rerio* embryos hatched in the 96
683 hpf mark are considered late hatchers whereas those hatched in 48 hpf are considered early
684 hatchers [95].

Based on the results shown in Fig 6, the majority of *Danio rerio* embryos hatched at 72 hpf. Embryos treated with 5% ethanol showed the highest number of hatching at 96 hpf. The trend in Fig 6 shows an increasing number of embryos hatching at 48 hpf upon increase in PE-MBS concentration. With these results, administration of statistical analysis of ANOVA indicated that there is a significant difference between the means and variances of the number of hatched *Danio rerio* within the 96 hour exposure to all treatments (S13 Appendix).

Fig 6. Cumulative number of hatched *Danio rerio* within 96 hour exposure to different treatments.

Data shown is based on the average of three replicates performed in the study. Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of cumulative hatching between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

All throughout the 96 hour exposure, results from Dunnet's test indicated that no significant difference in the means of the cumulative number of hatched individuals in *Danio rerio* treated with 0.01% Tween 80 and 1% DMSO with the negative control was present (S14 Appendix). Although these substances cause teratogenic and embryotoxic effects in high doses [96, 97], they were diluted in accordance to OECD guidelines to induce effects of negligible difference with the negative control while serving as appropriate solvents for the toxicant under study [17].

At 48 hpf, Dunnet's test revealed that no significant differences were noted in the means of the number of hatched *Danio rerio* treated with 20µg/L PE-MBS and 5% ethanol with the

negative control but there is a notable difference for the results garnered for *Danio rerio* treated with 200 and 2000 µg/L PE-MBS with the negative control. This indicates that high concentrations of PE-MBS induces early hatching in *Danio rerio* embryos. This physiological phenomenon may be a result of hypoxia caused by PE-MB. All vertebrates rely on diffusion for both gas exchange and respiratory gas transport, especially in the early stages of development [89]. Microbeads used in the study measured 300-355 µm in size whereas the diameter of the chorionic pores in *Danio rerio* measures less than 1 µm [88]. Possible adherence of PE-MB in the chorionic membrane of *Danio rerio* during embryogenesis may have resulted in clogged pores, hindered gas exchange and consequently, insufficient oxygen supply. As stated in a study by Burrgrgren and Pinder [89], 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 lack of oxygen [98]. These stress-induced responses as a result of hypoxic environment is also accompanied by premature hatchings since removing the resistance of the chorionic membrane is known to increase oxygen uptake in *Danio rerio* embryos [89]. Notably, recorded data revealed that despite hatching earlier than other treatment groups, *Danio rerio* treated with 200 and 2000 µg/L PE-MBS had lower survival rates after hatching (S4 Appendix). This reinforces the hypothesis that exposure to high doses of PE-MB is both teratogenic and embryotoxic.

For both 72 and 96 hpf, the means of the cumulative number of hatched individuals treated with 20 µg/L PE-MBS did not have a significant difference with the negative control, supporting early speculations that 20 µg/L PE-MBS is not sufficient enough to induce developmental delays nor premature hatching. However, Dunnet's test results for 5% ethanol garnered a significant difference since a number of embryos died before hatching. The same is true for *Danio rerio* treated with 200 and 2000 µg/L PE-MBS since polyethylene microplastics have the ability to

731 induce embryotoxic effects. Remarkably, a number of late hatchers were noted in *Danio rerio*
732 treated with 5% ethanol as ethanol is known to cause harmful complications and slow down certain
733 processes such as hatching and heart rate [97].

734 Results from Tukey-Kramer's test for the cumulative number of hatched individuals within
735 the 96 hour exposure revealed that all three concentrations of PE-MBS are significantly different
736 from each other thus indicating that the rate of premature hatching in *Danio rerio* is dose-
737 dependent and steadily increases depending on the dose administered (S15 Appendix).

738

739 **Teratogenicity**

740 Teratogenic endpoints are important to determine the teratogenic potential of a chemical
741 [99] and to generalize the response of *Danio rerio* towards this toxicant [81] of varying
742 concentrations. The most common malformations were edema, bent tail, bent body axis, and
743 collapsed swim bladder. Edema is defined by the accumulation of pellucid fluid in the pericardium
744 or in the yolk sac. A bent tail is observed in an abnormal, dorsoventral or lateral flexion of the tail
745 at the axial level of the caudal fin. A bent body axis is observed in an abnormal flexion of the
746 primary axis. Lastly, a collapsed swim bladder may be more unexpanded than the normal
747 phenotype of a *Danio rerio* swim bladder [96].

748

749

750

751 As shown in Fig 7, the average number of malformations observed in all treatments and
752 controls were in the following decreasing order: 2000 µg/L PE-MBS, positive control (5%
753 ethanol), 200 µg/L PE-MBS, 20 µg/L PE-MBS whereas 1% DMSO, and 0.01% Tween 80 garnered

the same value (S6 Appendix). The embryos in the negative control and the internal plate control did not show any malformations. With these results, administration of statistical analysis of the Kruskal-Wallis Test indicated that there is a significant difference between the means and variances of the number of malformations observed in *Danio rerio* within the 96 hour exposure to all treatments (S16 Appendix). The trend showed that increasing PE-MBS concentration resulted in an increased number of malformations as well.

Fig 7. Total number of malformations observed in *Danio rerio* for each treatment at 144 hpf.

Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of total number of malformations between *Danio rerio* at 144 hpf ($p < 0.05$). (*: $p < 0.05$).

As shown in Fig 8, edema had the highest number of incidents for each group, garnering a range of percent values from 62% to 100%. Bent body axis at 8% to 33% and bent tail with percent values of 21% to 24% came next while the collapsed swim bladder was the least observed teratogenic endpoint for all groups, garnering percent values from 3% to 13%.

Fig 8. Relative percentages of malformations observed in *Danio rerio* for each treatment at 144 hpf. Percentage shown is based on the average of three replicates performed in the study.

778 According to Ali et al. [96], one of the abnormalities found in *Danio rerio* subjected to
779 8000 mg/L ethanol was pericardial edema. Another study [100] found that even at lesser
780 concentrations of ethanol (1.5% and 2%), abnormalities such as bent body axis were observed in
781 *Danio rerio* embryos. That being said, all literature coincided with the observations in embryos
782 treated with 5% ethanol in this study (Fig. 9B, 10C, 11A, 11B). Dunn's Test (S17 Appendix) had
783 also indicated that the positive control, the 5% ethanol significantly differed with the negative
784 control.

785

786 Ali et al. [96] stated that *Danio rerio* embryos subjected to 200 mg/L Tween 80 exhibited
787 dispersed pigment cells, bent body axis, and branchial arch hypoplasia. However, since Tween 80
788 was diluted to lesser concentrations in this study, the mean observation found in *Danio rerio*
789 embryos subjected to the resulting concentration was too negligible to significantly differ with the
790 negative control (Fig. 9A, 10B, 11A). Meanwhile, DMSO was reported as a teratogen at higher
791 concentrations [101]. However, at lesser concentrations likened to 1%, embryos treated with 1%
792 DMSO did not exhibit significant teratogenicity (Fig. 9A, 10B, 11A). This finding also coincided
793 with other studies [101, 81]. Statistically, 0.01% Tween 80 and 1% DMSO both did not
794 significantly differ with the negative control as well (S17 Appendix).

795 Two hundred (200) and 2000 µg/L PE-MBS treatment groups significantly differed with
796 the negative control (S17 Appendix); however, concentration of 20 µg/L PE-MBS treatment was
797 too negligible to significantly differ with the negative control. With that said, polyethylene
798 microbeads may affect the body axis, body proportion and other morphological parameters of
799 aquatic organisms depending on PE-MBS concentration. Their sizes [102] may also be a factor
800 associated with malformation.

801 The Tukey Kramer Test indicated significant differences between all PE-MBS treatment
802 groups. Results also showed that the number of deformities increased upon increase of PE-MBS
803 concentration. This may be interpreted that higher concentration of PE-MBS induces greater
804 teratogenicity in *Danio rerio*. In spite of early speculation, polyethylene microbeads may have
805 caused disturbance to regulating barriers in internal water diffusion [5], possibly substantiating
806 increasing incidents of edema in increasing concentrations. Edema being the highest number of
807 type of malformation in all PE-MBS treatment groups, may be regarded as a symptom of hypoxia
808 in *Danio rerio* embryos, further substantiating that PE-MB may cause hypoxia [5]. It has also been
809 observed that sublethal stages of hypoxia can increase embryonic fish malformations by 77.4%
810 ultimately resulting in decline of species' fitness and aquatic populations [103].

811 Bent tails have also been reported in microplastic-treated *Danio rerio* adults at moderate
812 and high concentrations [104], coinciding with another article as well [105]. In the former study,
813 bent tails observed in polyethylene microplastic treated *Danio rerio* may be associated with
814 “knockdown of the cysteine-rich motor neuron 1 gene (*crim1*) or missense mutation in polycystin-
815 2(*pkd2*).” This gene encodes for the activation of the Ca^{2+} cation channel which is important in the
816 skeletal muscle excitation-contraction [106] that may be depicted in the tail movement of the
817 zebrafish. However, Kaleuff [107] recommends further investigation regarding whether exposure
818 to microplastics significantly changes the level of target gene expression and phenotype. Bent body
819 axes were also observed in polyethylene microplastic treated *Danio rerio* embryos [5]. Further
820 observation and findings are needed to associate these teratogenic effects to adhesion of
821 polyethylene microbeads to the external surface and to the gastrointestinal system of the *Danio*
822 *rerio* embryo; however a study of Malafaia et al. [5] associates these teratogenic effects to this
823 occurrence.

824 Collapsed swim bladders have also been evident in the 200 µg/L PE-MBS treatment but
825 more especially in the 2000 µg/L PE-MBS treatment (Fig. 9B). The swim bladder, an aid in making
826 upward hydrodynamic forces in prevention from sinking [108], was exhibited to be collapsed in
827 *Danio rerio*, and this may have resulted from hypoxia [107] possibly induced by 200 µg/L and
828 2000 µg/L PE-MBS. Collapsed swim bladders have also been observed in *Danio rerio* embryos
829 affected by nano plastics [11].

830 With substantiating the results from the Tukey Kramer Test indicating significant
831 differences between all PE-MBS treatment groups (S19 Appendix), it can be said that
832 teratogenicity increases with increasing PE-MBS concentration.

833

834 **Fig 9. Fresh mount of *Danio rerio* at 144 hpf showing collapse of the swim bladder. (A)**

835 shows normal development of the swim bladder (swb) observed in the negative control (RW),
836 0.01% Tween 80 % DMSO, and 20 µg/L PE-MBS. (B) shows collapsed swim bladder (cswb)
837 observed in the following treatments of increasing order: the positive control (5% ethanol), 200
838 µg/L PE-MBS, and 2000 µg/L PE-MBS.

839

840 **Fig 10. Fresh mount of *Danio rerio* at 144 hpf with different severities of yolk sac and**

841 **pericardial edema.**

842 (A) shows the normal development observed in the negative control (RW) with normal yolk sac
843 (ys) and pericardium (p). (B) exhibits mild yolk sac edema (myse) observed in treatments 0.01%
844 Tween 80 and 1% DMSO and mild pericardial edema (mpe). (C) shows severe yolk sac (syce)
845 and pericardial edema (spe) both observed in 200µg/L PE-MBS, 2000µg/L PE-MBS, and 5%

ethanol with the last two respective treatment and control groups exhibiting the most incidents of edema.

Fig 11. Fresh mount of *Danio rerio* at 144 hpf showing bent body axis and tail curvature.

(A) exhibits bent body axis (bba). (B) exhibits tail curvature (tc). Both malformations were observed in the positive control (5% ethanol) and in treatments, 200 and 2000 µg/L PE-MBS. Bent body axis was observed in the 20 µg/L PE-MBS.

Heartbeat

The heart rate of a developing zebrafish embryo is usually at 120-180 beats per minute (bpm) and it is usually visible at 48 hpf. It is a significant endpoint that should be observed to ensure tissue perfusion in all parts of the developing embryo [109].

As shown in Fig 12, the general trend was that the fastest bpm was always evident in the 96 hpf while the slowest bpm was observed in the 48 hpf for each treatment and control group. This is because the heart rate increases as development takes place [109]. As shown in the Fig 12, embryos in PE-MBS treatments steadily increased heart rate as the PE-MBS concentration increased. As for the control groups, each group showed steady increase of heart rate in exception to the positive control. Embryos in the positive control showed a slower rate of heartbeat as each succeeding 24th hour went by.

869 **S12 Fig. Heart rate (bpm) observed in *Danio rerio* for each treatment.**

870 Data shown is based on the average of three replicates performed in the study. Error bars indicate
871 standard error. Single-asterisk indicates a statistically significant difference of heart rate between
872 *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

873

874

875 But among all groups, the fastest average bpm was evident in embryos treated with 2000
876 $\mu\text{g/L}$ PE-MBS at 96 hpf (i.e., 203 bpm) while the slowest average bpm was observed in the positive
877 control at 96 hpf (i.e., 137 bpm).

878 With these results, statistical analyses of ANOVA indicated significant differences
879 between the means and the variances of each treatment (S20 Appendix). These results led to *post*
880 *hoc* analysis, namely the Dunnett's Test (S21 Appendix) and the Tukey Kramer Test (S22
881 Appendix). At all hpf, means of all treatments had significant differences with those in the negative
882 control in exception to ones in 0.01% Tween 80, 1% DMSO and 20 $\mu\text{g/L}$ PE-MBS. Meanwhile,
883 the latter test indicated significant differences between all PE-MBS treatments.

884 In a normal embryonic development of *Danio rerio*, the heart rate increases as development
885 takes place [109] as earlier mentioned. Increased heart rate had been observed in all treatments in
886 exception to 5% ethanol. Ethanol may cause decrease in size of ventricles and lessen the number
887 of cardiomyocytes in the heart of a developing zebrafish [110], leading to mortality and effects of
888 teratogenicity. Hallare et al. [81] also stated that ethanol greater than 1.5% concentration caused
889 developmental delays in heart beating. This finding also coincided with other studies [80,111].
890 Hence, 5% ethanol had a significant difference with the negative control for all hpf.

891 For other chemicals, means of heart rate in 0.01% Tween 80 and in 1% DMSO did not
892 significantly differ with the negative control. Tween 80 is known to have a relatively high toxicity
893 towards *Danio rerio*, even more than its toxicity to rodents because of its surfactant properties
894 [76]. But in comparison to this study, Tween 80 was diluted to a lesser concentration in accordance
895 with OECD guidelines [17] hence its concentration was found to be too negligible to cause a
896 significant difference with the negative control. High concentrations of DMSO ($\geq 1.5\%$ v/v)
897 were observed to induce brachycardia and pronounced arrhythmia; but
898 at lower concentrations, initial increase of average heart rate was
899 observed instead [81].

900 20 µg/L PE-MBS did not significantly differ with those in the negative control. This may
901 be interpreted that 20 µg/L PE-MBS was an insufficient dose that could not induce an irregularly
902 increased bpm in comparison to the bpm observed in the negative control; however, the other
903 higher PE-MBS concentrations induced a significantly increased heart rate in comparison to the
904 negative control that also exhibited an increase of bpm but at a steady rate. Results may be
905 interpreted that the higher the PE-MBS concentration, the more likely *Danio rerio* will be
906 subjected to cardiac toxicity. It is said that exposure to polyethylene causes cardiac toxicity, a term
907 defined by a greatly increased heart rate that may be attributed to physiological stress [112].

908 This significant increase of heart rate may also be associated with acute hypoxia
909 contributed by adherence of polyethylene microbeads to chorionic membranes as earlier stated.
910 One of the symptoms of hypoxia may be irregular, rapid heartbeat [113]. The study of Crail [112]
911 also showed significant increase of heart rate at delimited oxygen concentration. That being said,
912 means observed in 200 µg/L PE-MBS at 72 and 96 hpf and means observed in 200 µg/L PE-MBS
913 at 96 hpf have exceeded the regular heart rate of a *Danio rerio* embryo. This further substantiates

914 the occurrence of irregular, rapid heart rate in the zebrafish embryo at increasing concentrations
915 and at longer exposure to PE-MBS.

916 Tukey Kramer *post hoc* test (S22 Appendix) revealed that the heart rate of *Danio rerio*
917 treated with different concentrations of PE-MBS significantly differed from each other at all hpf.
918 Heart rate of zebrafish embryos increased and became more irregular upon exposure to increasing
919 concentrations of PE-MBS, implying that cardiac toxicity due to PE-MBS may be dose dependent.

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921

922 **Conclusions and recommendations**

923 The inclusion of polyethylene microbeads in personal care products such as facial washes
924 and cosmetics has captured the attention of the scientific community due to the deleterious effects
925 it poses on aquatic organisms. In this study, *Danio rerio* was chosen to be the representative model
926 due to its availability, high fecundity and great similarity with the human genome. Polyethylene
927 microbeads used in the study were based on measurements similar to actual commercial facial
928 cleansers that contained polyethylene. Results from the Fish Embryo Acute Toxicity Test revealed
929 that 20 µg/L did not have significant difference with the negative control in the observed
930 parameters (i.e., embryotoxicity, teratogenicity, hatching, and heartbeat), but 200 and 2000 µg/L
931 did, ascertaining that static exposure to high concentrations of polyethylene microbeads is
932 embryotoxic and teratogenic to *Danio rerio* embryos. Cases of mortality may be due to the soft
933 extraction of polyethylene microbeads using 1% DMSO that may have induced leaching of toxic
934 additives and Endocrine-Disrupting Chemicals (EDCs). In accordance with literature outside of
935 this study, these leached chemicals may have disrupted metabolic pathways [5, 85], alter gene
936 integrity [85, 86], and cause cell apoptosis [87]; hence possibly resulting in *Danio rerio* toxicity

937 during embryogenesis; however, this needs a more thorough study at gene level. Adherence of
938 polyethylene microbeads to the chorionic membrane may also have disrupted gas exchange and
939 induced hypoxia [5]. Hypoxia may have been the underlying cause of observed premature hatching
940 in *Danio rerio* which, in effect, increased occurrences of larval death and incidences of teratogenic
941 abnormalities such as edema, collapsed swim bladder, and bent body axes. Rapid and irregular
942 heart rate was also observed among *Danio rerio* embryos and may be associated with acute
943 hypoxia and cardiac toxicity caused by polyethylene microbead exposure.

944 The result obtained from the computation for the LC₅₀ is 2455.096 µg/L and is higher than
945 the treatment concentrations used in the study. Nonetheless, strong and urgent actions against the
946 production of facial cleansers containing PE-MB must be implemented to reduce the microplastic
947 pollution in bodies of water. Furthermore, investments and policy reforms on improving plastic
948 wastes management must also be enacted to minimize microplastic leaching into the aquatic
949 ecosystem from wastewater treatment plants. Through these concerted efforts, issues of
950 bioaccumulation and toxicity by microplastic (e.g., microbeads) pollutants may be mitigated or
951 prevented and consequently save the lives of both aquatic organisms and humans alike.

952 Since this study was only limited to polyethylene, different types of microbeads such as
953 polypropylene and polyamide and different sizes ranging less than 300-355 µm may be included
954 to broaden the study. It is also recommended that zebrafish exposed to microbeads may be further
955 observed at the gene level to investigate the possible occurrence of mutations and other alterations
956 such as hsp70, crim1, and pkd2 [105] that contribute to embryotoxicity and teratogenicity of the
957 zebrafish. New biomarkers can also be searched further that can be used to monitor the health of
958 aquatic habitat and its biota.

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960

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1321 **Figure Captions**

1322 **Fig 1. Lethal effects of PE-MBS on *Danio rerio* embryos within 96 hour exposure to**
1323 **different concentrations.**

1324 Data shown is based on the average of three replicates performed in the study. Error bars indicate
1325 standard error. Single-asterisk indicates a statistically significant difference of cumulative
1326 mortality between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

1327 **Fig 2. Relative percentages of toxicological endpoints observed in deceased *Danio rerio* at**
1328 **96 hpf.**

1329 Percentage shown is based on the average of three replicates performed in the study.

1330 **Fig 3. Toxicological endpoints observed in *Danio rerio*.**

1331 (A) normal development of *Danio rerio* at 48 hpf observed in the negative control (RW), 0.01%
1332 Tween 80, 80% DMSO, and 20 $\mu\text{g/L}$ PE-MBS. A. Embryo demonstrates eye bud (Eb), chorion
1333 (Ch), yolk (y), somites (s), and tail (t). 3 of the 4 toxicological endpoints denoting mortality: (B)
1334 lack of somite formation (*arrow*), (C) coagulation of eggs, and (D) non-detachment of tail
1335 (*arrow*) observed primarily in the positive control (5% ethanol), 200 $\mu\text{g/L}$ PE-MBS, and 2000
1336 $\mu\text{g/L}$ PE-MBS.

1337 **Fig 4. Concentration-Mortality curve in FET of *Danio rerio* treated with PE-MBS at 96**
1338 **hpf.**

1339 Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of
1340 cumulative mortality between *Danio rerio* at 96 hpf ($p < 0.05$). (*: $p < 0.05$).

1341 **Fig 5. Probit analysis for the estimation of LC₅₀ values of *Danio rerio* exposed to PE-MBS.**

1342 Analyzed results showed that the LC₅₀ is 2455.096 µg/L with 95% confidence limits. Error bars
1343 indicate standard error.

1344 **Fig 6. Cumulative number of hatched *Danio rerio* within 96 hour exposure to different
1345 treatments.**

1346 Data shown is based on the average of three replicates performed in the study. Error bars indicate
1347 standard error. Single-asterisk indicates a statistically significant difference of cumulative
1348 hatching between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

1349 **Fig 7. Total number of malformations observed in *Danio rerio* for each treatment at 144
1350 hpf.**

1351 Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of
1352 total number of malformations between *Danio rerio* at 144 hpf ($p < 0.05$). (*: $p < 0.05$).

1353 **Fig 8. Relative percentages of malformations observed in *Danio rerio* for each treatment at
1354 144 hpf.**

1355 Percentage shown is based on the average of three replicates performed in the study.

1356 **Fig 9. Fresh mount of *Danio rerio* at 144 hpf showing collapse of the swim bladder. (A)**

1357 shows normal development of the swim bladder (swb) observed in the negative control (RW),

1358 0.01% Tween 80 % DMSO, and 20 µg/L PE-MBS. (B) shows collapsed swim bladder (cswb)

1359 observed in the following treatments of increasing order: the positive control (5% ethanol), 200

1360 µg/L PE-MBS, and 2000 µg/L PE-MBS.

1361 **Fig 10. Fresh mount of *Danio rerio* at 144 hpf with different severities of yolk sac and**
1362 **pericardial edema.**

1363 (A) shows the normal development observed in the negative control (RW) with normal yolk sac
1364 (ys) and pericardium (p). (B) exhibits mild yolk sac edema (myse) observed in treatments 0.01%
1365 Tween 80 and 1% DMSO and mild pericardial edema (mpe). (C) shows severe yolk sac (syce)
1366 and pericardial edema (spe) both observed in 200µg/L PE-MBS, 2000µg/L PE-MBS, and 5%
1367 ethanol with the last two respective treatment and control groups exhibiting the most incidents of
1368 edema.

1369 **Fig 11. Fresh mount of *Danio rerio* at 144 hpf showing bent body axis and tail curvature.**

1370 (A) exhibits bent body axis (bba). (B) exhibits tail curvature (tc). Both malformations were
1371 observed in the positive control (5% ethanol) and in treatments, 200 and 2000 µg/L PE-MBS.
1372 Bent body axis was observed in the 20 µg/L PE-MBS.

1373 **S12 Fig. Heart rate (bpm) observed in *Danio rerio* for each treatment.**

1374 Data shown is based on the average of three replicates performed in the study. Error bars indicate
1375 standard error. Single-asterisk indicates a statistically significant difference of heart rate between
1376 *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

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1380 **Supporting Information**

1381 **S1 Table. Experimental setup for the Fish Embryo Acute Toxicity Test.**

1382 **S1 Appendix. Number of fertilized and unfertilized eggs collected during spawning.**

- 1383 **S2 Appendix. Toxicological endpoints observed in *Danio rerio* for each trial within the 96**
1384 **hour exposure.**
- 1385 **S3 Appendix. Cumulative number of deceased *Danio rerio* for each trial within the 96 hour**
1386 **exposure.**
- 1387 **S4 Appendix. Cumulative number of deceased *Danio rerio* embryo and larvae for each trial**
1388 **at the end of the-96 hour exposure.**
- 1389 **S5 Appendix. Cumulative number of hatched *Danio rerio* for each trial within the 96 hour**
1390 **exposure.**
- 1391 **S6 Appendix. Average number of malformations observed in *Danio rerio* embryos for each**
1392 **trial at 144 hpf.**
- 1393 **S7 Appendix. Average number of each kind of malformation observed in *Danio rerio* for**
1394 **each trial at 144 hpf.**
- 1395 **S8 Appendix. Average bpm of *Danio rerio* embryos exposed for each trial within the 96**
1396 **hour exposure.**
- 1397 **S9 Appendix. Single factor analysis of variance for the effect of different treatments to the**
1398 **mortality of *Danio rerio* within the 96 hour exposure .**
- 1399 **S10 Appendix. Dunnet's test for cumulative mortality of *Danio rerio* exposed to different**
1400 **treatments within the 96 hour exposure with 95% confidence intervals.**
- 1401 **S11 Appendix. Tukey HSD/Kramer test for cumulative mortality of *Danio rerio* treated**
1402 **with varying concentrations of PE-MBS within the 96 hour exposure.**
- 1403 **S12 Appendix. Calculation of LC₅₀ of PE-MB using probit analysis with 95% confidence**
1404 **limits**

- 1405 **S13 Appendix. Single factor analysis of variance for the effect of different treatments to the**
1406 **cumulative hatching of *Danio rerio* within the 96 hour exposure.**
- 1407 **S14 Appendix. Dunnet's test for cumulative hatching of *Danio rerio* exposed to different**
1408 **treatments within the 96 hour exposure with 95% confidence intervals.**
- 1409 **S15 Appendix. Tukey HSD/Kramer test for cumulative hatching of *Danio rerio* treated**
1410 **with varying concentrations of PE-MBS within the 96 hour exposure.**
- 1411 **S16 Appendix. Kruskal-Wallis Test for the malformations observed in *Danio rerio* exposed**
1412 **to different treatments at 144 hpf.**
- 1413 **S17 Appendix. Dunn's test for malformations observed in *Danio rerio* embryos at 144 hpf**
1414 **to different treatments ($p < 0.05$).**
- 1415 **S18 Appendix. Single factor analysis of variance for the effect of varying concentrations of**
1416 **PE-MBS to the number of malformations observed in *Danio rerio* at 144 hpf.**
- 1417 **S19 Appendix. Tukey HSD/Kramer test for malformations of *Danio rerio* treated with**
1418 **varying concentrations of PE-MBS at 144 hpf.**
- 1419 **S20 Appendix. Single factor analysis of variance for the effect of different treatments to the**
1420 **heart rate (bpm) of *Danio rerio* within the 96 hour exposure.**
- 1421 **S21 Appendix. Dunnet's test for the heart rate (bpm) in *Danio rerio* exposed to different**
1422 **treatments within the 96 hour exposure with 95% confidence intervals.**
- 1423 **S22 Appendix. Tukey HSD/Kramer test for the heart rate (bpm) of *Danio rerio* treated**
1424 **with varying concentrations of PE-MBS within the 96 hour exposure.**
- 1425 **S23 Appendix. Institutional Animal Care and Use Committee Letter of Approval.**

Fig 1. Lethal effects of PE-MBS on *Danio rerio* embryos within 96 hour exposure to different concentrations.

Data shown is based on the average of three replicates performed in the study. Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of cumulative mortality between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

Fig 2. Relative percentages of toxicological endpoints observed in deceased *Danio rerio* at 96 hpf.

Percentage shown is based on the average of three replicates performed in the study.

Fig 3. Toxicological endpoints observed in *Danio rerio*.

(A) normal development of *Danio rerio* at 48 hpf observed in the negative control (RW), 0.01% Tween 80, 80% DMSO, and 20 μg/L PE-MBS. A. Embryo demonstrates eye bud (Eb), chorion (Ch), yolk (y), somites (s), and tail (t). 3 of the 4 toxicological endpoints denoting mortality: (B) lack of somite formation (*arrow*), (C) coagulation of eggs, and (D) non-detachment of tail (*arrow*) observed primarily in the positive control (5% ethanol), 200 μg/L PE-MBS, and 2000 μg/L PE-MBS.

Fig 4. Concentration-Mortality curve in FET of *Danio rerio* treated with PE-MBS at 96 hpf.

Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of cumulative mortality between *Danio rerio* at 96 hpf ($p < 0.05$). (*: $p < 0.05$).

Fig 5. Probit analysis for the estimation of LC_{50} values of *Danio rerio* exposed to PE-MBS. Analyzed results showed that the LC_{50} is 2455.096 $\mu\text{g/L}$ with 95% confidence limits. Error bars indicate standard error.

Fig 6. Cumulative number of hatched *Danio rerio* within 96 hour exposure to different treatments.

Data shown is based on the average of three replicates performed in the study. Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of cumulative hatching between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).

Fig 7. Total number of malformations observed in *Danio rerio* for each treatment at 144 hpf.

Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of total number of malformations between *Danio rerio* at 144 hpf ($p < 0.05$). (*: $p < 0.05$).

Fig 8. Relative percentages of malformations observed in *Danio rerio* for each treatment at 144 hpf. Percentage shown is based on the average of three replicates performed in the study.

Fig 9. Fresh mount of *Danio rerio* at 144 hpf showing collapse of the swim bladder. (A) shows normal development of the swim bladder (swb) observed in the negative control (RW), 0.01% Tween 80 % DMSO, and 20 µg/L PE-MBS. (B) shows collapsed swim bladder (cswb) observed in the following treatments of increasing order: the positive control (5% ethanol), 200 µg/L PE-MBS, and 2000 µg/L PE-MBS.

Fig 10. Fresh mount of *Danio rerio* at 144 hpf with different severities of yolk sac and pericardial edema.

(A) shows the normal development observed in the negative control (RW) with normal yolk sac (ys) and pericardium (p). (B) exhibits mild yolk sac edema (myse) observed in treatments 0.01% Tween 80 and 1% DMSO and mild pericardial edema (mpe). (C) shows severe yolk sac (syse) and pericardial edema (spe) both observed in 200µg/L PE-MBS, 2000µg/L PE-MBS, and 5% ethanol with the last two respective treatment and control groups exhibiting the most incidents of edema.

Fig 11. Fresh mount of *Danio rerio* at 144 hpf showing bent body axis and tail curvature. (A) exhibits bent body axis (bba). (B) exhibits tail curvature (tc). Both malformations were observed in the positive control (5% ethanol) and in treatments, 200 and 2000 μg/L PE-MBS. Bent body axis was observed in the 20 μg/L PE-MBS

S12 Fig. Heart rate (bpm) observed in *Danio rerio* for each treatment.

Data shown is based on the average of three replicates performed in the study. Error bars indicate standard error. Single-asterisk indicates a statistically significant difference of heart rate between *Danio rerio* ($p < 0.05$). (*: $p < 0.05$).