Poll Res. 40 (2) : 379-390 (2021)

ISSN 0257–8050

ENVIRONMENTAL BIOMONITORING OF THE AQUATIC BODIES IN METRO MANILA, PHILIPPINES WITH RESPECT TO THE GENOTOXICITY POTENTIAL USING *IN VIVO* MOUSE BONE MARROW MICRONUCLEI TEST AND HEAVY METAL ANALYSIS

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(Received 12 October, 2020; accepted 13 November, 2020)

ABSTRACT

In the present study, *in vivo* mouse bone marrow micronucleus assay in combination with heavy metal analysis was used to compare the water quality of water channels namely- *estero de vitas* and *estero de paco*, which are part of the Pasig River System, Metro Manila with respect to their genotoxicity potential. As part of the strategy, the *esteros* are being rehabilitated to control pollution in the river systems whereby *estero de paco* was recently rehabilitated whereas *Estero de Vitas* is still largely neglected. There has been a lot of debate regarding the strategy adopted by the stakeholders to clean the esteros to revive the biologically dead pasgi river. This study aimed to find if there was any difference in the water quality brought about in the restored *estero de paco* as compared to the other *eseros* which are still not rehabiliated. The elevated levels of micronuclei observed in the erythrocytes of the genetic model, the *Swiss albino* mice exposed to water samples from both sources indicate the presence of genotoxic and hazardous pollutants in the water bodies of *estero de paco* and *estero de vitas*. Further, the water samples from *estero de vitas* was found to be far more genotoxic as compared to the water samples from *estero de paco* ($p<0.05$). The presence of micronuclei (genotoxicity) could be related to the presence of heavy metals in the water samples as well as the tissues of the mice as detected by the Atomic absorption spectroscopy (AAS). The results also suggest an improvement in the physico-chemical parameters such as the biochemical oxygen demand (B.O.D.), dissolved oxygen (D.O), and pH of the water samples from *estero de paco* as compared to *estero de vitas*. This study established that the restoration of the *esteros* can be an effective approach to control pollution of the Pasig river system hence mouse micronucleus test in combination with physico-chemical testing along with heavy metal analysis could be used effectively for regular environmental biomonitoring to control the pollution levels of water bodies flowing into the Pasig river system.

KEY WORDS : Genotoxicity, Mouse bonemarrow, Micronuclei, Heavy metals, *Swiss albino* mouse

INTRODUCTION

Pasig river system with its 47 tributaries or estuaries flowing through Metro Manila, Philippines was declared biologically dead and efforts have been going on to rehabilitate the river and improve its water quality (Asian Development Bank, 2012). Among many other initiatives, the ABS-CBN Foundation and the Pasig River Rehabilitation Commission launched a program called "*Kapit Bisig Para sa Ilog Pasig*" where it was decided to rehabilitate the estuaries that connect and drain into the Pasig River to contain the pollution levels in the water (Ramos, 2012; Pasig River Rehabilitation Commission, 2018). Under this program, attempts were made to rehabilitate some estuaries e.g *estero de paco* and *estero de san miguel, estero de vitas* which have been the recipient of contaminants, pollutants and hazardous substances including the heavy metals and thereby adding their contaminant load

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into the Pasig river system (Dioquino, 2014; Muana, 2013).

Heavy metals are genotoxic and cytotoxic as they cause DNA damage (Magaye *et al.,* 2012; Knasmuller, 1998; Hartwig, 1995).

The presence of micronuclei in the bone marrow of *Swiss albino* mice exposed to the water samples would be indicative of the DNA damage due to exposure to heavy metals or pollutants capable of causing genotoxicity. The genotoxicity of water can have detrimental health effects on the people especially those living close to the Pasig riverbanks and may lead to carcinogenicity due to DNA damage. Therefore, genotoxicity assessment is one of the essential components of the environmental safety evaluation to protect human and animal health and make the overall environment safe as it measures the DNA damage induced by the exposure to acontaminant. Essentially, the genotoxicity tests should be able to detect any of the three endpoints - gene mutations, structural chromosome aberrations, and numerical aberrations, as all of these could be the triggers of carcinogenesis and heritable genetic disorders (Madia, 2014).

Though a number of methods and assay systems are available for genotoxic hazard assessment, none of them are adequate enough to give complete information about the genotoxic status of a particular environment (Adler *et al.,* 2010). The aim of the present study, was to evaluate the suitability of using the combination of micronucleus assay in the *Swiss albino* mice along with heavy metal analysis using atomic absorption spectroscopy (AAS) as a model assay system to compare the genotoxic potential of the water samples from *estero de paco* with that of *esteros* de *vitas* to ascertain the effectiveness of the restoration efforts so far (Fig 1A and B). Should the water samples of *estero de paco* be found safer as compared to *estero de vita*s in terms of its genotoxicity, it can be established that the restoration efforts have been successful and any subsequent efforts to rehabilitate the inland waters will bring down the levels of pollution and restore the overall cleanliness of the Pasig river. To establish the correlation between the observed outcomes, the physico-chemical parameters such as BOD, COD and pH of water samples were also analysed.

METHODOLOGY

Sample Collection

The water samples were collected at the locations accessible at the bank of *estero de paco* (Figure 2A –

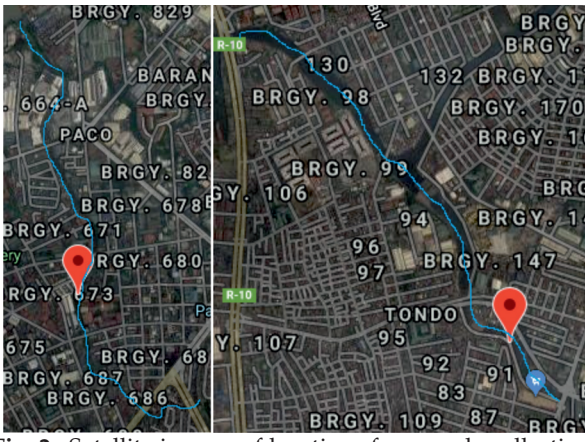

Fig. 2. Satellite images of locations for sample collection 2A- *estero de paco* and 2B- *estero de vitas*

Fig. 1A. A section of *Estero de Paco*

Fig. 1B. A section of *Estero de Vitas*

Fig. 1. Photographs of restored *estero de paco* (1A*)* and still polluted *estero de vitas* (1B).

GPS: 14. 579843, 120.993571) and *estero de vitas* (Figure 2B – GPS: 14.619613, 120.968984), both tributaries of the Pasig River System.

Treatment of Mice

Swiss Albino male mice (*Mus musculus*), 4-8 weeks old with an average weight range of 20-24g, were procured from the Pet Town Veterinary Clinic and Pet Supplies, Quezon City, Metro Manila. Since the gender of the mice is one of the variables in the micronucleus test with males found to be more sensitive to toxicological treatments, only males were used in the present study (Zuñiga *et al.,* 2001). The mice were acclimatized and maintained in laboratory conditions of 12 h dark and light cycle, temperature of 26.9 ± 6%C and were administered clean drinking water and standard rodent chow (Bio3000) *ad libitum*. All animal experiments were conducted in accordance with the standard guidelines on use of animals for experimental toxicology studies (CIOMOS, 2012). The oral administration of varying concentrations (5%, 10%, 25%, 50%, and 100% concentrations (v/v. water samples from *esteros*/tap water) of the *estero* water samples was carried out daily to five mouse per exposure group for 7 days (Alimba *et al.,* 2012). Similar treatment was concurrently given to the negative (tap water) and positive (methylmethanesulfonate, 4 mg/kg body weight) control groups.

Since the mode of administration of the test chemicals (intraperitoneal vs. oral gavage/*ad libitum*) have been identified to have an impact on the test results by the Collaborative Study Group for the Micronucleus Test under the Mammalian Mutagenicity Study Group (CSGMT/JEMSMMS) both modes of administration of water samples namely—*ad libitum* and intraperitoneal were used (Hayashi *et al.,* 1989). Two groups of 5 mice each, were administered with the same concentrations of water samples intraperitoneally and exposed to water samples *ad libitum.* For intraperitoneal mode, the mice were injected with 0.5 mL of water samples per day per mouse for 7 days at the same time of the day (Bakare, 2009). The negative control mice were similarly given tap water both *ad libitum* as well as intraperitoneally, while the positive control group was injected with MMS (methyl methanesulfonate, 4 mg/kg body weight) intraperitoneally.

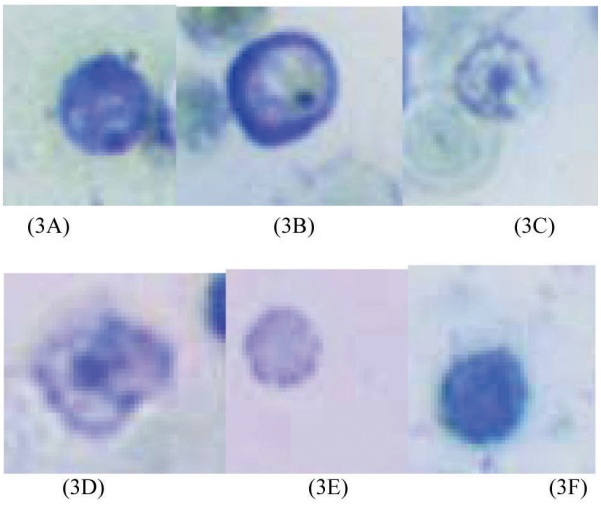

Fig. 3. Micronucleated (A-D) and non-micronucleated (E-F) polychromatic erythrocytes, at 1000x magnification, stained with May-Grunwald and Giemsa observed following exposure to water samples.

Micronucleus Assay

While carrying out the micronucleus test, the OECD guidelines on the testing of chemical substances were followed (OECD, 1997). Briefly, animals were sacrificed by cervical dislocation and their femur bones were surgically removed to flush out bonemarrow for the micronucleus test following the methods of Schmid, (1975) and Aaron *et al.,* (1989) with minor modifications. The bone marrow cells from the femurs were flushed into the Eppendorf tubes using 0.5 ml of fetal bovine serum (Invitrogen cat # 16170078). The cells were then centrifuged at 3000 rpm for 5 minutes and a smear was made on the pre-cleaned slides. After air-drying, the slides were fixed in methanol for 10 minutes, air dried and followed by staining them in May-Grunwald stain. Afterwards, the slides were stained with Giemsa for 3 minutes. The slides were coded and examined under an Olympus light microscope at 1000X magnification. At least 2000 cells per animal were scored for micronucleated polychromatic erythrocytes (MNPCE).

Laboratory Analysis

Physico-Chemical Analysis of water samples

The water quality of the two esteros was analysed by using three parameters namely pH level, amount of dissolved oxygen (DO) and the biochemical oxygen demand (BOD). The values obtained were compared with the prescribed primary parameters set by Department of Environment and Natural Resources, Philippines (DENR, 2016). The standard

Winkler titration method was used to calculate DO and BOD whereas the pH level was checked by using the pH strips (Department of Biology, 2015). All measurements were in accordance to the American Public Health Association's "Standard Methods for Examination of Water and Waste Water"(APHA, 2005).

Atomic Absorption Spectroscopy (AAS) of Water Samples and Mouse Tissue

The presence of heavy metals (Cu, Cd, Pb and Zn) in the water samples and mouse tissue samples was detected by the AAS using the atomic absorption spectrophotometer (model AA 6300 Shimadzu). The heavy metal analysis was carried out in accordance with the standard methods (Sharma and Tyagi, 2013; USEPA, 2006)

Acid Digestion of Water Samples

The acid digestion of the water samples was carried out by using the prescribed standard procedure where 5 ml HNO_3 was added to 50 ml of the water samples (Siraj and Kitte, 2013). The samples were boiled on a hot plate till only 20 ml of the solution was left. Further processing of samples involved cooling followed by further addition of 5 ml of HNO_3 and boiling the samples till only 10 ml was left. The sample thus obtained was filtered and diluted in a 100ml volumetric flask and stored in the refrigerator till further used.

Acid Digestion of Mouse Tissue

From each mouse, the liver, muscles, and bone marrow tissue were extracted. The liver and muscle tissues were placed separately in aluminum foil for drying. Bone marrow tissue was extracted following the procedure in the micronucleus assay, and samples were placed inside test tubes all samples were dried in an oven at 180 degrees Celsius for at least 2 hours. Afterwards, 0.1g of the liver and muscle tissues, and the whole sample for bone marrow tissue were processed for acid digestion using nitric acid for AAS (Akintujoye *et al.*, 2013).

Data Analysis/Statistical Tool

For the comparison of the negative control, 100% concentration of *estero de paco* and *estero de vitas*, and the positive control with each other, one-way ANOVA was used along with a post-hoc (Bonferroni approach) analysis. To compare the frequencies of micronuclei across concentrations, the same analysis was done for each mode of administration. The linear regression analysis, with respect to the control was done for different concentrations of water samples. For the comparison of the modes of administration per concentration, and overall concentrations, two-way ANOVA was used, as well as one-way ANOVA followed by a post-hoc (Bonferroni approach) analysis. Lastly, to analyse the results of heavy metal concentrations using atomic absorption spectroscopy, one-way ANOVA along with a post-hoc (Bonferroni approach) analysis was used.

RESULTS

An overall concentration-dependent increase was observed in the number of micronucleated erythrocytes in the bone marrow cells of the *Swiss albino* mice exposed to the water samples of *estero de paco* and *estero de vitas* in comparison to the control treatment at $P<0.05$ (Fig. 4. and 5). This indicates that the aquatic environments at each site possess genotoxic/mutagenic contaminants. Further the water samples from *estero de paco* were found to be less genotoxic as compared to *estero de vitas* as evident by the significant difference between the micronuclei. Further, the presence of heavy metals in the water samples as well as in the tissues of the mice which could be the probable source of this genotoxicity was detected by the AAS.

Analysis of Micronuclei Assay in the Bone Marrow Cells of Mice

The micronucleus assay showed the water samples, at 100% concentrations, collected from both locations were significantly genotoxic as compared to the negative control at $p<0.05$ both for the *ad libitum* as well as intraperitoneal administered treatments (Table 1 and 2). In the intraperitoneal administered treatment, the positive control and *estero de vitas* were not significantly different at $p>0.05$ (Table 2), further confirming that *estero de vitas* is highly genotoxic. Furthermore, since this was not detected in the *ad libitum* administered treatment (Table 1), it indicated that there is a difference between the two modes of administration. Further tests were subjected to statistical analysis to confirm if the difference is significant.

Analysis of the Frequency of Micronuclei Across Concentrations

A linear regression analysis confirmed a concentration-dependent increase in the

micronucleated polychromatic erythrocytes (MNPCEs) with increase in the concentrations of water samples for both *estero de paco* and *estero de vitas* as evident by the positive correlation observed with high R^2 values (Fig. 4 and 5). The *estero de paco* treated mice had the R^2 value of 0.7635 and the *estero de vitas* treated mice had an R^2 value of 0.9979 for *ad libitum* administered water samples (Fig 4). The intraperitoneally injected mice had an R^2 value of 0.8510 for *estero de vitas* and R-value of 0.9912 for *estero de paco* (Fig. 5). Overall, the high R^2 value in all treatments indicated the positive correlation between the general increase of micronuclei with an increase in concentration in each treatment.

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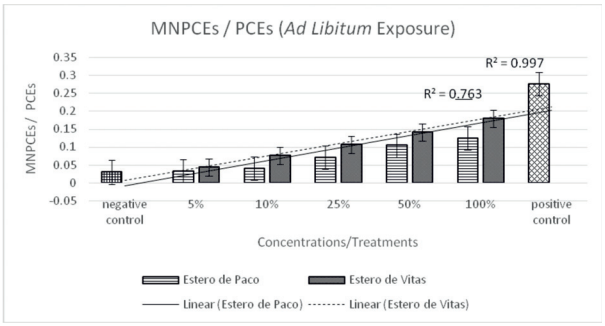

Fig. 4. Bar graph and linear regression analysis of concentration based increase in MNPCEs / Total PCEs with respect to negative control (tap water) and positive control (MMS) in mice exposed to water samples from two *esteros* through *ad libitum* exposure.

Comparison of Two Modes of Administration of the Water Samples

There was a difference between the genotoxicity effects of the water samples as a result of the difference in their route of administration with intraperitoneal route leading to significantly higher induction of micronuclei ($p<0.05$) as compared to *ad libitum* route for the same concentrations of water samples tested for both *estero de paco* (Table 3) as well

Table 1. Comparison of the frequency of micronuclei among the 4 treatments (negative control, positive control, 100% water concentration of *estero de paco* and *estero de vitas* in *ad libitum* and intraperitoneal exposure. (One-way ANOVA with post-hoc Bonferroni approach)

Treatment	Ave MN ± SD (AL)	Ave MN ± SD (IP)	Treatment Comparison	P-values (AL)	P-values (IP)
Negative Control	0.0302 ± 0.0011	0.0302 ± 0.0011	Negative Control vs. *estero de paco*	p<0.05 (0.000)	p<0.05 (0.002)
estero de paco (100% concentration)	0.1245 ± 0.0034	0.1434 ± 0.0081	Negative Control vs. *estero de vitas*	p<0.05 (0.000)	p<0.05 (0.000)
estero de vitas (100% concentration)	0.1800 ± 0.0142	0.2517 ± 0.0448	*estero de paco vs. estero de vitas*	p<0.05 (0.000)	p<0.05 (0.002)
Positive Control	0.2760 ± 0.0039	0.2760 ± 0.0039	Positive Control vs. *estero de paco*	p<0.05 (0.000)	p<0.05 (0.001)
			Positive Control vs. *estero de vitas*	p<0.05 (0.000)	p>0.05 (1.000)
			Negative Control vs. Positive Control	p<0.05 (0.000)	p<0.05 (0.000)

Table 2. Comparison between *ad libitum* and intraperitoneal administration of the *estero de paco* and *estero de vitas* water samples with respect to the induction of micronuclei in *Swiss albino* mice (Two-way ANOVA and one-way ANOVA with post-hoc Bonferroni approach)

Treatment	MNPCE/PCE ± SD				P-values		Intraperitoneal		
	Ad Libitum administration (*estero de paco*)	*Ad Libitum* administration (*estero de vitas*)	Intraperitoneal Injection administration (*estero de paco*)	Intraperitoneal Injection administration (*estero de vitas*)	*estero de paco* .	*estero de vitas*			
5%	0.0330 ± 0.0018	0.0431 ± 0.0027	0.0414 ± 0.0031	0.0638 ± 0.0159	*p>0.05 (1.000)		*p<0.05 (0.000)		
10%	0.0408 ± 0.0020	0.0763 ± 0.0037	0.0775 ± 0.0016	0.0950 ± 0.0086	*p<0.05 (0.018)		*p>0.05 (1.000)		
25%	0.0705 ± 0.0016	0.1064 ± 0.0103	0.1006 ± 0.0089	0.1613 ± 0.0062	*p<0.05 (0.000)	**p<0.05 (0.000)	***p<0.05 (0.000)	****p<0.05 (0.002)	*p<0.05 (0.027)	**p<0.05 (0.000)	***p<0.05 (0.000)	****p<0.05 (0.034)
50%	0.1041 ± 0.0080	0.1413 ± 0.0055	0.1247 ± 0.0068	0.2024 ± 0.0017	*p<0.05 (0.000)		*p<0.05 (0.009)		
100%	0.1245 ± 0.0034	0.1800 ± 0.0142	0.1435 ± 0.0081	0.2517 ± 0.0448	*p<0.05 (0.000)		*p<0.05 (0.002)		

*p-value between modes of administration per concentration (AL vs. IP per concentration)
**p-value between modes of administration (AL vs. IP)
***p-value between all concentration
****p-value between mode of administration and concentration

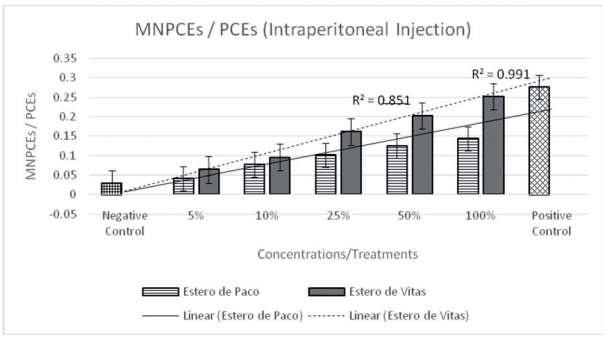

Fig. 5. Bar graph and linear regression of concentration based increase in MNPCEsTotal PCEs with respect to negative control (tap water) and positive control (MMS) in mice exposed to water samples from two *esteros* through intraperitoneal exposure.

as *estero de vitas* (Table 4). This observation was in tandem with other studies (Sutton and Boyd, 1993).

Physico-Chemical Analysis of Water Samples

The biochemical oxygen demand (B.O.D.), dissolved oxygen (D.O), and pH which are important parameters for the environmental assessment were used to compare the water quality of two water channels- *estero de paco* and *estero de vitas* with that of the standard values set up by the DENR. The BOD values of *estero de vitas* was much higher than the BOD values for water samples from *estero de paco* as well as exceeded the DENR standard values for class C (Table 5). The more acceptable BOD values from *estero de paco* water samples points to the successful rehabilitation of this *estero* and the need of constant monitoring to further improve the quality of water. The absence of any aquatic flora as well as the completely stagnant water with no flow may explain the low DO levels in the *estero de vitas* water samples.

Heavy Metal Analysis of Water Samples

For AAS analysis, a minimum detection limit of 0.05 ppm was established through standard readings prior to the heavy metal analysis of the water samples where the negative values for zinc and copper in the control samples, indicates the presence of the said heavy metals to be below 0.05 ppm (Table 3). The results suggest that the standards used in measuring the concentrations are too high for the detection of the two heavy metals. A minimum standard for the said heavy metals should be lower than 0.05ppm to produce positive values that would give a clearer understanding of the concentrations.

Further, the AAS confirmed the presence of heavy metals in the water samples from both *estero de paco* as well as *estero de vitas*. Zinc, cadmium and lead levels in the water samples from both *esteros* were higher than the recommended values for Class C standards by DENR. However, *estero de vitas* had the highest concentration of all heavy metals analysed, with a significant difference between the heavy metal contents in the water samples of *estero de paco* at ***p<0.05 (Table 6).

Heavy Metals Analysis of Mouse Tissue Samples

The AAS detected the presence of heavy metals namely cadmium (Cd), zinc (Zn), lead (Pb), copper (Cu) in the liver, muscle and bone marrow tissue of mice exposed to the water samples from *estero de vitas* (Table 7). The presence of high concentration of heavy metals in the mice exposed to *estero de vitas* as compared to *estero de paco* is corroborated by the higher numbers of micronucleated erythrocytes observed in the bone marrow cells as well (Table 3).

It is also noteworthy to mention, the presence of high levels of Pb in the control water samples as well as in the tissues of the control mouse group which is in agreement with some other studies where the presence of Pb in excess of allowable limits in the drinking water and soil samples at various locations in the Philippines was reported (Alam *et al.*, 2016; Fujimori *et al.*, 2012; Solidum and Solidum, 2012). Pb can pass through the blood – brain barrier and can accumulate in the brain leading to the damage to the central nervous system (Charlet, 2012). Hence,

Table 3. Comparison of the frequency of micronuclei among the 4 treatments (negative control, positive control, 100% water concentration of *estero de Paco* and *estero de vitas*) in intraperitoneal exposure. (One-way ANOVA with post-hoc Bonferroni approach)

Treatment	Average MN ± SD	Treatment Comparison	P-values
Negative Control	0.0302 ± 0.0011	Negative Control vs. *estero de paco*	p<0.05 (0.002)
estero de paco (100% concentration)	0.1434 ± 0.0081	Negative Control vs. *estero de vitas*	p<0.05 (0.000)
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Positive Control	0.2760 ± 0.0039	Positive Control vs. *estero de paco*	p<0.05 (0.001)
		Positive Control vs. *estero de vitas*	p>0.05 (1.000)
		Negative Control vs. Positive Control	p<0.05 (0.000)

Table 4. Comparison between *ad libitum* and intraperitoneal administration of the *estero de paco* water samples with respect to the induction of micronuclei in *Swiss albino* mice(Two-way ANOVA and one-way ANOVA with post-hoc Bonferroni approach)

Treatment	MNPCE / PCE ± SD					
	Ad Libitum administration (AL)	Intraperitoneal Injection administration (IP)	P-values			
5%	0.0330 ± 0.0018	0.0414 ± 0.0031	*p>0.05 (1.000)	**p<0.05 (0.000)	***p<0.05 (0.000)	****p<0.05 (0.002)
10%	0.0408 ± 0.0020	0.0775 ± 0.0016	*p<0.05 (0.018)			
25%	0.0705 ± 0.0016	0.1006 ± 0.0089	*p<0.05 (0.000)			
50%	0.1041 ± 0.0080	0.1247 ± 0.0068	*p<0.05 (0.000)			
100%	0.1245 ± 0.0034	0.1435 ± 0.0081	*p<0.05 (0.000)			

*p-value between modes of administration per concentration (AL vs. IP per concentration)
**p-value between modes of administration (AL vs. IP)
***p-value between all concentration
****p-value between mode of administration and concentration

further investigations with much larger sample size as well as more genetic models are needed to find the background levels of Pb in the other *esteros* in Manila city.

DISCUSSION

Micronucleus bone marrow assay in the *Swiss albino* mouse (used as model organism in the present study) is based on the principle of erythropoiesis in the bone marrow where the micronuclei are formed when the water samples during the cell proliferation, cause chromosome damage through the interaction of the genotoxins present in the water and the macromolecules of the cells resulting in the chromatid disjunction and spindle disruption (Krishna and Hayashi, 2000). Hence, the micronucleus assay has been recommended for both *in vitro* and *in vivo* routine toxicological assessment of chemicals and wastes (Cimino, 2006; ICPEMC, 1983). The overall statistically significant increase as well as concentration dependent increase in the

Table 5. Comparison between *ad libitum* and intraperitoneal administration of *estero de vitas* water samples with respect to the induction of micronuclei in Swiss albino mice(Two-way ANOVA and one-way ANOVA with post-hoc Bonferroni approach)

Treatment	MNPCE / PCE ± SD					
	Ad Libitum administration (AL)	Intraperitoneal Injection administration (IP)	P-values			
5%	0.0431 ± 0.0027	0.0638 ± 0.0159	*p<0.05(0.000)	**p<0.05	***p<0.05	****p<0.05
10%	0.0763 ± 0.0037	0.0950 ± 0.0086	*p>0.05 (1.000)	(0.000)	(0.000)	(0.034)
25%	0.1064 ± 0.0103	0.1613 ± 0.0062	*p<0.05 (0.027)			
50%	0.1413 ± 0.0055	0.2024 ± 0.0017	*p<0.05 (0.009)			
100%	. 0.1800 ± 0.0142	0.2517 ± 0.0448	*p<0.05 (0.002)			

*p-value between modes of administration per concentration (AL vs. IP per concentration)
**p-value between modes of administration (AL vs. IP)
***p-value between all concentrations
****p-value between mode of administration and concentration

Table 6. Comparison of the physicochemical parameters and heavy metal profile of water samples: negative control, *estero de paco*, and *estero de vitas* versus DENR Class C Standard (One-way ANOVA with post-hoc Bonferroni approach)

	Treatments				p-values			
	Control	*estero de paco*	*estero de vitas*	DENR Class C Standard	*p-value	**p-value	***p-value	****p-value
B.O.D (mg/l)	3	8	-NA-	7	-	-	-	-
D.O. (minimum; mg/l)	4	5	-NA-	5	-	-	-	-
pH	7	7.5	6.7	6.5-9.0	-	-	-	-
Cadmium (ppm)	0.0191	0.9807	2.6487	0.005	p<0.05 (0.000)	p<0.05 (0.005)	p<0.05 (0.000)	p<0.05 (0.000)
Copper (ppm)	-0.0669	-0.0713	0.0204	0.02	p<0.05 (0.010)	p<0.05 (0.037)	p>0.05 (1.000)	p<0.05 (0.017)
Lead (ppm)	0.3384	1.3535	3.5868	0.05	p<0.05 (0.001)	p>0.05 (0.086)	p<0.05 (0.003)	p<0.05 (0.004)
Zinc (ppm)	-0.2004	1.4817	5.8928	2	p<0.05 (0.000)	p<0.05 (0.000)	p<0.05 (0.000)	p<0.05 (0.000)

*p-value between DENR standard, *estero de paco* and *estero de vitas* water samples
**p-value between DENR standard and *estero de paco* water samples
*** p-value between DENR standard and *estero de vitas* water samples
**** p-value between *estero de paco* and *estero de vitas* water samples

micronuclei and MNPCE observed in the bone marrow cells of the mouse exposed to the water samples from the *esteros* is an indication that this assay as standardised in the present study for the purpose of environmetal biomonitoring was sensitive enough to detect the difference between the genotoxicity of the water samples from different sources.

The higher numbers of micronuclei induced by the intraperitoneal route as compared to the *ad libitum* route is as accepted since intraperitoneal route is fast and more efficient, and is favored over other methods to deliver the test chemicals/toxins for toxicological studies and short-term assays (Alabi *et al.,* 2013). The two plausible explanations for the higher number of micronuclei induced through intraperitoneal injection as compared to the *ad libitum* are- firstly the intraperitoneal injection route delivers more precise and accurate dosage of the test substance which ensures relatively more accurate induction of causal effects of the test substance. Secondly, the intraperitoneal route of exposure is known to cause acute stress to the laboratory animal thereby increasing the overall response and impact of the test substance (Baek *et al.,* 2013; Drude *et al.,* 2011).

The presence of heavy metals in the water samples is not unexpected as the continued mismanagement and the disposal of untreated organic waste may have led to the toxins including the heavy metals leaching out in to the waters of *estero de Vitas*, especially from the solid waste dumpsites located at Smokey Mountain (Yoshimura *et al.,* 2015). The heavy metals are also known to accumulate in different tissues and cause genotoxicity in terms of chromosomal damage and micronuclei both *in vivo* and *in vitro* conditions besides other health hazards (Morales, 2016; Li *et al.,* 2018). Since the liver is the main detoxification organ and accordingly it accumulates high concentration of heavy metals such as Cu, Pb therefore for the heavy metal analysis, liver tissue was used in the present study (López-Alonso *et al.,* 2006; Mercer, 2001). The lower genotoxic potential of the *estero de paco* water samples was confirmed by the lower concentrations of the heavy metals present except Zn as compared to the tissues of mice exposed to water samples from *estero de vitas.* Since the average of the heavy metal concentration in all three tissues was taken for statistical analysis the difference in the concentrations was statistically insignificant (Table 8).

The heavy metals are known to act synergistically to induce DNA damage triggered by oxidative stress and free radical formation and induce the MNPCE formation (Wu and Ding, 2016; Henkler *et al.,* 2010).

Table 7. Accumulation of heavy metals in tissues— bone marrow (B), muscle (M) and liver (L) of Swiss albino mice treated with water samples from *estero de paco* and *estero de vitas*

Metal	Negative Control			*estero de paco*			*estero de vitas*		
	L	M	B	L	M	B	L	M	B
Cd	-0.0045	-0.0153	-0.0379	0.000	0.0116	0.0055	0.0205	0.0238	0.0477
Cu	0.0298	0.0152	0.0352	0.1774	0.0258	0.0464	0.2894	0.0350	0.0556
Pb	0.7865	0.0215	0.2824	0.4089	0.4944	0.1195	0.5468	0.6764	0.6626
Zn	0.4121	0.2245	0.0726	0.5185	0.1105	0.0587	2.2025	0.7876	1.3851

Table 8. Comparison of heavy metal concentrations in tissues (average values from all three tissues— bone marrow, muscle and liver) of mouse treated with water samples from *estero de paco*and *estero de vitas*(One-way ANOVA with post-hoc Bonferroni approach)

Metal	Control	*estero de paco* (mean concentration in ppm ± SD)	*estero de vitas* (mean concentration in ppm ± SD)	P-values			
Cd	-0.0192 ± 0.0170	0.0058±0.0058	0.0307±0.0148	*p<0.05 (0.0115)	**p>0.05 (0.192)	***p<0.05 (0.012)	****p>0.05 (0.191)
Cu	0.0267 ± 0.0103	0.0832±0.0822	0.1267±0.1413	*p>0.05(0.4761)	**p>0.05(1.000)	***p>0.05(0.730)	****p>0.05(1.000)
Pb	0.3635 ± 0.3889	0.3409±0.1965	0.6286±0.0712	*p>0.05(0.3692)	**p>0.05(1.000)	***p>0.05(0.749)	****p>0.05(0.648)
Zn	0.2363 ± 0.1701	0.2292±0.2518	1.4584±0.7103	*p<0.05(0.0230)	**p>0.05(1.000)	***p<0.05(0.046)	****p<0.05(0.045)

*p-value between all samples
**p-value between control and *estero de paco*
***p-value between control and *estero de vitas*
****p-value between *estero de paco* and *estero de vitas*

The presence of xenobiotics including heavy metals in the leachates and effluents are known to cause abnormal cellular functions and pathological disorders (Bakare *et al.,* 2013). The damaged cells are either eliminated by programmed cell death (apoptosis) or accidental cell death (necrosis) (Pulido and Parrish, 2004). In this study, though the genotoxicity induced by other contaminants and toxins present in the water samples of the two *esteros* cannot be ruled out, the synergistic chemical combinations of these heavy metals could be attributed to be harmful enough to cause the observed genotoxicity. The significantly lower concentrations of the heavy metals in the water samples of *estero de paco* as compared to *esteros de vitas* confirmed that the restoration efforts to clean up the *estero de paco* has been successful hence it is recommended that rehabilitation of the *estero de vitas* as well as other *esteros* in the Metro Manila should be undertaken urgently to control the overall pollution levels of the Pasig River.

CONCLUSION AND RECOMMENDATIONS

The mouse bone marrow micronucleus assay results confirmed the genotoxic potential of water bodies as evident by positive correlation with high R^2 value where the number of micronuclei increased with an increase in concentration of water samples from both esteros with maximum genotoxicity observed at 100% concentration. The water samples from *estero de paco* were found to be less genotoxic as compared to *estero de vitas* as apparent by the significant difference between the micronuclei, better physico- chemical parameters like BOD, DO and pH and the heavy metal profile confirming that the restoration of *estero de paco* has been successful to some extent. The heavy metals present in the water samples as well as the mouse tissue could be the possible source of elevated levels of micronuclei which was detected by AAS. The route of administration with i*ntraperitoneal* route, inducing more micronuclei as compared to *ad libitum*, can affect the genotoxicity effects of the water samples for this reason the mode of administration should be specified while carrying out genotoxicity experiments using laboratory animals. Hence, in accordance with the results of the study, the rehabilitation of *estero de Paco* is highly recommended to be a model program for the rehabilitation of the rest of the estuaries/esteros flowing into Pasig River sytem, to bring down the pollution levels in this river as well as other rivers with esterso in the Philippines.

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