The use of *in vivo* mouse bone marrow micronuclei test and Atomic absorption spectroscopy for environmental biomonitoring of the aquatic bodies in and around *Metro Manila*, *Philippines*

Zeba F Alam* and Mikaella Justin Umali
Biology Department, College of Science, De La Salle University, Taft Avenue, Manila, Philippines
***Corresponding author- zeba.alam@dlsu.edu.ph**

Abstract: The combination of *in vivo* mouse bone marrow micronucleus assay with atomic absorption spectroscopy has been used as an assay system for environmental biomonitoring of the polluted water bodies specifically the two *Esteros namely- Estero de Vitas* and *Estero de Paco*, belonging to thr Pasig River System, Philippines. 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. 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 observed genotoxic effects of the water samples appeared to be related to the physicochemical characteristics studied using Atomic absorption spectroscopy, which showed the presence of heavy metals in the water samples from both sources. The AAS technique was also used to detect the presence of heavy metals in the mouse tissue exposed to the water samples from two locations to further confirm the probable source of genotoxicty. The results establish that the mouse micronucleus test in combination with AAS can be used effectively for environmental biomonitoring. The lower genotoxicity potential of *Estero de paco* clearly demonstrates that the restoration of the *Esteros* can be an effective approach to control pollution of the water bodies especially the Pasig river system.

Keywords Genotoxicity; Mouse bonemarrow; micronuclei; Heavy metals

1. INTRODUCTION

The Philippines, the fastest growing economy in the Association of Southeast Asian Nations (ASEAN) (World Bank 2018) has been struggling with environmental degradation due to lack of comprehensive environmental policy and mismanagement in terms of implementation of guidelines and rules (Asian Development Bank 2009). The Pasig river with its 47 tributaries or estuaries which flows through Metro Manila, was declared biologically dead and efforts have been going on to clean the river and improve the water quality of the Pasig River System (Asian Development Bank 2012). One of the approaches to tackle the pollution levels in the Pasig River, started by the ABS-CBN Foundation and adopted by the Pasig River Rehabilitation Commission (Pasig River Commission, 2018), was the launching of 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). Under this program attempts were made to rehabilitate two estuaries namely-- *Estero de Paco* and *Estero de San Miguel*. A number of contaminants are present in the polluted water bodies, one of them being the heavy metals that can be genotoxic and cytotoxic by causing DNA damage (Knasmuller et al. 1998; Hartwig 1995). Though the restoration efforts have led to the improvement in the physico-chemical parameters of the water (Muana, 2013), it is of paramount importance to evaluate the genotoxic and cytotoxic potential of the waters of these *esteros* due to their proximity to the human dwellings. 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 a contaminant. 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. 2011).

Therefore the overall environmental biomonitoring should have a battery of tests/methods including the genotoxicity/mutagenicity tests to keep track of the environment's status. Hence in the present study, suitability of using the micronucleus assay in the *Swiss albino* mice and the heavy metal analysis using atomic absorption spectroscopy was evaluated as a model assay system for environmental biomonitoring. Both methods in combination were used to compare the genotoxic potential of the water samples from *Estero de Paco* with that of *Estero de Vitas* and further ascertain the affectivity of the restoration efforts of various stakeholders.

2. METHODOLOGY

2.1 Collection of Samples

The water samples were collected at the locations which are accessible along the banks of *Estero de Paco* and *Estero de vitas.*

2.2 Treatment of Mice

Swiss Albino male mice (*Mus musculus*), 4-8 weeks old with an average weight range of 20-24g, were used as the test subjects for the experiments. The mice were acclimatized and maintained in laboratory conditions of 12 h dark and light cycle, temperature of 26.9 ± 6◦C and had access to clean drinking water and standard rodent chow (Bio3000) *ad libitum*. A standard procedure of oral administration of 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 (methyl methanesulfonate, 4 mg/kg body weight) control groups.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 0.5ml of water samples per day per mouse for 7 days at the same time of the day (Bakare et al. 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.

2.3 Micronucleus Assay

Animals were sacrificed by cervical dislocation and their femur bones were surgically removed to flush out bone marrow for the micronucleus test. The micronucleus test was performed according to 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. The slides were air-dried once again, and then stained with the May-Grunwald stain for 5 minutes. 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).

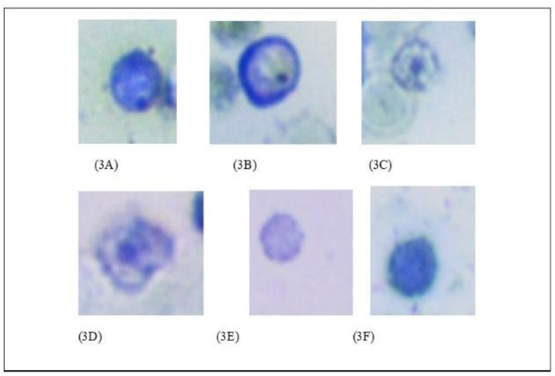

Figure 1. Micronucleated (A-D) and non-micronucleated (E-F) polychromatic erythrocytes, at 1000x magnification, stained with May-Grunwald and Giemsa observed following exposure to water samples.

2.4 Laboratory Analysis

Physico-Chemical Characteristics

To compare the physico-chemical properties of water samples from the two *esteros*, three parameters were analyzed— pH level, amount of dissolved oxygen (DO) present and the biological oxygen demand (BOD).

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

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

Data Analysis/Statistical Tool

The analysis off results was carried out by using one way ANOVA, along with post-hoc (Bonferroni apprach). To study the cocnetration dependent efftects linear regression analysis was performed.

3. RESULTS AND DISCUSSION

The results indicate that there was an overall concentration-dependent increase in the number of 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 $P<0.05$ (Fig1, 2 and Fig 3). 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 Atomic absorption Spectroscopy detected 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 the genotoxicity (Table 1 and 2). A difference in the overall effect was observed based on the mode of administration of the treatment.

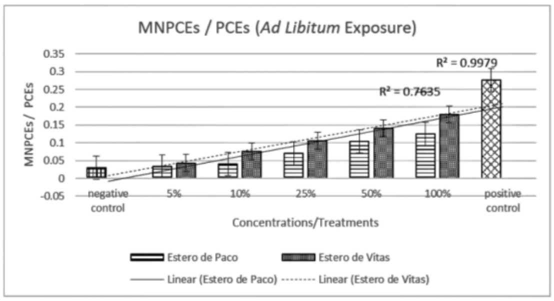

Figure 2. Bar graph and linear regression analysis of concentration based increase in MNPCEs / Total PCEs with respect to negative control (tap water) and positive control (MMS) in mice exposed to water samples from two *esteros* through *ad libitum* exposure

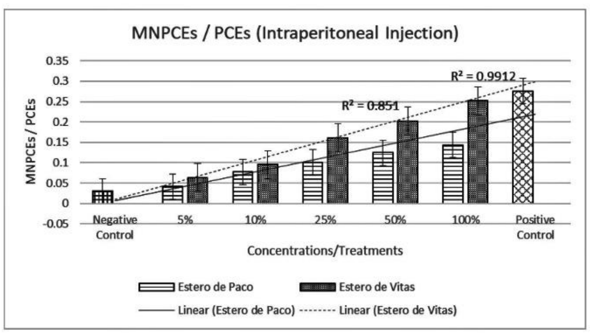

Figure 3. Bar graph and linear regression of concentration based increase in MNPCEs Total PCEs with respect to negative control (tap water) and positive control (MMS) in mice exposed to water samples from two *esteros* through intraperitoneal exposure.

Table 1. Comparison of the 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-value			
	Control	Estero de Paco	Estero de Vitas	DENR Class C Standard	*p-value	**p-value	***p value	****p-value
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 Esteri de Vitas water samples

Table 2. Accumulation of heavy metals – cadmium (Cd), copper (Cu), lead (Pb), and zinc (Zn) in various 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

4. CONCLUSION AND RECOMMENDATIONS

The mouse bone marrow micronucleus assay and physico-chemical analysis of water samples along with the heavy metal detection in the water samples and mouse tissue, clearly establish that the rehabilitation efforts to control and improve the water quality of *Estero de Paco* have been effective to some extent in terms of genotoxicity. Since no *in vivo* rodent assay can detect the genotoxicity status on its own, the combination of mouse bone marrow micronucleus test with heavy metal analysis using AAS can effectively be used for environmental biomonitoring. Lastly, 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 of Pasig River, as well as the other polluted bodies of water in the Philippines.

5. REFERENCES

Aaron CS, Sorg R, Zimmer D. 1989. The mouse bone marrow micronucleus test: evaluation of 21 drug candidates. Mutat Res 223: 129–140.

ABS-CBN News. 2018. DENR vows 'swift action' on garbage shipment from S. Korea. Available from: https://news.abs-cbn.com/news/11/13/18/denr-vows-swift-action-on-garbage-shipment-from-s-korea

Adler S, Basketter D, Creton S, Pelkonen O, van Benthem J, Zuang V, Andersen KE, Angers-Loustau A, Aptula A, Bal-Price A, Benfenati E, Bernauer U, Bessems J, Bois FY, Boobis A, Brandon E, Bremer S, Broschard T, Casati S, Coecke S, Corvi R, Cronin M, Daston G, Dekan, W, Felter S, Grignard E, Gundert-Remy U, Heinonen T, Kimber I, Kleinjans J, Komulainen H, Kreiling R, Kreysa J, Leite SB, Loizou G, Maxwell G, Mazzatorta P, Munn S, Pfuhler S, Phrakonkham P, Piersma A, Poth A, Prieto P, Repetto G, Rogiers V, Schoeters G, Schwarz M, Serafimova R, T€ahti H, Testai E, van Delft J, van Loveren H, Vinken M, Worth A, Zaldivar JM. 2011. Alternative (non-animal) methods for cosmetics testing: current status and future prospects-2010. Arch Toxicol 85(5), 367e485. http://dx.doi.org/10.1007/s00204-011-0693-2.

Alimba CG, Bakare AA, Aina OO. 2012. Liver and kidney dysfunction in Wistar rats exposed to municipal landfill leachate. Resources Environ 2: 150–163.

Asian Development Bank. 2012. Cleaning up the Pasig River in Manila, Philippines. Available from: https://www.adb.org/features/pasig-river-clean

Asian Development Bank. 2009. Country environmental analysis for the Philippines. Available from: https://www.adb.org/documents/country-environmental-analysis-philippines

Asian Development Bank. 2012. Philippines: Pasig River environmental management and rehabilitation sector development program. Available from: https://www.adb.org/sites/default/files/evaluation-document/36027/files/pvr-187.pdf

Bakare AA, Alabi OA, Gbadebo AM, Ogunsuyi OI, Alimba CG. 2013. *In vivo* cytogenotoxicity and oxidative stress induced by electronic waste leachate and contaminated well water. Challenges 4: 169–187.

Hartwig A. 1995. Current aspects in metal genotoxicity. Bio Metals 8: 3–11.

Knasmuller S, Gottmann E, Steinkellner H, Fomin A, Pickl C, Paschki A, God R, Kundi M. 1998. Detection of genotoxic effects of heavy metal contaminated soils with plant bioassays. Mutat Res 420: 37–48.

Muana R. 2013. Private sector initiative in the *Estero de Paco* project: a case study on Manila waterways urban redevelopment plan.

Department of Civil Engineering. Global Hydrology and Water Resource Engineering Laboratory. Urban and Regional Policy and Planning. Available from: https://www.academia.edu/3618635/Private_Sector_Initiative_in_the_Estero_de_Paco_Project_A_Case_Study_on_Manila_Waterways_Urban_Redevelopment_Plan

Pasig River Rehabilitation Commission. 2018. History. Available from: http://prrc.gov.ph/index.php/pasig-river/history

Ramos RR. 2012. Led streetlights brighten up *Estero de Paco*. Available from: http://abscbnpr.com/led-streetlights-brighten-up-estero-de-paco/

Schmid W. 1975. The micronucleus test. Mutat Res 3: 9–15.

Sharma, B. and Tyagi, S. 2013. Simplification of Metal Ion Analysis in Fresh Water Samples by Atomic Absorption Spectroscopy for Laboratory Students. Journal of Laboratory Chemical Education, 2013; 1(3): 54-58.

USEPA - United State Environmental Protection Agency. 2006. Acid digestion of sediments sludge and soils, Method-3050B. USEPA: Washington, DC. www.epa.gov/safewater/mcl.html.

World Bank. 2018. Philippines growth to remain strong despite global uncertainty. Available from: http://www.worldbank.org/en/news/press-release/2018/07/13/philippines-growth-to-remain-strong-despite-global-uncertainty