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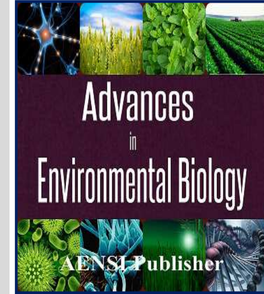

Genotoxic Stress Induced By Intensive Aquaculture Activities In Taal Lake (Philippines) On Circulating Fish Erythrocytes Using the Comet Assay and Micronucleus Test

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ABSTRACT

The present study determined the genotoxic potential induced by intensive fish cage aquaculture in Taal Lake (Philippines) on Nile tilapia (*Oreochromis niloticus*) erythrocytes using the comet assay and micronucleus test. Water and sediment samples were collected from three sampling sites namely: Gonzales, Bañaga, and Balakilong, which are non-aquaculture, moderate aquaculture, and heavy aquaculture sites, respectively. The highest incidence of tail moments, micronuclei, and nuclear abnormalities was detected in Balakilong site. The nuclear abnormalities of erythrocytes included blebbed, notched and lobed nuclei. Both assays (comet assay and micronucleus test) showed concordance by establishing similar gradient of severity based from cumulative results: Balakilong > Bañaga > Gonzales. Using *post-hoc* analyses, the two aquaculture sites showed no significant differences in both mean tail moment and frequency of nuclear abnormalities. On the other hand, significant differences were observed when both aquaculture sites were compared to the non-aquaculture site. Although no specific cause-effect relationships were established, the genotoxicity data indicate association between the level of unionized ammonia and copper, which have settled into the sediments from unconsumed feeds. Tilapia, which feeds heavily in bottom sediments, is in direct contact with the contaminants and can take in considerable levels of ammonia. The detection of copper even in non-aquaculture site indicates that that contamination has spread widely in the lake. Results of the study have demonstrated that both assays are effective as field biomarkers for investigating the harmful effects of fish cage aquaculture on lake quality.

KEYWORDS: genotoxicity, fish cage, ammonia, heavy metals, sediment quality, Tilapia

INTRODUCTION

To satisfy the increasing demand for food, construction of fish cages wherein fish fry are grown and fed in cages until they reach market size has become a common practice in developing countries like the Philippines. Natural lakes therefore, become prone to severe pressure as they are made to accommodate overstocking and intensive feeding; the latter, leads to the elevation of organic pollutants such as ammonia, and heavy metals such as copper and zinc. Aside from fish, the organic enrichment associated with aquafarming has been reported to affect diversity of benthic community and biodiversity loss and increase in opportunistic species [1]. In our previous study [2], we have shown that the deteriorating water and sediment quality due to fish aquaculture has

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detrimental effects on fish embryos. However, pollutant effects cannot only be observed at the levels of fish populations but more so at the molecular and genetic levels. Understanding the genotoxicity potential to caged fish is, therefore, of high ecotoxicological relevance.

Fish species have continued to draw attention among ecotoxicologists who are interested in assessing the impacts of water- and sediment-borne contaminants [3]. Fish maintain ecosystem stability by playing major roles in aquatic food-webs. There is a strong connection between fish and human health since fish provide the main source of animal protein for those living in coastal regions. Although it is still difficult to establish a direct link between the ecological effects of pollution and human health, the consumption of contaminated fish grown in cages could provide the conceptual basis for this connection. The increased frequency of genotoxic damage in bottom-feeding fish inhabiting polluted environments may serve as early warning indicators of carcinogenic hazards to humans. Hence, assessing the state of health of fish is important for protecting human economics and health.

Monitoring genotoxicity in the environment using indigenous organisms as sentinels requires sensitive assays. The Comet Assay (Single Cell Gel Electrophoresis) detects DNA damage in single cells induced by mutagens and other environmental pollutants dissolved in water. It is now more commonly used in genotoxicity assessment and environmental biomonitoring because of its sensitivity, relative simplicity and rapidity [4, 5, 6]. The Nile tilapia (*Oreochromis niloticus*) is a suitable species as it is the primary aquaculture produce of Taal Lake and, because like other fish, it can metabolize, accumulate, and store pollutants directly from contaminated water [7]. Erythrocytes of fish species are more preferably used in the comet assay due to their sensitivity to the genotoxic pollutants in the aquatic environment [4].

Exposure to genotoxic agents may also result in the formation of chromosomal aberrations, giving rise to lagging whole chromosomes or fragments, which lack spindle attachment organelles (kinetochores or centromeres). These lagging chromosomes and acentric fragments are excluded from the daughter nuclei during cell division, resulting in the formation of an additional, and notably smaller, nucleus within the cytoplasm known as the micronucleus. Micronuclei are considered markers of abnormal cell division involving chromosomal breakage, missaggregated chromatin or mitotic interference, events thought to be associated with an increased risk for cancer [8, 9].

Two aquaculture sites Balakilong, Laurel and Bañaga, Agoncillo, and a non-aquaculture site Gonzales, Tanauan were selected for the present study. The Nile tilapia (*Oreochromis niloticus*), being the primary fish species cultured in Taal Lake, a benthopelagic organism, and an established test species for genotoxicity studies was used as the test organism.

By using erythrocytes extracted from Nile tilapia (*O. niloticus*), we have investigated the genotoxicity potential of chemical stressors associated with intensive fish cage aquaculture. Specifically, we have used the Comet assay and micronucleus test to determine and compare the levels of DNA damage and chromosomal aberrations (eg., micronuclei) in fish sampled from aquaculture sites (Banaga and Balakilong) and non-aquaculture site (Gonzales). In addition, we have discussed the observed genotoxic effects with the existing physical and chemical parameters on the sites.

MATERIALS AND METHODS

Water and Sediment Sampling:

Three sampling sites within Taal Lake were selected for this study (Figure 1). The first two sites (Balakilong and Banaga) are characterized by heavy and moderate aquaculture activities, respectively whereas the third (Gonzales) is an open water fishing site. The same sites were recommended by the Bureau of Fisheries and Aquatic Resources (BFAR) [10] and have been used in a related study by Hallare et al. [2].

Water samples from each site were collected in polyethylene plastic jars and were stored in the dark with ice prior to chemical analyses. Near sediment samples from zero to 5 cm from the lake bottom were also collected using a sediment grabber. Sediment samples were stored in pre-washed and properly labelled polyethylene jars in the dark and with ice prior to chemical processing and testing. Wet sediment samples were freeze-dried at -30˚C, stored in polyethylene jars at 4˚C in the dark prior to chemical analysis.

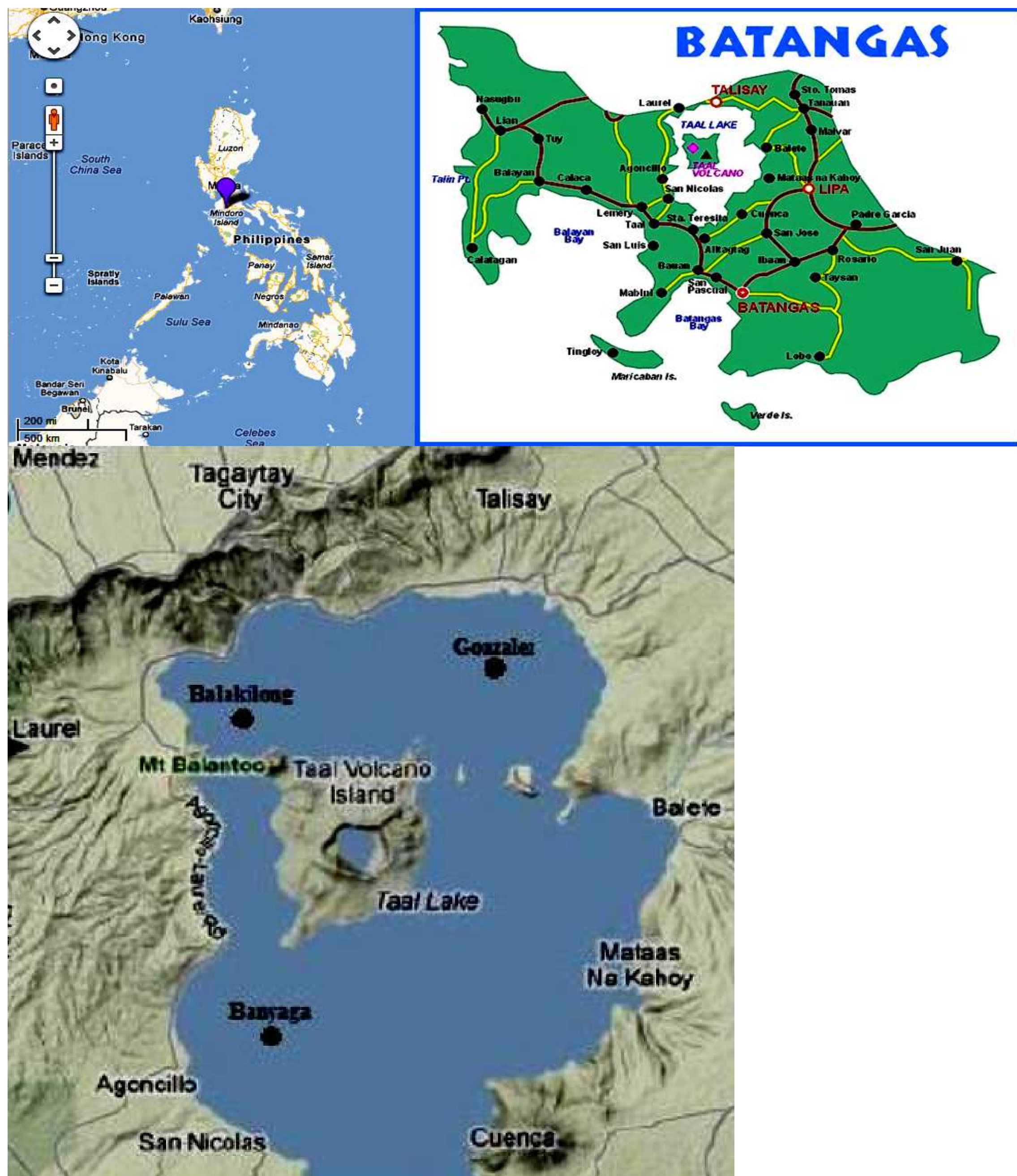

Fig. 1: (a) Map of the Philippines; (b) Map of the province of Batangas; (c) Study sites that differ in the intensity of aquaculture activities along Taal Lake: Gonzales, the non-aquaculture site in Tanauan City, and the aquaculture sites: Bañaga (or Banyaga) and Balakilong in the municipalities of Agoncillo and Laurel, respectively.

Erythrocyte Sampling:

Fifteen adult specimens of *Oreochromis niloticus* were harvested from each of the study sites Balakilong, Bañaga, and Gonzales. Five adult specimens were randomly selected for blood extraction from each group. The selection basis for the captured Nile tilapia was not restricted by the gender of the fish. The number of specimen tested is based on the statistical recommendation made by [11] of at least 5 samples for the Kruskal-Wallis test as applied for the comet assay.

The collection of blood samples from *O. niloticus* was performed *in situ* as to ensure that the results obtained are a direct consequence of exposure to Taal Lake water and sediments only. Caudal punctures were performed on the specimens to obtain 1mL of blood in citrated tubes. The tubes were placed in a test tube rack inside a small iced cooler and stored in the dark to protect the harvested cells from sun exposure and damage caused by physical handling. The samples were then transported to the laboratory for the comet assay.

Comet Assay Procedures and Analysis:

The comet assay was performed in accordance with the guidelines provided in the CometAssay® Reagent Kit (Catalog # 4250-050-K) adapted for the neutral comet assay for Tris-borate-EDTA buffer (TBE). Blood samples were centrifuged at 800 rpm for 10 minutes. The plasma was removed from the suspension and the resulting mixture was resuspended with 1000µL of 1X PBS. The mixture was then diluted to obtain erythrocytes at $1x10^{-5}$/mL. About 50 µL of cells at $1x10^{-5}$/mL were combined with 500 µL Comet LM Agarose (*Trevigen*) at 37˚C. Fifty µL of the resulting mixture was then obtained and gently pipetted to the CometSlide™ (*Trevigen*). This was done to immobilize the cells prior to electrophoresis. The pipetting was performed in triplicate per sample. The slides were then chilled at 4˚C for 10-15 min or until a clear ring was formed between each comet slide border and the agarose mixture. The chilled slides were then treated with a pre-chilled Lysis Solution (*Trevigen*) by immersion at 4˚C for 30 min in order to remove membranes and histones from the DNA. The slides were then washed by immersing in 50mL pre-chilled 1X TBE at 4˚C for 15 min. A Gel XL Ultra V-2 horizontal electrophoresis unit was used to perform the assay. The slides were aligned equidistant from the electrodes and 1X TBE buffer was added up to no more than 0.5cm above the slides. This is to prevent the LMA mixture from losing its adhesion to the CometSlide™. Voltage was applied at 1 volt per cm, and the electrophoresis was run for 40 min. Excess TBE was drained and the slides were then transferred for immersion in distilled water for 5 min. The slides were then transferred and immersed in 70% ethanol for 5 min, dried for 10 to 15 min, and stored in the dark at room temperature with desiccant. Prior to viewing 100µL of diluted SYBR Green I Nuclei Acid Gel Stain (*Trevigen*) was pipetted to each slide. The slides were then refrigerated at 4˚C for 5 min and dried in the dark at room temperature.

Slides were independently coded and without knowledge of the code, the investigators examined them using a fluorescence microscope at high magnification (*Olympus System Microscope BX43 with an Olympus Reflected Fluorescence System*), equipped with a B-3^A (excitation λ = 240-490nm; emission barrier: λ = 520 nm) and a 40x objective lens (UPLFLN PH). The cell images were recorded using a high sensitivity digital camera (*Olympus DP72*). The nuclei were analysed using an imaging software (*cellSens*). A total of 100 erythrocytes per slide were randomly selected and then scored for DNA tail moments (tail length multiplied by fluorescence intensity in tails) using an automatic image-analysis system (*TriTek CometScore™ Freeware*).

The DNA damage recovery test data for tilapia obtained from the three lakeshore towns of Gonzales, Bañaga and Balakilong were compared using the *Kruskal-Wallis test* at a 5% significance level. To further determine the significance of difference in the mean tail moments among the three sample sites, a post-hoc analysis without using a *Bonferroni* correction was perfomed [12]. This was done by calculating for the eta-squared (η^2) effect size estimate [13].

Micronucleus Test Procedures and Analysis:

Fish samples were collected from each site with the same criteria as that for the comet assay. Then five fish per site were randomly selected to be used for testing. For each fish, blood samples were taken by caudal puncture using citrated syringes. The extracted blood was immediately smeared on a clean glass slide. After 24 hours, the material was fixed in absolute methanol for 10 minutes and was stained with 5% Giemsa for another 20 minutes [8]. The micronucleus was identified using the criteria cited in a study from Ray *et al*. [14]. To be considered a true micronucleus the object must be (1) containing a nuclear material (2) completely separated from the parent nucleus; (3) less than 1/5 of the parent nucleus; (4) staining lightly; (5) fragmented; (6) circular to oval in shape; (7) found within 4-fold the shortest axis of the parent nucleus; and (8) not more than two present in the cell. All the micronuclei which satisfy the following criteria were counted.

Cells with two nuclei present were considered as nuclear abnormalities, specifically, binucleates. Other nuclear abnormalities of the erythrocytes were classified as blebbed, notched and lobed nuclei [8, 15]. The frequency of micronucleus and nuclear abnormalities was expressed per 2000 erythrocytes. For the statistical analysis of the data obtained from both parameters, a One-way Analysis of Variance (ANOVA) using Randomized Complete Block Design (RCBD) with 95% confidence level was used to verify if the environmental condition of the Taal Lake and other factors significantly increased the micronuclear frequency and the nuclear abnormality frequency.

The frequency count of the two treatment groups (Bañaga and Balakilong) was compared to that of the control group (Gonzales) using Dunnett's Test. To further determine the significant difference in mean frequency count among the three groups, Tukey's HSD (Honestly Significant Difference) Test was used.

Results:

The physico-chemical analysis of water samples from the three sites is shown in Table 1. The obtained temperature, pH values, and dissolved oxygen values for all three sites are within the Department of Environment and Natural Resources (DENR) [16] criterion for Class C waters suitable for propagation and growth of fish and aquatic resources.

For all three test sites, total nitrogen in water was obtained to be 0.63 mg/L, 0.94 mg/L, and 1.73 mg/L for Gonzales, Balakilong, and Bañaga respectively. Total phosphorus in water was detected in each of the three sites in the aforementioned order at 1.58 mg/L, 1.87 mg/L, and 1.64 mg/L. Total ammonia was detected at all three sites at 0.39 mg/L. The total dissolved solids were at 987 mg/L, 972 mg/L, and 953 mg/L; whereas the total suspended solids were detected to be less than 2.0 mg/L for all three sites. Cadmium and lead were not detected in the waters from all three sites despite the detection limits set at 0.005 mg/L and 0.03 mg/L, respectively. For copper, 0.02 mg/L were detected in Balakilong, and in the

Table 1: Physico-chemical parameters of water samples from the three sites in Taal Lake.

Parameter	Gonzales, Tanauan	Bañaga, Agoncillo	Balakilong, Laurel	DENR Standards [16]	Aquaculture Tolerance Range [17, 18,19,20,21]
Exact Location	N 14º 4.069' E 121º 4. 342'	N 14 º 0.832' E 120 º 57.159'	N 14º 3.838' E 120º 56.266'	-	-
DO (mg/L)	7.36	6.35	6.37	>5	>5.5
pH	8.26	8.16	8.10	6.65-8.5	6.5-9.0
Temperature (˚C)	29.35	29.75	29.88	28.3	26-28
Copper (mg/L) [a]	0.02	ND[b]	0.02	<0.05	0.02
Cadmium (mg/L)[a]	ND[b]	ND[b]	ND[b]	<0.01	0.01-0.5
Lead (mg/L) [a]	ND[b]	ND[b]	ND[b]	<0.05	0.0032
Zinc (mg/L) [a]	0.05	0.05	0.05	<0.05	<.05
Total P (mg/L)	1.58	1.64	1.87	0.05-0.1	0.1
NH_4-N (mg/L)	0.39	0.39	0.39	0.18	<0.025
Unionized Ammonia/ UIA	0.4476	0.4476	0.4476	<0.60	<0.019

[a]Flame Atomic Absorption Spectrometry (Section 3111), "Standard Methods for the Examination of Water and Wastewater", 16th edition, American Public Health Association (APHA), 1015 Fifteenth Street NW, Washington, DC 20005; [b]Not Detected

Table 2: Physico-chemical parameters of sediment samples from the three sites in Taal Lake.

Parameter	Gonzales, Tanauan	Bañaga, Agoncillo	Balakilong, Laurel	Aquatic Tolerance Standards [2, 21, 22, 23]
Moisture Content (%) [b]	0.76	2.25	1.97	-
Organic Matter (%)	0.36	0.26	2.34	-
Organic Carbon (%)	0.21	0.15	1.36	<5
NH_4^+-N (mg/L)	3.53	14.14	22.97	-
NH_3-N [f] (mg/L)	3.33	13.35	21.68	-
NO_3-N (mg/L)	1.77	Trace	1.84	
Unionized ammonia (NH_3) [f] (mg/L)	3.96	16.02	26.02	0.019
Ionized ammonia (NH_4^+) [f] (mg/L)	4.33	20.83	33.83	-
Total ammonia [f] (mg/L)	8.29	36.85	59.85	100
Cadmium [a,c] (mg/kg)	ND[e]	ND[e]	ND[e]	4.2
Copper (mg/kg) [a]	20.2	52.7	29.9	<18.7
Lead (mg/kg) [a,d]	ND[e]	ND[e]	ND[e]	35
Zinc (mg/kg) [a]	55.2	53.6	45.2	<90.0

[a]Flame Atomic Absorption Spectrometry. US EPA Test Methods for Evaluation of Solid Wastes, Physical and Chemical Methods, CD ROM SW-846, 1994.
[b]Gravimetric. US EPA Test Methods for Evaluation of Solid Wastes, Physical and Chemical Methods, CD ROM SW-846, 1994.
[c]Detection limit for cadmium (1 mg/kg); [d]Detection limit for lead (5 mg/kg); [e]Not Detected
[f]Computed based on USEPA (1999) Guideline

non-aquaculture site Gonzales, but not in Bañaga. Across all sites, 0.05 mg/L of zinc was detected. For the sediments, cadmium and lead were not detected in the three sample sites. However, copper was detected at 25.9 mg/kg in Gonzales, 29.0 mg/kg in Balakilong and 52 mg/kg in Bañaga (Table 2).

The Comet Assay:

The mean tail moments were recorded for all three sites with Site 1 Gonzales as the non-aquaculture site. Representative comet images from the Gonzales, Balakilong and Bañaga specimens are shown in Plate 1.

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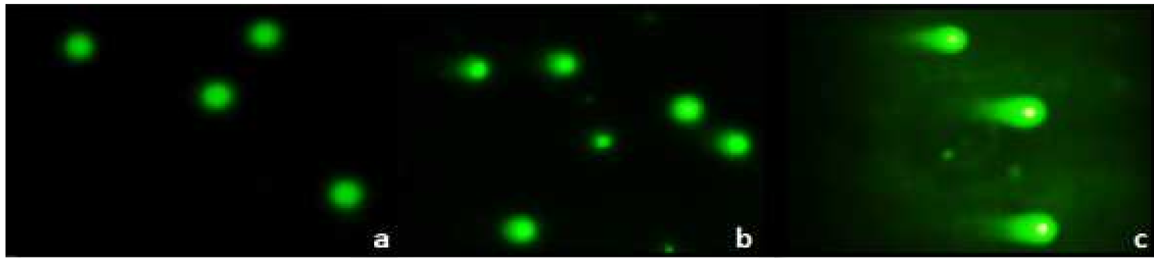

Plate 1: Comet assay results obtained from *Oreochromis. niloticus* erythrocytes from the non-aquculture site (a) Gonzales, and from the aquaculture sites (b) Bañaga and (c) Balakilong.

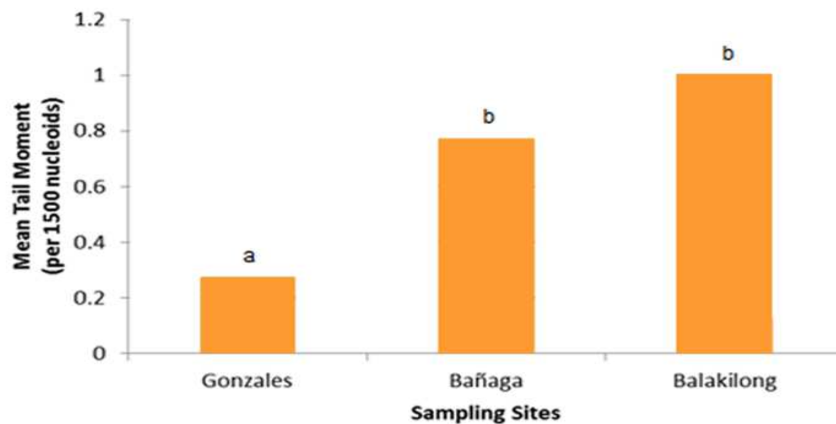

Fig. 2: shows the mean tail moment and the mean tail moment rank for the 1500 erythrocytes analysed per site. The Kruskal-Wallis Test was performed on the tail moments of the erythrocytes sampled from each site. The IBM SPSS Statistics 20 was used to perform the subsequent statistical analyses.

The mean tail moment of erythrocyte nucleoids obtained from Balakilong (1.004558586) was the highest compared to those from Bañaga (0.771479733) and Gonzales (0.273308374). The obtained significance is much less than the set $p < 0.05$ and based on the effect of size estimate for all three sample sites, 26.0% of the variability in tail moment is accounted for by the difference in sample site. This thereby relates to a large effect accounted for by the site parameter, according to the guide by Cohen [24].

To determine where the differences lie, the Kruskal-Wallis test was performed between the following site groups: the non-aquaculture site Gonzales and the aquaculture site Bañaga, the non-aquaculture site Gonzales and the aquaculture site Balakilong, and the two aquaculture sites Bañaga and Balakilong. In the post-hoc analysis for the tail moments from Gonzales and Bañaga, the significance is much less than the level of significance set at $p < 0.05$. The statistical test shows that there exists a significant difference between the two sample groups. Employing the effect size estimate for the data from Gonzales and Bañaga, 20.4% of the variability in tail moment is accounted for by the difference in sample site. This can be interpreted as a large effect of the site parameter on tail moment for these two groups.

The analysis for the tail moments showed that there exists a significant difference between the two sample groups, Gonzales and Balakilong. Employing the effect size estimate for the data from Gonzales and Balakilong, 35.1% of the variability in tail moment is accounted for by the difference in sample site. The post-hoc analysis for the tail moments showed that there exists an insignificant difference between the two sample groups, Bañaga and Balakilong. Employing the effect size estimate for the data from the two aquaculture sites, only 3.1% of the variability in tail moment is accounted for by the difference in sample site.

The Micronucleus Test:

The mean micronucleus and nuclear abnormality frequencies were recorded for all three sites. Plate 2 shows representative micronuclei present in samples obtained from Gonzales, Balakilong, and Bañaga.

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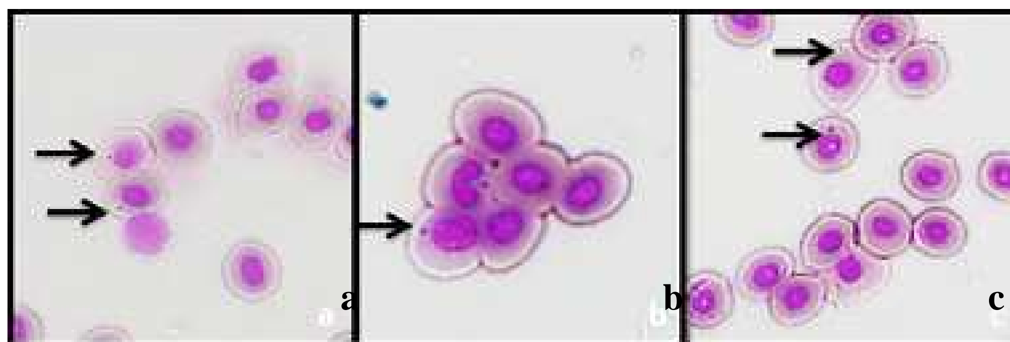

Plate 2: Photomicrographs of micronuclei from circulating erythrocytes of *O. niloticus* obtained from (a) Gonzales, (a) Bañaga, and (c) Balakilong.

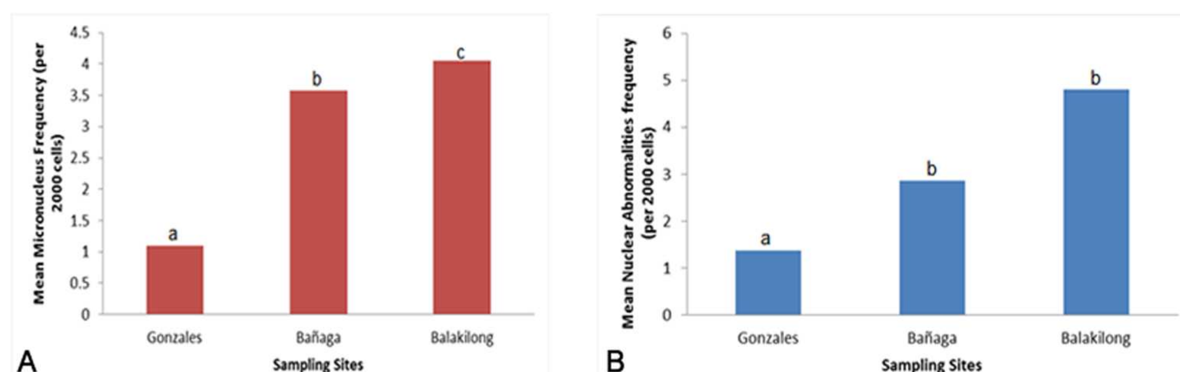

Fig. 3: (A) Shows mean micronucleus frequencies of *O. niloticus* erythrocytes, whereas (B) shows the mean nuclear abnormality frequencies of *O. niloticus* erythrocytes from three sampling sites in Taal Lake. Differences in letters indicate significant differences using the Tukey's HSD Test.

The mean frequency counts for the three sites were compared using multiple comparison Post hoc tests. Tukey's HSD test showed that the mean micronucleus frequency for all the three sites is statistically significant. On the other hand, Tukey's HSD test for mean nuclear abnormality showed that the mean comparison between M1 –M2 (Gonzales and Bañaga) and M1-M3 (Gonzales and Balakilong) are statistically significant while that of M2-M3 (Bañaga and Balakilong) is not. Figure 3 shows the mean micronucleus and nuclear abnormalities frequencies between three sites, Gonzales, Bañaga and Balakilong.

Discussion:

Physico-Chemical Analysis:

The chemical analysis of water and sediments shows that Taal Lake falls under Class C status [16]. According to section 68 of DENR Administrative Order, this makes the lake beneficial for the following uses: (1) fishery water for the propagation and growth of fish and other aquatic resources; (2) recreational water Class II (boating, etc.) and (3) industrial water supply Class I for manufacturing processes after treatment. This research did not aim to sort out the specific pollutants responsible for the detected genetic damages; the objective was to measure the physico-chemical parameters and to determine the presence and levels of contaminants in the water samples. From the results, specific pollutants may be pointed out as the causative agents of the observed genotoxicity in the circulating erythrocytes.

The Comet Assay and Micronucleus Test:

The results showed an increase in the migration (i.e damage to DNA) in *Oreochromis niloticus* obtained from both aquaculture sites as compared to non-aquaculture site (Plate 1). Because of this relatively greater fragmentation, the DNA fragments from the aquaculture sites move more rapidly in the agarose gel when subjected to an electrophoretic run, thereby allowing the characteristic comet shape to develop (Plate 1). Based on the statistical analysis conducted on the three sample groups, there exists a significant difference in the tail moments across the three sample groups. This reveals significant genotoxic effects of water and/or sediments from aquaculture sites (Bañaga and Balakilong) compared to the water and/or sediments from non-aquaculture site (Gonzales).

In the post-hoc analyses conducted between the non-aquaculture site and the two aquaculture sites, significant variability for each comparison was observed. The effect size estimate obtained may be interpreted as a large effect of the site parameter on tail moment for these two groups. The fragmentation of DNA from aquaculture site samples is generally more pronounced as compared to the Gonzales samples. This is consistently observed in the comparisons of the samples from the Balakilong and Bañaga sites against samples from the Gonzales site. The difference in the degree of DNA fragmentation may be indicative of the differences in environmental quality between the aquaculture sites and the non-aquaculture site.

On the other hand in the post-hoc analysis between samples from the two aquaculture sites, a small to negligible variability was observed. This variability can be interpreted as a small to insignificant effect of the site parameter on tail moment for the two aquaculture sample groups. This absence of significant difference in the analysis between the samples from Balakilong and Bañaga indicates that the environmental quality between the two aquaculture sites does not vary significantly.

Despite the ability of the comet assay to detect double-strand breakage, and to some extent single-strand breakage in cell nucleoids, the test is not able to specifically identify the clastogenic agent responsible for the damages observed. This research can only hypothesize based on the results from the physico-chemical analyses that the particularly elevated levels of ammonia complexed with copper in the water samples may account for the difference in DNA fragmentation and tail moment between the samples from the two aquaculture sites and the samples from the non-aquaculture site. It is also modest to assume that the detection of overall genotoxic damage is due to multiple contaminants other than those measured in the present study.

Both micronucleus and nuclear abnormalities were seen in all three sites. However, aquaculture sites also revealed higher and statistically significant difference in frequencies as compared to the non-aquaculture site. The presence of micronucleus in erythrocytes indicated the presence of pollutants that are able to degrade the water quality along Taal Lake. Several studies [6,8,9] relate the presence of different pollutants with the formation of micronucleus. It noteworthy that despite the absence of cadmium and lead in the sediment samples, unionized ammonia (NH_3) and the heavy metal copper were detected in elevated levels.

Obiakor et al. [25] studied the genotoxicity of copper, zinc and their binary mixtures using both comet assay and micronucleus test. Their results revealed that the highest frequency of micronucleated erythrocytes was seen in fishes exposed to Cu and Zn mixtures. Earlier observations on genotoxicity of Cu have been reported also by [26] and [27], with the latter elucidating the genotoxicity of copper sulphates to planaria by means of comet assay. Non- specific binding of Cu2+ cations to essential sites in the enzyme molecules is presumed to be the cause of inhibition of DNA repair enzymes, elevated levels of DNA strand breakage and chromosome fragmentation. A newer study by [28,29] indicated that contamination due to copper exposure caused an approximately three-fold increase in reactive oxygen species (ROS) production, a poorer antioxidant response element (ARE) binding ability, and an increased caspase-3 signaling-modulated DNA fragmentation in the fish muscle. It can be assumed that Cu and Zn act as aneugens, which induce aneuploidy resulting in chromosomal rearrangement and micronucleus formation [25]. This further reflects the formation of micronuclei in the circulating erythrocytes of fishes from all three sites with elevated levels of Cu (Table 1).

Unionized ammonia (NH_3), the toxic form of ammonia, was also detected in the samples from the three sites (Table 1). The presence of NH_3 can cause the degradation of microtubular-associated proteins, which causes alterations of the microtubular network, inducing mitotic spindle damage, hence MN formation [30]. Table 2 shows that Balakilong has the highest amount of NH_3 in sediments and likewise has the most number of micronucleus and nuclear abnormalities. Sediments can accumulate large quantities of ammonia that maybe taken up by benthic fish, such as tilapia, which could then elicit biomarker responses from the fish [31], especially at conditions of low salinity and elevated temperature [32]. Just like copper, ammonia has been demonstrated to induce intracellular reactive oxygen species (ROS), which subsequently lead to DNA damage and cell apoptosis [33].

Correlation of Data Using the Genotoxicity Assays:

Among the sampling sites, Balakilong has the greatest DNA damage and amount of chromosome aberrations as revealed by the Comet Assay and Micronucleus Test. Bañaga showed slightly lower results for both tests, but still significantly different from the samples from Gonzales. The gradient established by cumulative results of Comet Assay and Micronucleus Test is as follows: Balakilong > Bañaga > Gonzales. Both tests identified Balakilong as the heavier aquaculture site compared to the other aquaculture site Bañaga. Both tests were able to establish a significant difference between the data obtained from the non-aquaculture site Gonzales and the aquaculture sites Balakilong and Bañaga.

It is important to note that despite the sensitivity of both tests in detecting DNA damage, the source of clastogenic agent cannot be immediately identified due to the non-specific nature of the Comet assay and the Micronucleus Test. Moreover, the incidence of strand breaks is essentially reversible as revealed in a study by Russo et al. [6] on the *Gambusia holbrooki*. The study indicates that there are effective molecular mechanisms of repair determining a decrease of DNA damage after removal from the natural environment. Despite the

reversibility of strand breaks and chromosomal aberrations, the presence of pollutants and clastogenic agents enhances the occurrence of these genotoxic effects on the tilapia cells. This therefore points out that the observed strand breaks in the comet assay and the observed chromosomal aberrations in the micronucleus test may have resulted from the sum or combined interactions of separate genotoxic agents such as ammonia, zinc, phosphorus and/or copper.

The use of traditional physico-chemical parameters in the evaluation of water and sediments while helpful, does not give a complete picture of the current ecological status of the environment. It is therefore recommended that these methods be complemented using biomarkers. More specifically, fish- based systems because they utilize the top vertebrate predators in the food chain, allowing for a more representative study on the effects of metabolism and bioaccumulation of pollutants [34]. While the effects are not immediately noticed on the macroscopic level, results from tests such as the comet assay and micronucleus test allow for a more sensitive detection of damages caused by environmental pollutants.

Taal Lake has already been categorized under DENR Class C [2,16]. The increase in pollutant levels in Taal Lake due to improper aquaculture practices such as overstocking, and overfeeding may further contribute to the eutrophication of Taal Lake.

The Taal Lake is not only inhabited by fish from aquaculture farms; the endemic species of freshwater sardine, freshwater snake, and other coexisting species present in the lake face environmental threats due to overexploitation of fishery resources, introduction of pollutants, and the introduction of invasive species such as tilapia, propagated by fish farmers [7,35,36]. Continuing aquaculture practices pose a greater threat to the already dwindling biodiversity of species currently existing in Taal Lake. Moreover, the increase in the levels of pollutants may affect not only the biodiversity and lake water quality, but also the quality of fish species farmed on fish cages in Taal Lake.

Conclusion:

The present study determined the genotoxicological effects of intensive fish cage aquaculture in Taal Lake (Philippines) using comet assay and micronucleus test on Nile tilapia (*O. niloticus*) erythrocytes. Results of the genotoxicity assays showed that DNA fragmentation, micronucleus formation, and the presence of other nuclear abnormalities are related to the overstocking and intensive feeding practices in the aquaculture sites. Higher levels of ammonia (especially in its unionized form), copper and zinc in the Balakilong and Bañaga were detected across all sites. The increase in pollutant levels in Taal Lake due to improper aquaculture practices such as overstocking, and overfeeding may further contribute to the deterioration of Taal Lake. As pollutant effects eventually extend from the molecular level into higher levels of biological organization, stressors linked to intensive aquaculture practice, if unmediated, may eventually render the lake unable to sustain the lives dependent on it. Overall, the present study has further confirmed the potential value and usefulness of the two bioassays (comet assay and micronucleus test) for environmental biomonitoring of aquatic systems that are overburdened by intensive aquaculture activities.

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