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Cytogenetic Analysis of Zebra Fish (*Danio rerio*) Exposed to Water Samples collected from Different Areas of Pampanga River

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Abstract

Toxicity in different bodies of water is widespread due to the presence of both genotoxic and cytotoxic components such as heavy metals that originate from different sources, in which anthropogenic factors play a major causative role. Due to these problems, biomonitoring was developed to keep things in check and to know firsthand the danger of these toxic substances in living organisms. This study aimed to assess the level of toxicity of water samples collected from Pampanga River. Three specific stations were chosen: Macabebe (T1), Sulipan (T2) and Candaba (T3). Zebra Fish (*Danio rerio*) were exposed to these collected water for seven (7) days along with three other controls: T0 (Zebra Fish exposed to Purified Water), T+1 (Zebra Fish exposed to water with 1ppm Copper) and T+2 (Zebra Fish exposed to water with 1ppm Lead). Three zebra fish were exposed to different treatment for 7 days. Gills were collected from the specimen and subjected to cytogenetic procedures to acquire c-metaphase cells.

Cytogenetic analysis revealed the occurrences of chromosomal abnormalities such as Ring Chromosome, Chromatid Gap, Chromatid Break, Endoreduplicated Chromosome and Chromosome fragments in c-metaphase. Analysis of variance revealed the significant occurrence of chromosomal abnormalities in each station.

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Duncan multiple range test shows that the two positive controls (T+1 and T+2) produced the highest means of chromosomal abnormalities followed by the three sampling stations (T1, T2 and T3). The (T0) negative control did not produce any occurrence of chromosomal abnormalities. Based on the results T+2 (7.667) has the highest occurrence of chromosomal abnormalities followed by T+1 (6.667), T3 (3.33), T1 (2.333) and lastly, T2 (2.000).

Results suggest that the water samples from the three stations are able to produce chromosomal abnormalities indicating the presence of DNA intercalating agents in Pampanga River.

Keywords: chromosomal abnormalities; cytogenetics; heavy metals; ring chromosome.

1. Introduction

Environmental issues are common problems faced by different nations all around the world. These are considered to cause a universal crisis for different living organisms from the simple to the more complex ones. These problems range from air to land and even the bodies of water, which constitute about 71% of the earth's surface, have not been spared [1]. Contaminations on bodies of water are widespread in marine and freshwater ecosystems. Water pollution is a medium in which serious human diseases can be spread and one factor which diminishes water quality [2]. Polluted waters may have different kinds of contaminants which may be carcinogenic, mutagenic and/or teratogenic. Heavy metals are contaminants which could be introduced into the aquatic systems. These are being utilized in a variety of ways in industries, agriculture, food processing and households and in many forms resulting to its alarming rate of accumulation [3]. Heavy metals are a threat to human health because they have been proven to be highly toxic, are not disintegrated as well as decomposed in the environment and can bioaccumulate along the food chain [4]. Most heavy metals that can be found in freshwater are cadmium, mercury, zinc, copper, nickel, lead and chromium [5]. Of these, lead, cadmium and mercury were proven to have an adverse effect on humans [6]. Effects of heavy metals include the reduction or inhibition of enzyme activity, intercalation of DNA, and modification of biomolecules which may result in cellular abnormalities [7]. For instance, copper shows inhibitory effects in DNA repair in a freshwater planarian [8]. Lead can cause chromosomal abnormalities such as ring chromosome, breakage and gaps. Mercury can induce genetic damage reflected in the cytogenetic and molecular profile of some freshwater organisms [7]. Pampanga river is noted for some contamination of heavy metals such as lead and cadmium [7], arsenic [9,10] and copper [10,11,12]. Nucleus alteration and micronuclei formation on Nile tilapia and mud fish collected from Pampanga River were noted also in the studies of De Guzman [10] and Esteban [12].

Cytogenetic analysis can elucidate more the toxicity profile of a water system. Bio-indicators such as Zebra fish can be used to further measure the toxicity [13]. As toxicity can be indicated by genetic damage, zebra fish epithelial tissues are the best source of chromosome collection for cytogenetic analysis. Hence, this study was conducted to determine the cytogenetic/chromosomal damaging effect of collected water samples from selected areas of Pampanga River on zebra fish (*Danio rerio*) which will reflect the toxicity of water station on the genetic material level.

2. Methodology

2.1. Research Design

This study was done to assess the occurrence of chromosome abnormalities found on the gill cells zebra fish (*Danio rerio*) which was exposed to different water samples collected from three (3) specific stations of Pampanga River, which were found to be positive for heavy metals such as lead and copper. The gills and fins of the zebra fish were used in chromosomal analysis. No analysis of heavy metals was conducted on water sample. The basis for choosing the station is the heavy metal analysis done by De Guzman in 2015.

2.2. Experimental Procedure

A total of thirty-five (35) zebra fish was divided into six (6) groups and exposed a span of seven (7) days to different treatments for as follows: T0 (purified water), T1 (water collected from Danga River, Macabebe Pampanga), T2 (water collected from Sulipan River, Apalit Pampanga), T3 (water collected from Dukma River, Candaba Pampanga), T+1 (water with 1ppm copper) and T+2 (water with 1ppm lead). Cytogenetic analysis was done after the exposure time. A total of 50 c-metaphase cells were used for analysis of chromosomal aberrations. Chromosomal abnormalities on the gill cells of the zebra fish were scored and statistically analyzed using Analysis of Variance and LSD test.

2.3. Maintenance of Zebra fish

The Zebra fish was grouped according to their treatment, with one aquarium containing five (5) Zebra fish, each aquarium representing one (1) treatment. The aquaria, each with a uniform dimension of 15 x 10 x 12 inches, were filled with the treatment water to ¾ of their capacity. The set-up was kept at a place with a room temperature (25°C – 28°C) and a water change of 15% (using the same treatment water) was done every four (4) days for seven (7) days period. Flake foods were fed to the Zebra fish twice a day.

2.4 Cytogenetic Analysis Technique for Zebra Fish

After the seventh-day period exposure time, the zebra fish were transferred for six (6) hours in 0.01% colchicine solution. Then, the *D. rerio* were sacrificed. Fins and gills were removed and placed in a hypotonic treatment solution of 0.4% KCl for about 60 to 75 minutes. Subsequently, the gills and fins were then exposed to a fixative solution (3:1 methanol:glacial acetic acid). The tissues were then cleansed and digested using 65% glacial acetic acid. Giemsa was then added during the preparation of the slides.

2.5 Chromosome and Karyotype Analysis

With the aid of a binocular electric microscope, 100 C-metaphase mitotic cells per experimental animal were examined for chromosomal aberration. The chromosome spread was checked under LPO and HPO. The chromosomal aberrations such as breaks, gaps, dicentric, polycentric, desperalized, complex sticky, and rings (centric and acentric) were identified and counted under the oil immersion objective. For analysis and

documentation, the slides were photographed using digital camera (Panasonic). The karyotype was prepared to assess further abnormalities. The photos, under a dark background, were used to determine the chromosomal structure in detail.

3. Results and Discussion

3.1 Normal Chromosome Spread and Karyological Profile of Normal Male Zebra Fish

Figures 1 and 2 below present the normal karyotype and normal spread of T0 normal Zebra Fish chromosome, respectively. The total chromosome number of Zebra Fish is 2n=50. Nearly all chromosomes are metacentric or submetacentric, with only a few acrocentric chromosomes [14]. Some homologous pairs may vary a little in length but this can be explained by technical factors or due to condensation delays.

Figure 1: Normal Karyotype

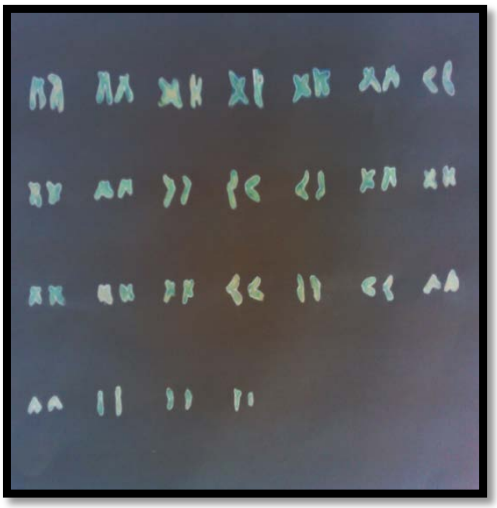

Figure 2: Normal Chromosome Spread

3.2 Different Chromosomal abnormalities of Danio rerio exposed to Different Treatments

There were five different chromosomal abnormalities observed in this study. These are ring chromosome (Figure 3), chromatid break (Figure 4), chromatid gap (Figure 5), endoreduplication (Figure 6), and fragmentation (Figure 7).

Ring chromosome occurred in T2 with a mean of 1.33 and T+2 with a mean of 0.67. A ring chromosome occurs due to deletion of both ends of the chromosome leading to rotational mechanism of a chromosome or a long fragment of chromosome (Medina, 2009). Chromatid breaks were noticed in T1 with a mean of 1.33, T3 with a mean of 2.33, T+1 with a mean of 1.33, T+2 with a mean of 2.67. Breakage of chromosomes is formed via disruption of the phosphodiester linkage leading to detachment of a certain fragment. Chromosomal gap was observed in T+1 and T+2 with a mean 0.67 for both.

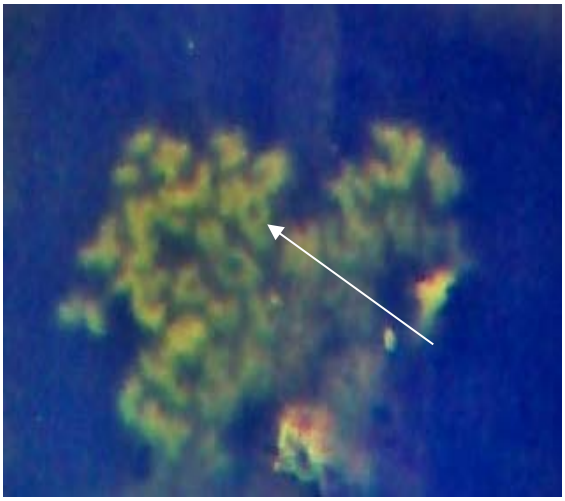

Figure 3: Ring Chromosome

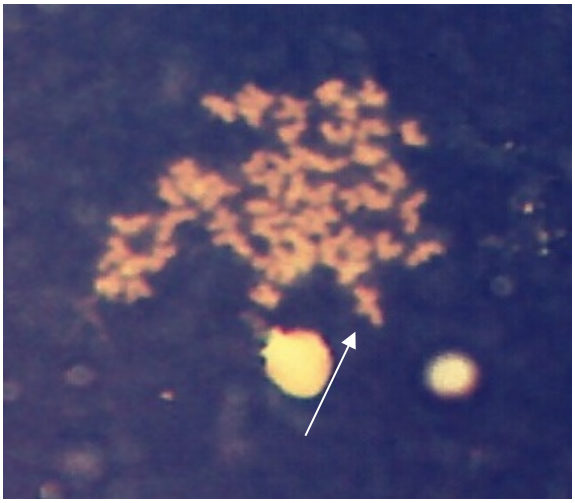

Figure 4: Chromatid Break

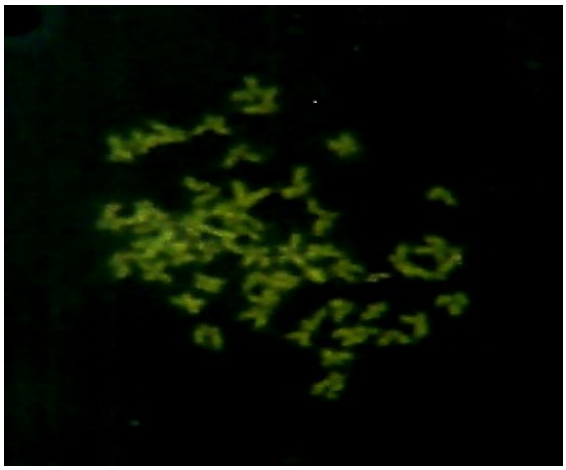

Figure 5: Chromosome Gap

Figure 6: Endoreduplication

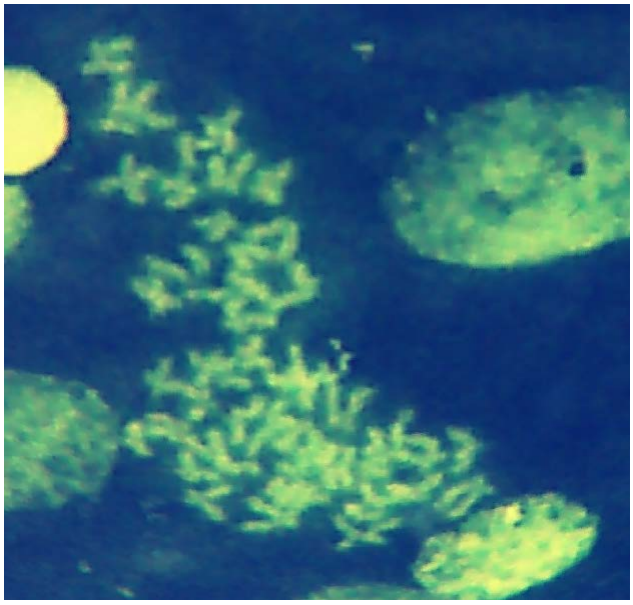

Figure 7: Chromosome Fragment

Endoreduplication was noticed in Zebra Fish that were exposed to T1 and T3, each with a mean of 0.67. This type of abnormality is formed due to rapid irregular DNA replication producing irregular shaped chromosomes. On the other, there is fragmentation observed in T1,T2, T3, T+1 and T+2 with the mean of 0.33, 0.67, 0,33, 4.67 and 4.0, respectively. Fragments are products of segmental breakage of the chromatid or both.

Chromosomal abnormalities were associated with different toxic water contaminants such as lead and copper (Gracilla and Bagunu, 2011). Lead and copper were identified to exist in Pampanga River by DOST (2005), and Esteban and De Guzman (2015). These two heavy metals are known to inhibit DNA synthesis and their intercalating activity hinders the proper formation of DNA thus the formation of different chromosomal aberrations. Deletions, translocation and inversion of DNA are effects of heavy metals that may explain why chromosome breaks and gaps can be observed. These gaps or breakages leave the chromosome sticky at the site of fissure. These sticky ends explain the formation of ring chromosomes as they may attach one end to the other. In addition, because of the intercalating activity of the heavy metals, they affect the bonds between nucleotides thus leading to their separation from one another resulting in gaps, breaks and even fragmented chromosomes.

3.3 Occurrence of Ring Chromosome of Danio rerio exposed to Different Treatments

Table 1: ANOVA of Ring Chromosome

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	4.667	5	0.9333	F (5, 12) = 3.360	P = 0.0396
Residual (within columns)	3.333	12	0.2778		
Total	8.000	17			

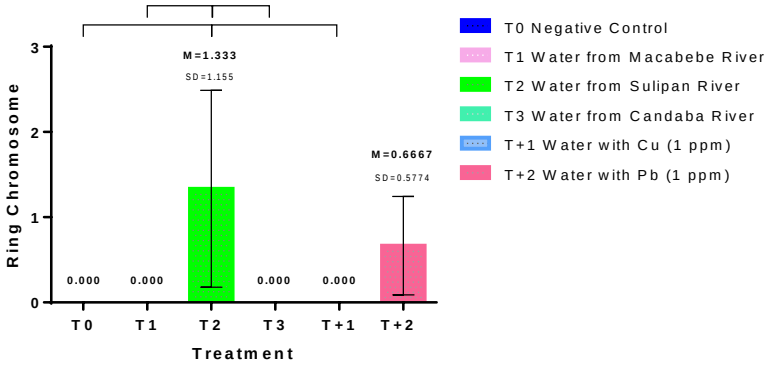

Figure 8: Graphical representation of the means of ring chromosome

There is significant occurrence of ring chromosome in different treatments as reflected in the analysis of variance (Table 1) with F (5, 12) = 3.360 and p-value of 0.0396 which is lower than the alpha value of 0.05. Duncan Multiple range test revealed the significant differences among the treatments. As shown in Figure 8 the highest production of ring chromosome was noticed in T2 (mean= 1.33) which is significantly different from T+2 with a mean 0.67 gaining the second highest mean. This suggests that both water samples possess the ability to induce chromosomal ring formation. In T0, T1, T3 and T+1, there is no occurrence of ring chromosome signifying that the water samples do not possess the ability to induce ring formation.

3.4 Occurrence of Chromatid Break in Danio rerio exposed in Different Treatments

Table 2: ANOVA of Chromatid Break

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	16.44	5	3.289	F (5, 12) = 14.80	P < 0.0001
Residual (within columns)	2.667	12	0.2222		
Total	19.11	17			

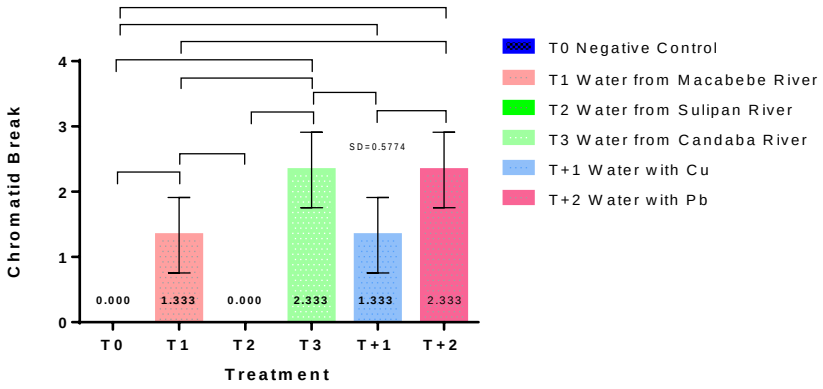

Figure 9: Graphical Representation of Chromatid Break

The ANOVA results, as seen in Table 2, show that with F (5, 12) = 14.80 and p < 0.0001, the differences in the number of chromosomal breaks among the six treatments are statistically significant at the 0.05 level of significant.

Figure 9 above clearly presents the significant differences among the various treatments based on Duncan's Multiple Range Test. The highest mean recorded was found in both T3 and T+2 with the same mean (m=2.33). The absence of statistically significant difference between T3 and the T+2 indicates that the water from Candaba River has a capacity comparable to the lead control to cause chromatid breaks to the zebra fish.

3.5 Occurrence of Chromatid Gap in Danio rerio exposed in Different Treatments

Table 3: ANOVA of Chromatid Gap

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.778	5	0.3556	F (5, 12) = 0.8000	P = 0.5705
Residual (within columns)	5.333	12	0.4444		
Total	7.111	17			

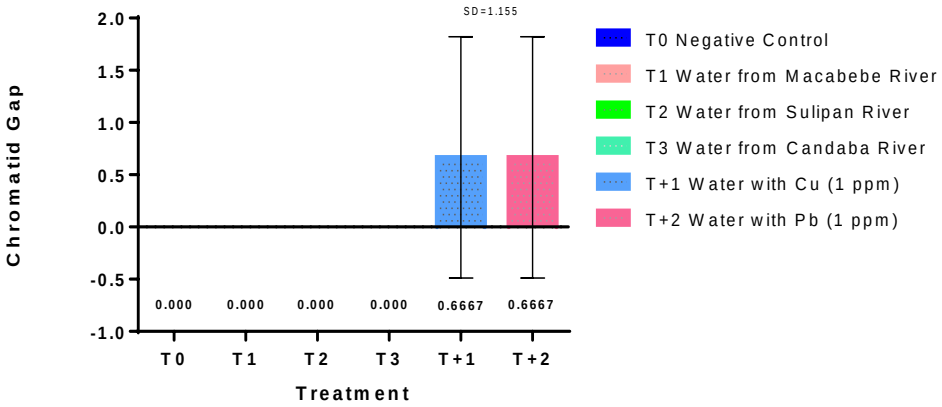

Figure 10: Graphical Representation of Chromatid Gap

The ANOVA results, as shown in Table 3, reveal that at the 0.05 level of significance, with F (5, 12) = 0.8000 and p= 0.5705, the occurrence of chromosomal gap among the six treatments is not significant statistically. Based on Figure 10 above, even though no significant occurrence of chromatid gap was noted, the fact that there is an occurrence might also suggest that T+1 and T+2 could produce this type of abnormality. This implies that the quantity of lead and copper, even though it is noted to be present in water collected from T1 (Macabebe), T2 (Sulipan) and T3 (Candaba), is insufficient to produce this kind of chromosomal abnormalities. The probable reason why the chromosome gap is insignificant statistically is that it can be repaired faster compared with other chromosomal abnormalities (Medina as cited by Gracilla, 2010).

3.6 Occurrence of Endoreduplication in Danio rerio exposed to Different Treatments

Table 4: ANOVA of Endoreduplication

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	1.778	5	0.3556	F (5, 12) = 3.200	P = 0.0458
Residual (within columns)	1.333	12	0.1111		
Total	3.111	17			

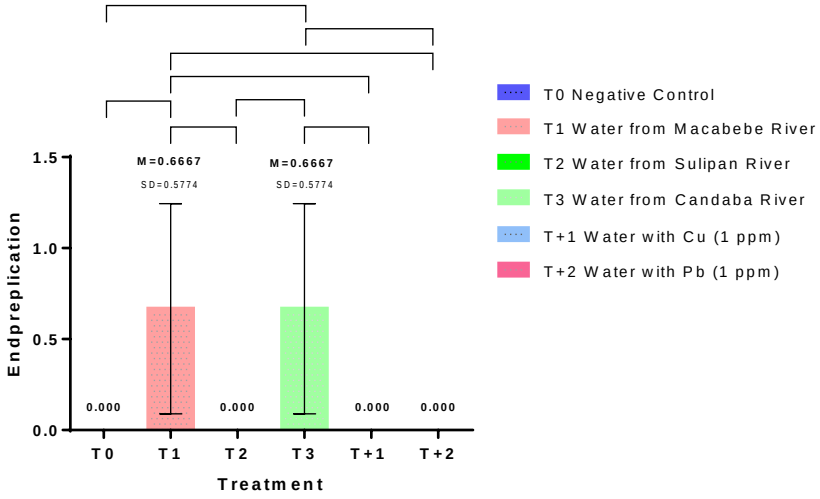

Figure 11: Graphical Representation of Endoreduplication

Table 4 shows that there is a significant occurrence of endoreduplication with F (5, 12) = 3.200 and a p-value of 0.0458, which is lower than the alpha value of 0.05. Duncan multiple range test revealed that T1 and T3, each with mean of 0.67, are significantly different from T0, T2 and T+1 and T+2 which does not have any occurrence of endoreduplication. The fact that endoreduplication exist only in T1 and T3, but not in other treatments, suggests that there could be other contaminants that caused the endoreduplication aside from the previously identified heavy metal contaminants (lead and copper).

3.7 Occurrence of Fragmentation in Danio rerio exposed in Different Treatments

Table 5: ANOVA of Fragmentation

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	65.33	5	13.07	F (5, 12) = 14.70	P < 0.0001
Residual (within columns)	10.67	12	0.8889		
Total	76.00	17			

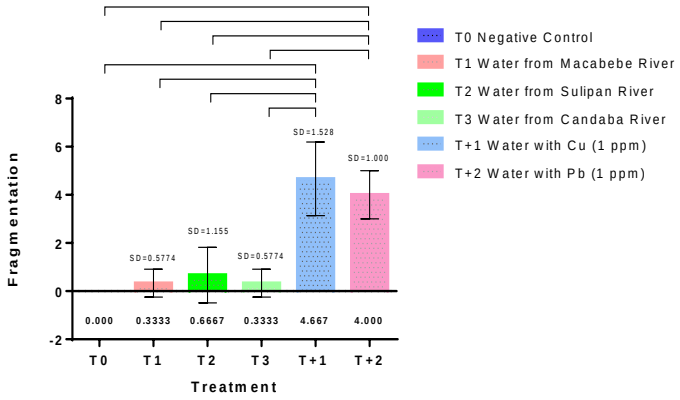

Figure 12: Graphical Representation of Fragmentation

The ANOVA results, as shown in Table 5, reveal that with F (5, 12) = 14.70 and p< 0.0001, the incidence in the number of fragmentation among the treatments are statistically significant. Thus, at the 0.05 level of significant, there is evidence of statistically significant differences in the occurrence of fragmentation. T+1 has the highest mean which is comparable to T+2 which has the second highest mean. The positive controls show significant difference when compared with T1, T2, T3 and T0, as indicated in Figure 12. Even though there is a significant difference no 0 values were obtained except for the negative control (T0). These results suggest that T+1 and T+2 can cause fragmentation on the chromosomes.

3. 8 Total Chromosomal Abnormalities of Danio rerio Exposed to Different Treatments

Table 6: ANOVA of Total Chromosomal Abnormalities

ANOVA table	SS	DF	MS	F (DFn, DFd)	P value
Treatment (between columns)	129.3	5	25.87	F (5, 12) = 29.10	P < 0.0001
Residual (within columns)	10.67	12	0.8889		
Total	140.0	17			

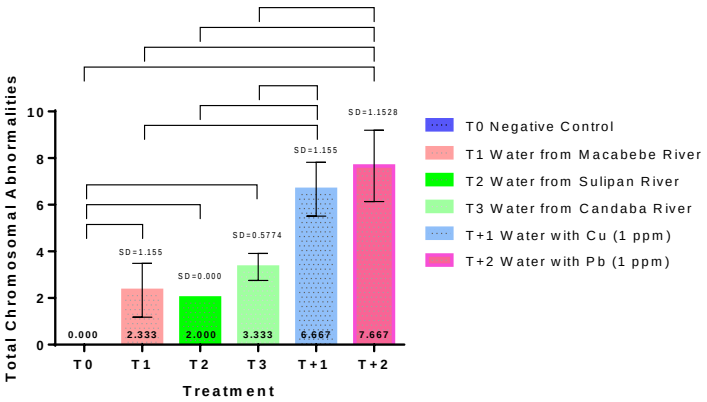

Figure 13: Graphical Representation of Total

Table 6 shows that the differences in the overall occurrence of chromosomal abnormalities are significant with F (5, 12) = 29.10 and a p< 0.0001. Duncan multiple range test revealed that there is significant difference among the treatments. Figure 13 shows the graphical representation of the mean of different treatments in overall chromosomal abnormalities. In general, both positive control (T+1 with mean of 6.67and T+2 with mean of 7.67) are significantly different with T1 (mean = 2.333), T2 (mean=2.0) and T3 (mean=3.33) and T0 (mean=0). The T0 (negative control) does not produce chromosomal aberration in general, and is not comparable with T1, T2 and T3. This suggests that the water samples from Pampanga River possess a chromosomal damaging activity on *Danio rerio* that is comparable with water treatment containing 1ppm of both lead and copper.

The fact that the water samples from Pampanga River produced chromosomal abnormalities suggest that toxic substances continue to exist in the water. The previously identified toxic substances, such as lead and copper (Esteban and De Guzman, 2015), are still existing in the river and may be the cause of the overall production of chromosomal abnormalities. Chromosomal abnormalities that were found in the positive controls as well as from the collected water samples from Pampanga River (T1, T2, T3) were chromatid break, ring chromosome and chromosome fragments. Chromatid gaps are only found in the positive control while the endoreduplication is only found in the T1, T2 and T3.

The study by Arya et al. (2013) on the root cell of *Allium cepa* and *Viciafaba*, Harabawy et al. (2014) on Nile tilapia, and Nagoankar et al. (2015) have proven both the genotoxic and cytotoxic effects of lead and copper since these heavy metals produce chromosomal abnormalities. Effects of these heavy metals include the reduction or inhibition of enzyme activity, intercalation of DNA and the modification of biomolecules (Medina, 1990 as cited by Gracilla, 2010) which can cause chromosomal abnormalities.

Study 9. Presence and Absence of chromosomal abnormalities on the different treatments

Table 7: Comparative Summary on the presence of chromosomal abnormalities in different water treatments

Type of Chromosomal Abnormalities	**T0**	**T1**	**T2**	**T3**	**T+1**	**T+2**
Ring	-	-	+	-	-	+
Chromatid Break	-	+	-	+	+	+
Chromosome Gap	-	-	-	-	+	+
Endoreduplication	-	+	-	+	-	-
Chromosome Fragments	-	+	+	+	+	+

Chromosomal abnormalities such as ring found in the positive control (water with 1 ppm Cu) were also found in T2 (Sulipan, Apalit River). This suggests that the ring formation maybe attributed to the presence of the two heavy metal lead and copper as previously reported by Canaria (2008) and De Guzman (2015). That chromatid

break occurs in both positive control (T+1 and T+2), T1 and T3 signifies the presence of heavy metals. Chromosome fragments occurred in both positive control and in T1, T2 and T3 but not in T0. This supports the data above on the other chromosomal abnormalities.

Endoreduplication occurs only in T1 and T3. This suggests that there might be other contaminants that caused this chromosomal aberration because it is a unique abnormality not found on other treatments.

On the other hand, chromatid gap is produced only in the two positive controls suggesting that both possess the capability of inducing the abnormality. This type of abnormality repairs easily as compared to other abnormalities.

Chromosome gap and ring formation were observed in Nile Tilapia collected from the same area as reported by Gracilla and Bagunu (2011). The present result strongly supports the findings of Gracilla and Bagunu (2011) and shows that the three areas of the Pampanga River still possess toxicity at the cytogenetic level. Thus, the non-occurrence of this abnormality in other treatment might due to the strong repair potential of chromosome gap.

4. Conclusion

There are chromosomal abnormalities on gill cells of Zebra Fish (*Danio rerio*) exposed to collected water samples from Pampanga River. The types of chromosomal abnormalities induced by the water sample in Zebra Fish (*Danio rerio*) are ring, chromosome break, endoreduplicated chromosomes, and chromosome fragments. There is significant similarities on chromosomal aberration, particularly ring, break, and fragmentation. There is also notable difference, particularly on the endoreduplicated chromosomes, suggesting that there might be other toxic substances responsible for these specific abnormalities. Finally, it can be concluded that Pampanga River continues to be contaminated with heavy metals, which pose a continuing threat not only to Nile tilapia but also to other organisms thriving in that ecosystem. Human beings are also under threat because of the bioaccumulation of heavy metals in the fish that they consume from the river.

5. Recommendation

The presence of abnormalities in zebra fish sample exposed to collected waters from Pampanga River reflects the toxicity of the three stations.

From the findings of this study and previous reports on the presence of heavy metals, it is recommended that bioaccumulation studies be conducted and actions and programs be designed to remove or reduce the pollutants of Pampanga River.

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