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Genotoxicity Mutation in the Nile tilapia - *Oreochromis niloticus* (Linnaeus, 1758) Exposed to Sub-Lethal Doses of Selected Water Pollutants

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ABSTRACT

Environmental pollution from heavy metals (HM) poses serious threats to aquatic lives. Fish are particularly vulnerable to sub-lethal doses of HM and may lead to genetic mutations and long-term ecological impacts. The study assessed the cytogenetic effects of sub-lethal doses of HM—cadmium (Cd), copper (Cu), iron (Fe), and lead (Pb)—on *Oreochromis niloticus* from Uta Ewa Creek, receiving aluminium effluent in southeastern Nigeria. *O. niloticus* samples were exposed to three different sub-lethal concentrations (25, 50, and 75%) of Cd, Cu, Fe, and Pb for 14 days, while a group (control) was unexposed. Random amplified polymorphic DNA (RAPD-PCR) was performed using four polymorphic markers (with 60–70% polymorphism) to evaluate DNA from all fish groups and to identify genetic mutations associated with HM exposure. Genetic differentiation (Gst) in RAPD markers was also examined. RAPD profiles revealed increasing DNA damage in *O. niloticus* as HM concentrations rose. Cd showed the highest genotoxicity, followed by Cu, Fe, and Pb, compared to control. The control group had 100% GST yield, Pb samples had 30.72% while Cd had 4.33%. These indicated that *O. niloticus* bioaccumulate these HM with all tested doses causing significant alterations to blood DNA nucleotide sequences. Thus, Uta Ewa Creek contains HM at levels that are genotoxic to local fish populations. Therefore, the efficacy of RAPD-PCR as a tool for detecting genotoxic effects of environmental pollutants in aquatic species was affirmed and there is need for monitoring and regulating metal pollution in aquatic ecosystems to protect biodiversity and ecological health.

INTRODUCTION

Fish are one of the main aquatic organisms in the food chain that can accumulate large amounts of certain metals above the levels in the aquatic environment from the diet (via the gastro-intestinal tracts) and their ambient environment (water and sediments) via the respiratory organs (gills) in aquatic organisms **(Oguzie & Okosodo, 2008; Ambedkar & Muniyan, 2011; Bawuro *et al.*, 2018)** since they occupy high trophic levels **(Moruf & Akinjogunla, 2018)**. Moreover, they are important source of protein and essential nutrients **(Blasco *et al.*, 1998; Agah *et al.*, 2009; Akinjogunla *et al.*, 2017;**

2021). Therefore, they are used for assessment of environmental pollution **(Borkovic *et al*., 2008; Akinjogunla & Lawal-Are, 2020)**.

Oreochromis niloticus is an example of finfishes that are used for assessment of environmental toxicology (presence or absence of pollutants/contaminants) **(El-Batrawy, 2013; Almisherfi *et al*., 2023)**. This is because they can accumulate high levels of pollutants in water without mass mortality **(Ahmed *et al*., 2020; Whenu *et al*., 2021)**. They are readily available; widely distributed for repetition and comparison of research; most importantly, they are handy, rugged and can be cultured or reproduced in the laboratory **(El-Sayed, 2015; Alm-Eldeen *et al*., 2018; Abdelzaher *et al*., 2022)**.

There is a major global concern of heavy metal pollution in aquatic environments. There can be bioaccumulation of these heavy metals in the tissues of fish **(Adeyemi-Ale *et al.*, 2021)** and the accumulation of these metals may also flow to the non-target organisms (consumers) (**Ajala *et al*., 2022)**, depending on the level of accumulation or type of heavy metal. This may lead to transmissible mutate ones **(Osman, 2014; D'Costa *et al*., 2017)**. Unfavourable consequences such as reproductive capacities, morphological abnormalities, mutation (alteration) of the gene cells, and abnormal enzymatic actions could be some of the results of the subsequent uptake of heavy metals in the food chain by aquatic organisms and man **(Obaroh *et al.*, 2015)**.

Relevant studies have been carried out to investigate the level of metal bioaccumulation in aquatic organisms, water and sediments **(Bahnasawy *et al*., 2009; Garg *et al*., 2009; Authman *et al*., 2013; Akinrotimi *et al*., 2015; Ayoola & Taoreed, 2015; Samuel *et al*., 2015; Onwuteaka *et al*., 2015; Abdelaiz *et al*., 2019; Moruf & Akinjogunla, 2019; Abiaobo *et al*., 2020; Akinjogunla & Lawal-Are, 2020; Adeyemi-Ale *et al.*, 2021; Shilla & Sawe, 2021; Akinjogunla *et al*., 2023)** and also the genotoxic effects of acute and sub-chronic exposure of some heavy metals to various animal models **(Wang *et al.*, 2009; Zhang *et al.*, 2010; Monteiro *et al.*, 2011; Varotto *et al.* 2013; Nicosia *et al.*, 2015; Ambreen & Javed, 2016; Sohail *et al.*, 2017)** which induced reduction of growth rate, mortality, oxidative stress, damaged DNA and affected embryogenesis.

Several assays and biomarkers have been used to assess the level of genotoxic impacts of these heavy metals on fish **(Farombi *et al*., 2007; Salem *et al.*, 2014)**. Recently, more sensitive and selective assays through advances in molecular biology have been developed **(Livingstone *et al*., 2000; Chevre *et al*., 2003; Sarkar *et al*., 2006; Adedeji *et al*., 2012; Tajik-Esmaelili *et al*., 2017)** among which the rapid amplified polymorphic DNA (RAPD) have been extensively used in this regard. RAPD is a robust, powerful polymerase chain reaction (PCR)-based technique, anchored on random genomic segment amplification, able to detect DNA damage and mutations in different organisms including fish **(Callejas & Ochando, 2001; Rocco *et al.*, 2014)** viz. *O. niloticus* **(Mahboob *et al.*, 2018; Anwar *et al.*, 2022)**.

Understanding the nature of heavy metal-induced effects at the molecular level could provide the required information to proffer options for remediation when such event arises. Since the genetic materials (DNA) composition is similar in all living organisms, a treated *O. niloticus* DNA sample was used in this study to determine the possible mutagenic effects of Cd, Cu, Fe and Pb in the nucleotides sequence rearrangements using Random Amplified Polymorphic DNA (RAPD-PCR) technique. Hence, this study aimed to investigate the potential genotoxic effect of heavy metals pollution in this economically, commercially and commonly consumed fish species, *O. niloticus*.

MATERIALS AND METHODS

Sampling area and samples collection

Uta Ewa Creek is a tributary of Imo River that receives influx of aluminum effluents from Aluminum Smelter Company (ALSCON) and domestic waste discharges from residential estates around the Creek. Imo River lies on latitude 4º30'32" North of the Equator and longitude 7º30'30" East of the Greenwich Meridian. Uta Ewa Creek is surrounded by a semi-rural community whose major occupations are fishing and farming and the inhabitants of this community depend mainly on rain and surface water as the only source of drinking and for domestic purposes **(Abiaobo *et al*., 2020)**.

The Nile tilapia, *O. niloticus* (Fig. 1) was selected for this heavy metal doses response study because it is an economically and commercially important finfish in Akwa-Ibom State. This species is widely consumed by a large populace due to its palatability and availability all year round. A total of 30 healthy (by physical observation) adult-sized *O. niloticus* with an average weight of 48.6±2.14g and an average length of 17.1± 0.81cm were collected. During the peak of dry season, the samples were collected with water from the creek in two (2) 20L open troughs and transported alive to the laboratory for analysis. The specimens were allowed to acclimatize to the laboratory water conditions (temp. - 27.0°C, pH of 6.5, salinity - 5‰) for 14 days under a 12-hour photoperiod and fed *ad libitum* with a commercial diet. The water was changed once a day to reduce fecal contamination. The fish were treated according to the guidelines of the Fisheries and Hydrobiology Unit, Department of Zoology, University of Ilorin, Nigeria.

Fig. 1. Whole mount *Oreochromis niloticus* from Uta Ewa Creek, Akwa Ibom state, Nigeria

Experimental design

Semi-static test conditions were followed for this experiment according to the **OECD (2019)** guidelines. The *O. niloticus* fish were distributed in groups, each containing 10 individuals for dose-response studies and control. Concentrations of the selected water pollutants – cadmium (Cd); iron (Fe), copper (Cu) and lead (Pb) were based on the presence and levels of these environmental metals in the water and sediment samples of the Uta Ewa creek, as reported by **Ekpo and Ukpong (2014)** and *O. niloticus* samples by **Abiaobo *et al.* (2020)**.

For each pollutant, three (3) different concentrations in the range 25, 50 and 75% were respectively chosen denoting a range of doses. Fe (Iron EDTA) concentrations were prepared in the range of 1.8, 3.6, and 5.4mg/ L; Cd (from cadmium chloride) and Cu (from copper sulphate) concentrations were prepared in the range of 0.5, 1.0, 1.5mg/ L each; and Pb from lead nitrate in the range of 5.2, 10.4, and 15.6mg/ L. All the reagents were the products of Sigma-Aldrich®. The fish were exposed to the pollutants for a period of 14 days. The water was discarded and renewed daily along with the dose of the respective pollutant. Another group was maintained in parallel without any pollutant exposure and was used as the negative control.

Blood sampling and DNA extraction

Blood samples were drawn from the caudal vein under sterile conditions from a total of 52 fish (four samples from each concentration). The needle was run as deep as possible through the middle line just behind the anal fin in a dorso-cranial direction. DNA was extracted and purified from whole blood collected samples using a Blood-Animal-Plant DNA preparation kit (Jena Biosci., Germany) according to the manufacturer's protocol. The quality and quantity of extracted DNA samples were verified using 260nm (Nanodrop^{TM} 1000 spectrophotometer, Thermo Scientific., Waltham, MA, USA). The concentrations of extracted DNA samples were adjusted to 25µg/ µL for PCR amplification a QIAamp® DNA blood mini-Kit (Qiagen Germany).

RAPD-PCR amplification

PCR amplification was performed using four (4) commercially available decamer random primers (1 – OPCO4; 2 – OPCO2; 3 – OPBO6 and 4 – OPBO5), chosen arbitrarily based on the guanine and cytosine (GC) content of 60 - 70% polymorphism for these experiments (Table 1). A total reaction volume of 15µl contained 5µg genomic DNA, 0.2 UM of each primer, 1x of *Taq* polymerase buffer, 2 units of *Taq* polymerase (Fermentas). PCR amplification was performed under cycling conditions of 96°C for 4min, followed by 35 cycles of 94°C for 30sec, 55°C for 1min, 72°C for 1min terminated with elongation at 72°C for 10min.

Gel electrophoresis

Length and purity of the PCR products were evaluated by 1.5% (w/v) agarose gel containing ethidium bromide (0.5ug/ml) in 1X Tris Borate EDTA buffer at 95 volts

Table 1. Sequence and amplified band size range of RAPD markers used on DNA of *O. niloticus*

Primers number	Nucleotide Sequences (5' – 3')	Size range (bp)	GC (%)	PIC
1	CCGCATCTAC	497 – 2039	60	0.747
2	GCCAGATCAG	438 – 1985	60	0.661
3	TGCTCTGCCC	302 – 2288	70	0.634
4	TGAGGGCCGT	229 – 2083	70	0.786

Keys: A: Adenine, T: Thymine, G: Guanine and C: Cytosine, PIC – Polymorphic.

Information content

The bands were visualized under ultraviolet light (UV light) using a D-digit gel scanner trans-illuminator and the gels were photographed using a digital gel documentation system (Bio-Rad, USA). DNA fragment sizes were estimated by their comparison with standard molecular size markers.

Estimate of genetic differentiation (Gst %)

Genetic differentiation (Gst) was calculated by using **Nei (1987)** formula:

$$Genetic\ diff.(Gst) = 1 - \frac{Hs}{Ht}$$

Where, Hs is sample gene diversity
Ht is total gene diversity

Statistical analysis

Statistical analyses were conducted using SPSS software, version 16. To identify significant differences between the control and treated groups, a one-way ANOVA was performed. All analyses were evaluated at a significance level of $P<0.05$.

RESULTS

RAPD profile evaluation of selected heavy metal sub-lethal dose treatments in *O. niloticus*

The RAPD profile generated using the four commercially available decamer primers are shown in Fig. (2). The profile shown represent the RAPD profiles for *O. niloticus* in the control and the different pollutants. The banding patterns of *O. niloticus* in the pollutants clearly exhibited differences, while those of the control group were not changed. Polymorphism and monomorphism bands of the different treatment schedules of the heavy metals in the blood of *O. niloticus* are summarized in Table (2), where Pb50% and Fe75% showed the highest number of polymorphic bands (11), closely followed by Primer 2 with sub-lethal doses of 50% Cd and Cu, Primer 3 with sub-lethal

doses of 50% Cd and 75% Fe and Primer 4 with 75% Cd and 25% Fe with 10 polymorphic bands representation each. For cadmium (Cd) treatments, Primer 2 had the highest representations of polymorphic bands of 29, followed by Primer 3 with 27 representations, Primer 4 with 21 and Primer 1 with 18 polymorphic bands representation.

Table 2. RAPD banding patterns of *Oreochromis niloticus* blood by heavy metal treatments using selected decamer primers

	Primer 1		Primer 2		Primer 3		Primer 4	
Heavy metals/ Treatment	a*	b*	a*	b*	a*	b*	a*	b*
Cd*25%*	6	0	7	1	8	0	2	1
Cd*50%*	6	1	10	0	10	0	9	0
Cd*75%*	6	0	12	0	9	0	10	0
Sub Total	18	1	29	1	27	0	21	1
Cu*25%*	5	2	8	1	4	0	9	0
Cu*50%*	5	2	10	1	7	0	9	0
Cu*75%*	6	0	8	1	9	0	8	2
Sub Total	16	4	26	3	20	0	26	2
Pb*25%*	6	1	8	1	6	1	8	1
Pb*50%*	5	2	11	1	6	1	7	2
Pb*75%*	6	3	8	0	9	0[	8	1
Sub Total	17	6	27	2	21	2	23	4
Fe25%	3	1	5	0	6	0	10	2
Fe*50%*	5	2	9	1	9	0	9	3
Fe*75%*	6	0	11	0	10	1	7	0
Sub Total	14	3	25	1	25	1	26	5
Grand Total	65	14	107	7	93	3	96	12

***Note**: a* - Polymorphic bands; b* - monomorphic bands; Cd – Cadmium; Cu – Copper; Pb- Lead; Fe – Iron; 25%, 50% and 75% - sub-lethal does treatment schedules; Primer 1 – OPCO4 ; primer 2 – OPCO2; primer 3 – OPBO6; primer 4 – OPBO5*

All the primers had 1 representation for the monomorphic bands, except for Primer 3. Copper (Cu) had double highest representation of 26 polymorphic bands in Primers 2 and 4, followed by Primer 3 with 20 polymorphic band representations and Primer 1 with 16 polymorphic bands. The highest monomorphic band representations were found in Primer 1 (4 bands), followed successively by Primer 2 with 3 bands and Primer 4 with 2 bands while no monomorphic band was found in Primer 3.

Lead (Pb) has a wide gap representation of 27 polymorphic bands representation in Primer 2, followed by Primer 4 with 23 polymorphic bands.

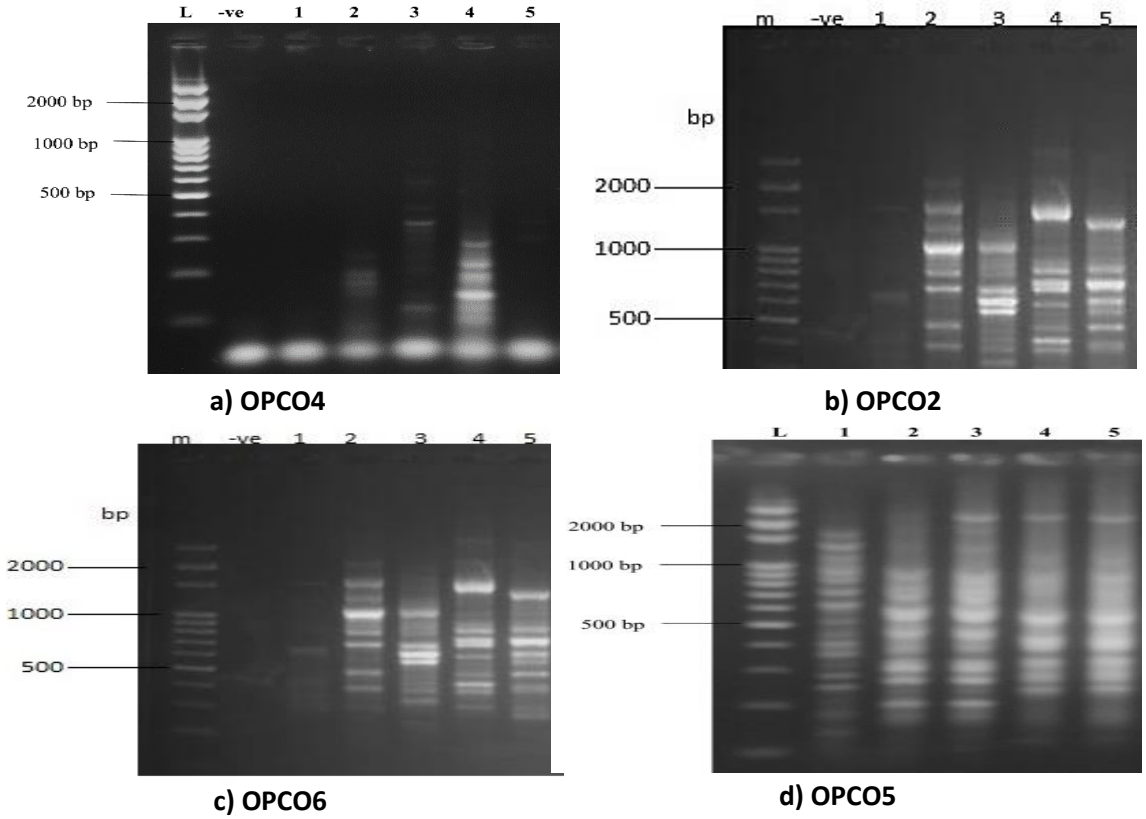

Fig. 2. a, b, c and d: Detection of PCR products of primers on 2% agarose gel electrophoresis on *O. niloticus*

***Key**: M / L: DNA ladder; -ve: negative control; 1: Cadmium treated O. niloticus; 2: Copper treated O. niloticus; 3: Lead treated O. niloticus; 4: Iron treated O. niloticus; 5: Non treated O. niloticus*

The highest monomorphic band counts of 6 were found in Primer 1, while the lowest monomorphic band counts of 2 were found in Primers 2 and 3. For Iron (Fe) sub-lethal dose treatments, Primer 4 had the highest polymorphic and monomorphic band representations of 26 and 5, respectively.

In summary, polymorphic bands occurred in this order: Primer 2 (107 bands) > Primer 4 (96) > Primer 3 (93) > Primer 1 (65) while the monomorphic bands were represented in this order: Primer 1 (14) > Primer 4 (12) > Primer 2 (70) > Primer 3 (3).

Effect of heavy metals on genetic differentiation (Gst) in *O. niloticus*

Estimation of Gst yielded by RAPD markers in *O. niloticus* is illustrated in Fig. (3). The estimated Gst yield for control treatment was at 100% for the species. The highest % Gst yield was for lead (Gst_{Pb}) in *O. niloticus* with 30.72%, copper (Gst_{Cu}) at 17.89%, iron (Gst_{Fe}) at 14.81%, while the least was cadmium (Gst_{Cd}) with 4.33%.

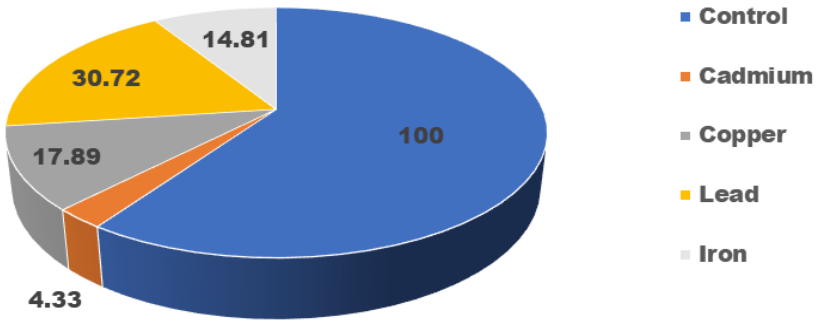

Fig. 3. Estimate of genetic differentiation (Gst %) yield by RAPD markers in *O. niloticus* exposed to heavy metal

In Fig. (4), the trend showed a decline in Gst as the sub-lethal doses (25, 50 and 75%) increases for all evaluated heavy metals (Cadmium, copper, lead and iron) in *O. niloticus*. The highest and lowest Gst evaluated for all metals were recorded for cadmium (Gst_{cd}) at 78.27 and 4.42% at sub-lethal doses of $Cd_{25\%}$ and $Cd_{75\%,}$, respectively, while evaluation of Gst for Iron (Gst_{Fe}) had the closest margin of 12.94 and 11.46% at $Fe_{50\%}$ and $Fe_{75\%}$ sub-lethal treatment doses, respectively.

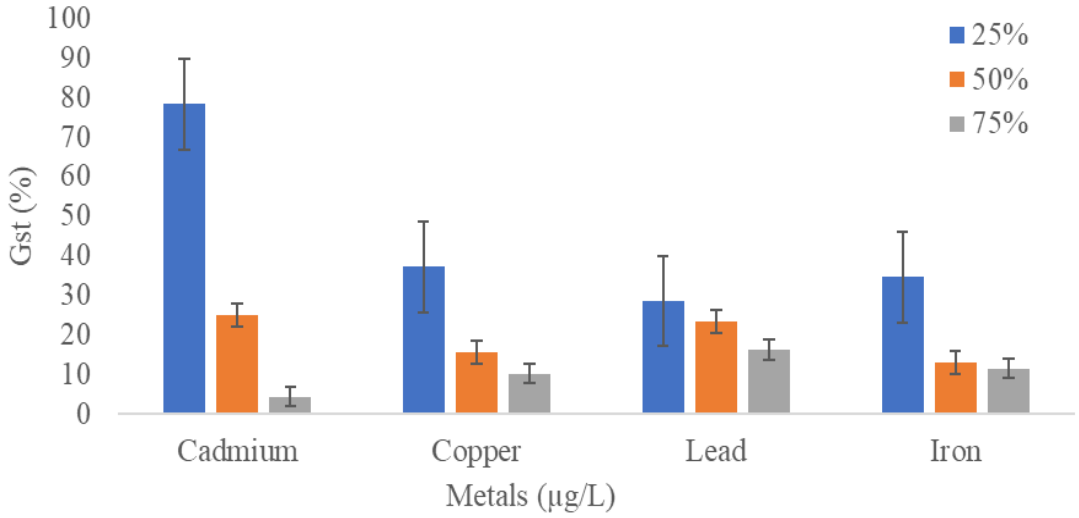

Fig. 4. Gst of *O. niloticus* in evaluated heavy metal sub-lethal doses

DISCUSSION

The findings of this study underscore the potential genotoxicity of heavy metals present in Uta Ewa Creek, a critical aquatic habitat supporting diverse biota and serving economic and ecological roles. The study focused on *O. niloticus* (Nile tilapia), chosen as a bioindicator due to its ecological ubiquity, economic significance, and sensitivity to environmental changes. By examining sub-lethal doses of cadmium (Cd), copper (Cu), iron (Fe), and lead (Pb), the research revealed significant genetic alterations, pointing to the severe genotoxic impact of these metals. This aligns with previous studies indicating that fish, occupying high trophic levels, bioaccumulate heavy metals, leading to toxicological and genetic consequences **(Ambedkar & Muniyan, 2011; Bawuro *et al.*, 2018)**.

Heavy metal pollution is a critical environmental issue globally, particularly in industrial and urban areas where water bodies often serve as repositories for effluents. Uta Ewa Creek is no exception, with its proximity to industrial activities and agricultural runoff contributing to heavy metal contamination. These metals enter aquatic systems through direct discharge, leaching from soils, and atmospheric deposition, subsequently being absorbed or ingested by aquatic organisms. Fish like *O. niloticus* are particularly vulnerable, as their gill membranes, skin, and alimentary canal serve as sites for metal uptake **(Fernandes *et al.*, 2008)**.

The bioaccumulation of heavy metals in fish is of concern, due to its cascading effects on the food chain. Studies indicate that these metals accumulate in fish tissues, leading to adverse physiological effects, including oxidative stress, immune suppression, and genotoxicity. For instance, cadmium has been shown to impair enzymatic functions

and disrupt cellular homeostasis, resulting in genetic mutations **(Liu *et al*., 2005; Kumar *et al*., 2015)**. Similarly, lead exposure disrupts calcium metabolism, causing neurological and genetic impairments in fish and higher organisms **(Shahzad *et al*., 2018)**.

Among the metals studied, cadmium emerged as particularly genotoxic, with increasing DNA damage observed with rising concentrations. This finding corroborates earlier research where cadmium exposure induced genetic mutations and chromosomal aberrations in various aquatic organisms. Cadmium's high genotoxicity can be attributed to its ability to generate reactive oxygen species (ROS), leading to oxidative damage and DNA strand breaks. Furthermore, cadmium can replace essential metals in metalloenzymes, disrupting cellular functions and exacerbating genetic instability **(Jin *et al*., 2003; Kumar *et al*., 2015)**.

Other metals such as copper and lead, while essential in trace amounts, also exhibited genotoxic effects when present at higher concentrations. Copper, an essential trace metal, becomes toxic at elevated levels, inducing lipid peroxidation and DNA cross-linking. Lead, on the other hand, is a non-essential metal with no biological role, and its toxicity arises from its ability to mimic calcium, disrupting cellular signaling pathways and DNA repair mechanisms **(Shahzad *et al*., 2018)**.

This study employed Random Amplified Polymorphic DNA (RAPD)-PCR analysis to assess the genotoxic effects of heavy metals on *O. niloticus*. RAPD-PCR is a molecular tool that detects DNA sequence changes by amplifying random segments of the genome. The resulting polymorphic bands provide a snapshot of genetic alterations, making it a sensitive and cost-effective method for monitoring genotoxicity.

In this study, the polymorphism observed with primers 2 and 4 was particularly notable, indicating significant genetic damage. The effectiveness of RAPD-PCR in identifying nucleotide sequence rearrangements has been demonstrated in various studies, reinforcing its value as a biomonitoring tool. For instance, **Aksakal and Esim (2015)** used RAPD-PCR to detect genetic damage in fish exposed to heavy metals. On the other hand, **Salem *et al*. (2014**) employed the technique to assess the genotoxic effects of industrial effluents. These studies, along with the current findings, underscore the versatility and sensitivity of RAPD-PCR in environmental genotoxicity research.

The genotoxic effects of heavy metals on the Nile tilapia have far-reaching ecological and economic implications. Genetic damage in fish can impair vital physiological functions, including reproduction, growth, and immunity, ultimately reducing population viability. This is particularly concerning for Uta Ewa Creek, where *O. niloticus* plays a critical role in the food web and supports local fisheries. The destabilization of fish populations can have cascading effects on the aquatic ecosystem, as fish contribute to nutrient cycling and energy flow. Furthermore, the bioaccumulation of heavy metals in fish poses a direct threat to human health. Fish are a major dietary protein source in many regions, and the consumption of contaminated fish can lead to

metal toxicity in humans, manifesting as neurological, renal, and cardiovascular disorders **(Alipour *et al.*, 2014)**.

The findings of this study highlight the urgent need for stricter regulation of industrial effluents and agricultural runoff to mitigate heavy metal pollution in Uta Ewa Creek. Despite existing environmental regulations, enforcement remains a challenge, often due to inadequate monitoring and limited public awareness. Strengthening regulatory frameworks and investing in pollution monitoring infrastructure are essential steps toward addressing this issue.

Community engagement is equally critical. Raising awareness about the ecological and health risks associated with heavy metal pollution can empower local communities to advocate for better environmental practices. Educational programs and stakeholder involvement in conservation initiatives can foster a collective effort to protect Uta Ewa Creek and its biodiversity.

While this study provides valuable insights into the genotoxic effects of heavy metals on *O. niloticus*, further research is needed to unravel the molecular mechanisms underlying these effects. Understanding the pathways through which heavy metals induce DNA damage can inform the development of biomarkers for early detection of genotoxicity in aquatic organisms. Additionally, exploring the potential for genetic adaptation or resistance in fish populations exposed to heavy metals could provide new perspectives on ecosystem resilience.

Molecular-level investigations should also focus on the interactions between heavy metals and other environmental stressors, such as temperature fluctuations, hypoxia, and pathogen exposure. Such multi-stressor studies are crucial for understanding the compounded effects of pollution in dynamic aquatic ecosystems.

CONCLUSION

The study underscores the severe genotoxic effects of heavy metals in Uta Ewa Creek, with significant implications for aquatic biodiversity and human health. *O. niloticus*, as a bioindicator, revealed genetic alterations when exposed to cadmium, copper, iron, and lead, highlighting the pervasive impact of metal pollution. RAPD-PCR analysis proved to be a robust tool for detecting genetic damage, providing a basis for biomonitoring and environmental assessment.

Addressing heavy metal pollution in Uta Ewa Creek requires a multifaceted approach, combining stringent regulation, community engagement, and scientific research. Protecting this vital aquatic habitat is essential not only for preserving its ecological functions but also for safeguarding the health and livelihoods of local communities. As industrialization and urbanization continue to threaten aquatic ecosystems worldwide, the findings of this study serve as a call to action for sustainable environmental management.

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