Egyptian Journal of Aquatic Biology & Fisheries
Zoology Department, Faculty of Science,
Ain Shams University, Cairo, Egypt.
ISSN 1110 – 6131
Vol. 25(3): 883 – 899 (2021)
www.ejabf.journals.ekb.eg

Biochemical, Histopathological, and Genetic Impacts of River Nile Pollutants on the Nile tilapia (*Oreochromis niloticus*)

Anas A. Heiba[1*], Amal M. Yacoub[2&4], Ebrahim A. Sabra[1], Reham M. Abd El-Azeem[3], Medhat H. Hashem[1]

1. Animal Biotechnology Department, Genetic Engineering and Biotechnology Research Institute (GEBRI), University of Sadat City (USC), Sadat City, Egypt.
2. National Institute of Oceanography and Fisheries, Cairo, Egypt.
3. Environmental Biotechnology Department, GEBRI, USC, Sadat City, Egypt.
4. Faculty of Science, Taif University

[*]Corresponding Author: anas-heaba@hotmail.com

ARTICLE INFO

Article History:
Received: April 17, 2021
Accepted: June 2, 2021
Online: June 30, 2021

Keywords:
Pollutants,
Biochemical,
Histopathological,
Genetic,
SSR (Simple sequence repeats),
Oreochromis niloticus,
River Nile.

ABSTRACT

Water pollution is one of the most principal environmental and public health problems in the River Nile. In the present study, aims were directed to evaluate the biochemical, histopathological, and genetic effects of El-Rahawy Drain pollutants on the Nile tilapia (*Oreochromis niloticus*). Hence, during the summer of 2018 and winter 2019, fish samples were collected from two locations (upstream and downstream) of El-Rahawy drain in the River Nile at the Rosetta Branch. Biochemical studies (Total Protein, Albumin, Globulin, A/G, total lipids, Cholesterol, AST, and ALT) were carried out for the specimens and important results were revealed. Compared with the fish caught from the upstream, a deterioration was detected in the health status of the downstream- fish; displaying more extreme histopathological and molecular variations. Using SSR (simple sequence repeats) to detect the molecular effects of water pollutants on *O. niloticus*, molecular markers indicated genetic differences in fish samples from the same sex in the different studied locations. Consequently, to prevent disease outbreaks and aquatic ecosystem disturbances, the Rosetta Branch is recommended to have an intensive water-quality monitoring program, and wastewater treatment as well.

INTRODUCTION

All over the world, freshwater resources such as rivers are polluted with a variety of solid and liquid wastes. In Egypt, the River Nile has become polluted due to the discharge of untreated waste, dumping of industrial effluents, and run-offs from agricultural fields, making it a model for a polluted ecosystem to assess biomarker responses in fish **(Osman 2012; Hashem *et al.*, 2020a).** The River Nile is Egypt's most important source of water. It bifurcates at a distance of 25 km (north of Cairo) into the Rosetta and Damietta branches forming a delta (**Abdel-Satar *et al.*, 2017**). Rosetta

Branch is about 225 kilometers long with an average depth of 2.0–2.3 meters. Agricultural, commercial, and domestic wastewaters, as well as anthropogenic activities, all contribute to a high microbial load on the Rosetta Branch in addition to the organic and inorganic pollutants **(Othman *et al.*, 2020)**. Therefore, serious negative impacts on the branch environment have been detected (**Hashem *et al.*, 2020a**).

The Nile tilapia is the sixth most cultured fish in the world (**Reantaso, 2017; Hashem *et al*., 2020b**). It can be used to estimate the Nile pollutants and is one of the most major biomonitors for water pollution (**Hamdoon *et al*., 2002a, b; Begum, 2004**). The pollutants directly enter the River Nile fish through the gills or the intestines. Then, through the bloodstream, pollutants are distributed throughout the body, where blood cells are the first receptors **(Sadauskas-Henrique *et al.*, 2011)**. Remarkably, blood biomarkers have been over used in fisheries science because being crucial in toxicological research, environmental monitoring, and predicting fish health conditions. **(Bitten-Court *et al.*, 2003)**. As a result, fish blood shows observable physiological changes faster than any other physiological evaluation parameter **(Ezeri *et al.*, 2004).** It also reacts to changes in other tissues as a result of pollution exposure **(Ruas *et al*., 2008)**. Since blood parameters react to low doses of contaminants, hematological and biochemical variables have recently emerged as promising biomarkers for measuring the effects of aquatic pollution in fish. Blood parameters are critical in diagnosing the structural and functional state of fish exposed to environmental contaminants because they are considered good physiological biomarkers of the entire body **(Seriani *et al*., 2011)**. Hematological and biochemical variables' responses to environmental stressors are usually unspecific. Nonetheless, by indicating the general physiology and health status of fish, they can provide useful information in impact assessment studies **(Beyer *et al*., 1996).**

The muscular system makes up the majority of a teleost's body. Locomotion, synchronized movement of skeletal components, blood pumping, and peristaltic constriction of visceral organs and their associated structures are some of their roles in the overall body (**Saad *et al*., 2012**). The epidermis of skin has recently been shown to contain naturally bacteriolytic substances as well as a constantly responding immune system, where, the affected epidermis losing its protective function as well as its osmotic barrier ability (**Kadry *et al*., 2015**).

Genome identification and selection have progressed rapidly with PCR technology help. A large number of markers that require only a small quantity of DNA have been developed. Each marker method has its advantages and disadvantages **(Shehata *et al*., 2009)**. Molecular markers such as inter simple sequence repeats (ISSR) and simple sequence repeats (SSR) markers are vital tools for monitoring fish populations **(Rashed *et al*., 2008; Hashem *et al.*, 2020a)** and fish species genetic variability **(Saad *et al*., 2009)**. The SSR marker is a co-dominant molecular marker that can distinguish between homozygotic and heterozygotic samples and also have a large number of alleles.

Hashem *et al*. (2020a) found that water quality parameters (nutrient salts and heavy metals) were increased at downstream of El-Rahawy Drain in addition to the depletion of dissolved oxygen. Additionally, fish samples showed severe histological and molecular alterations in downstream compared to upstream by using liver tissues and PCR-ISSR markers, respectively.

Thus, the present study aimed to evaluate the biochemical, histopathological (skin and muscles), and genetic effects (by using SSR molecular markers) of El- Rahawy Drain pollutants on the Nile tilapia; *Oreochromis niloticus*.

MATERIALS AND METHODS

1- Study area

The River Nile pollutants are derived from different uncontrolled sources such as industrial wastewater, agricultural drainage, in addition to municipal wastewater **(Al-Afify and Abdel-Satar, 2020)**. Rosetta Branch receives heavy pollutants, untreated municipal and agricultural drainage water, containing a high amount of organic matter and heavy metals from El-Rahawy Drain. To examine the polluted water, fish samples were collected from two locations (upstream and downstream) of El-Rahawy drain at Rosetta Branch of River Nile during the summer of 2018 and winter 2019 (Fig. 1).

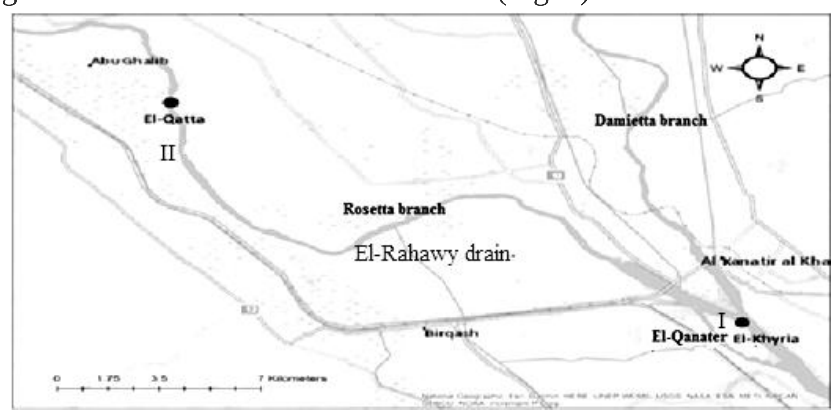

Fig. 1: A map showing the sampling locations in (I) Upstream and (II) Downstream of El-Rahawy drain at Rosetta Branch.

2- Biochemical studies

2.1 Sampling and preparation of blood serum

The fish were caught from the selected locations and dried from excess external water with filter paper. The blood samples were taken by cutting the tail of the fish. Blood samples were taken by severance of the caudal peduncle of fish and collected into small sterilized vials. The blood was left to clot and then centrifuged at 3000 r. p. m for 10 minutes to obtain serum. Supernatant serum was obtained using a micropipette model (Labystems K 33071) for biochemical studies.

2.2 Blood serum analysis

- **Serum total proteins and albumin**

Kits for the determination of total proteins and albumin (Sigma-Aldrich) were used.

- **Globulin content**

The globulin content was obtained by the succeeding equation:

Globulin content = Protein content – Albumin content.

- **Serum total lipid and cholesterol**

This was conducted by using serum cholesterol and total lipid Kits (Diamond diagnostic kits, USA).

- **Alanine aminotransferase (ALT) and Aspartate aminotransferase (AST)**

For the determination of serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine, Spectrum Diagnostics kits, Egypt were used.

3- Histopathological studies:

Skin and muscle samples obtained from *O. niloticus* were carefully removed then fixed in 10% formalin, dehydrated in ascending grades of alcohol and cleared in xylene. The fixed tissues were embedded in paraffin wax and sectioned at 5 microns by using Euromex Holland microtome. Sections were stained according to Harris Hematoxylin and Eosin method **(Bernet *et al.*, 1999)**, examined microscopically and photographed by using a microscopic camera.

4- SSR Molecular Markers

4.1 DNA extraction

Fish muscle tissues were used for DNA extraction through a genomic DNA extraction kit (G-Spin) from iNtRON Biotechnology, Inc., Korea. This kit was used for rapid isolation of the total DNA of 20 fish samples from the two locations (10 samples from each, 5 males and 5 females).

4.2 SSR primers

Eight SSR marker loci were screened from 20 individuals to detect the differences between the samples from the two studied locations (up and downstream of the drain). The PCR primers that were used are shown in Table (1) as described by **Eshel *et al.* (2012).**

Table 1: Simple Sequence Repeats Markers (SSR) Primers Sequences that were Used with *O. niloticus* Individuals from the Two Studied Locations.

Primer	Forward Primer	Reverse Primer
1	TGCTCTCACTGCTGAGCAAA	CGCAAATGTTAGGCCAGAAA
2	AAGACCCGTTCTTCGTCGTC	TTCATTCCACCTGCTCCAAA
3	GTGAGGCAAGTCCGGTTTCT	TGATCCACGGCGTATTGAGT
4	GTGGGCAAAAACAAGCCATT	TGTTTCAGTGTGAACGTGTGTG
5	AGGCCTTTCATCGCTGTTTT	ACCCTGTAGATGAGCGCAAA
6	AAGGGAAAGTGGCTCAGCTC	GTTGCTTCCCCACAGTTTCA
7	AGGAGAAGTCGCAGGTGACA	GGCACAGTTGCCTGGTACAT
8	CGAGCTGCTTTGTTGTCTGA	CGAACCGAAAATGAGAATGC

4.3 PCR amplification conditions

The PCR conditions were as follows: 5 mins at 94°C followed by 60 sec at 94°C, 45 sec at 55°C, and 60 sec at 72°C for 30 cycles and a final step for 10 mins at 72°C. PCR products were tested by using 3% agarose gel electrophoresis. 100 bp DNA ladder plus (iNtRON Biotechnology, Inc. Korea) was used to detect the obtained PCR products. The electrophoresis run was achieved at 75 V in the DNA electrophoresis unit (Bio-Rad) for 90 mins.

4.4 Molecular markers data analysis

The SSR banding patterns were scored as (1) for the appearance band and (0) for the disappearance one. Data matrices were analyzed using the Numerical Taxonomic and Multivariate Analysis System program (NTSYS), version 2.1, Applied Biostatistics Inc. (**Rohlf, 2000**). Similarity coefficients were applied for dendrogram construction by using the UPGMA (Unweighted Pair Group Method with Arithmetic Average) as well as the SAHN (Sequential Agglomerative Hierarchical Nested Clustering) routine in the NTSYS program.

RESULTS AND DISCUSSION

1. Biochemical studies

The variations of biochemical parameters of the studied *O. niloticus* fish are tabulated in Table (2).

Table 2: Variations of Biochemical Parameters of *O.niloticus* Fish Collected from Two Different Seasons.

Blood Parameter	Summer season		Winter season		Ahmed *et al.* (2019)	Mohamed *et.al.* (2020)
	upstream	Downstream	upstream	Downstream		
Total protein(g/dl)	3.80	2.5	3.30	2.10	3.7- 6.3	-
Albumin (g/dl)	1.85	0.70	1.30	0.55	1.8 -1.1	-
Globulin (g/dl)	1.95	1.50	2.00	1.55	-	-
,A/G ratio	0.92	0.46	0.65	0.35	-	-
Total lipids (mg/dl)	450.00	850.00	420.00	950.00	-	-
Cholesterol(mg/dl)	75.50	175.50	109.25	196.60	81-189	136-202.9
AST (IU/ml)	48.00	220.00	72.50	288.00	58.6 -275	15.2 – 37.5
ALT (IU/ml)	42.50	95.50	55.40	150.50	29 -68	14.4 – 25.4

Biochemical studies have been used as an indicator of the health status of fish and an important indicator to address the effect of pollutants on fish (**Ahmed, 2012; Bayomy *et al*., 2017; Hashem, 2020a**).

Total serum protein (T.S.P.)

The important function of total serum protein is the maintenance of osmotic balance between the circulating blood and tissue spaces (**Ahmed *et al*., 2019**). The results declared that the T.S.P. has lower values for *O. niloticus* samples collected from downstream than the upstream as shown in Table (2). Furthermore, those values were lower in winter than in summer. The decrease in the total serum protein in the *O. niloticus* collected from downstream may be due to the changes taking place in serum globulin metabolism as a result of different pollutants discharged from El-Rahawy Drain as reported in the study of **Tayel *et al*. (2014)**.

Total serum albumin (T.S.A.) and Total serum globulin (T.S.Gl.)

The determination of albumin and globulin were taken into consideration in the present investigation, as variation in total serum protein is more or less due to the changes taking place in the serum globulin metabolism as a result of water pollution **(Ahmed, 2012**). The values of albumin for the selected fish were within the range obtained by **Ahmed *et al.* (2019)** as shown in Table (2). The decrease in albumin and globulin for fish living downstream (sewage water) may be explained as a result of the increase in metabolic rate of albumin and globulin due to the decrease or depletion in dissolved oxygen. This explanation agrees with that of **Tayel *et al.* (2008, 2013)**.

Total serum lipids and Cholesterol

It was observed that the obtained values of total serum lipids and cholesterol were higher downstream than those recorded upstream (Table 2). In addition, the total serum lipids and cholesterol were recorded high level in winter, where the decrease in flow level of the Nile in winter tends to concentrate the pollutant as ammonia (**Ahmed *et al*., 2019; Mohamed *et al.*, 2020).**

Alanine aminotransferase level (ALT) and **Aspartate aminotransferase level (AST)**

The lysosomal membranes which are very sensitive to many pathogenic factors are disrupted thus their enzymes are released causing degeneration and vacillation of the cytoplasm of liver cells (**Bayomy and Mahmoud, 2007**). The aminotransferase alanine and aminotransferase aspartate are two important key enzymes considered as sensitive markers to evaluate hepatocellular damage and some hepatic diseases (**Aly *et al.*, 2003**)**.** The present data showed that the values of ALT and AST obtained from fish collected from downstream were higher than those obtained from upstream (Table 2). On the other hand, those values recorded the highest level during winter for both locations coinciding with the decrease in the flow level of the Nile. The values of ALT and AST of the *O. niloticus* fish collected from the two studied locations were higher than the ranges obtained in the study of **Mohamed *et al*. (2020)**. Those results showed a general trend of an increase in ALT and AST activities. Moreover, findings indicated that the pollutants of the Nile water affect the liver cells as evidenced by the alterations that occurred in both AST and ALT activities. Additionally, the increments in the ALT and AST activities could be considered as indicators for liver damage (**Ibrahim and Mahmoud, 2005; Bayomy *et al*., 2017**). Furthermore, it may be attributed to sewage wastes of the El-Rahawy Drain and increasing of organic pollutants, ammonia, and heavy metals in the River Nile **(Ahmed, 2012).**

2. Histopathological studies

Histopathology is used as a sub-lethal test for evaluating the toxic effect of water pollutants on fish **(Yacoub *et al.*, 2020**; **Tayel *et al.*, 2020; Hashem *et al*., 2020a).**

Skin and muscles

Normally, the skin of *O. niloticus* fish is composed of epidermal, dermal, and hypodermal layers. The skin covers the muscles' layer, which is composed chiefly of segmental myomeres. Each myomere is regarded as muscle and its fibers are parallel to the long axis of the body **(Tayel *et al.*, 2018)**. Skin and muscles of *O. niloticus*, collected from the investigated area, suffered from many pathological alterations.

Morphologically, the skin and muscle of *O. niloticus* fish inhabiting the water of investigated area showed absence of scales and destroyed dorsal fin, especially in specimens collected from the downstream location.

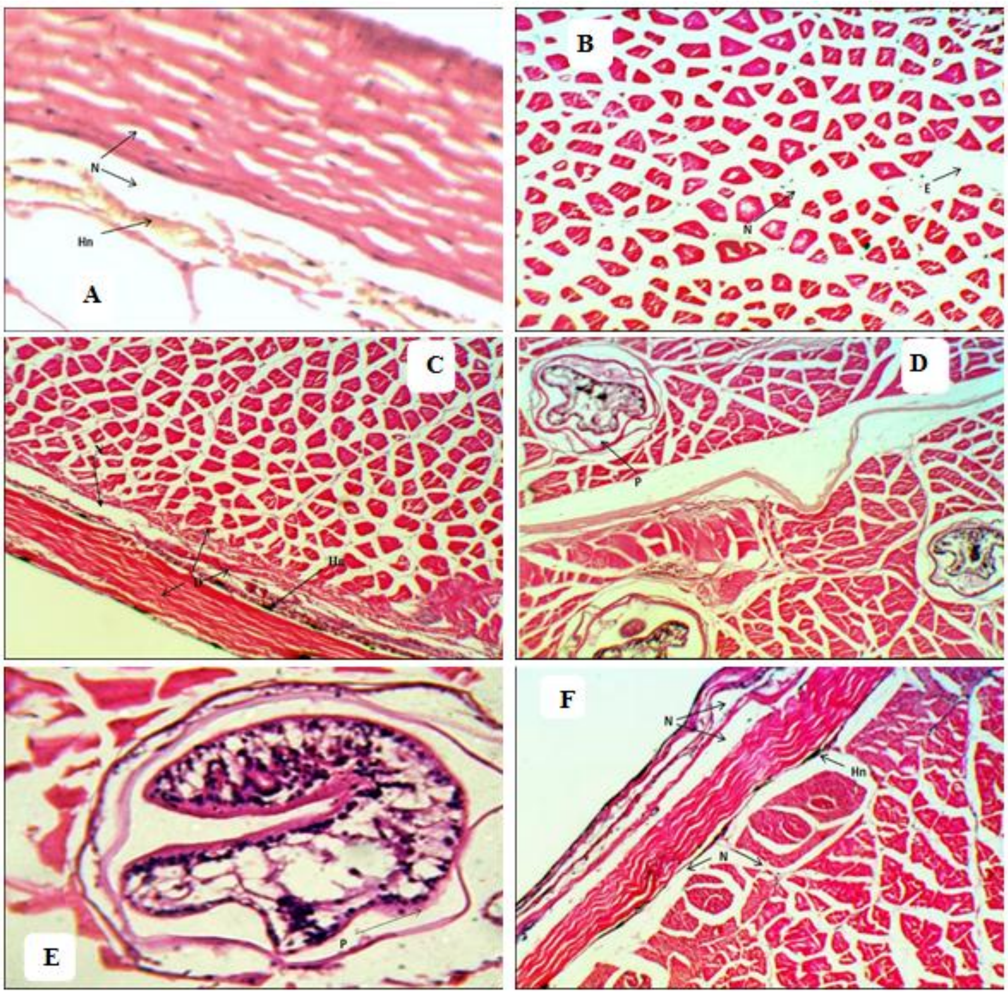

Fig. 2: Vertical Section (V.S.) in Skin and Muscles Sections of *O niloticus* Collected from Upstream and Downstream Regions of El-Rahawy Drain Discharge Point at Rosetta Branch. (H&E) X400. Showing:

a) Necrosis (N) in dermal & hypodermal layers. Hemosiderin (Hn) in hypodermal layers.
b) Necrosis (N) and edema (E) in the muscles layer.
c) Degeneration (D) in skin & muscle layers and necrosis (N) & hemosiderin (Hn) in the connective layer.
d) Parasitic form (P) in the muscle layer.
e) Parasitic form (P) in the muscle layer.
f) Necrosis (N) in epidermal, dermal, hypodermal, and muscle layers. Hemosiderin (Hn) beneath dermal layers.

Histological alterations of skin and muscles of *O. niloticus* fish collected from the investigated area were summarized in degeneration, necrosis, edema, and hemosiderin accumulation in all skin and muscle layers for samples obtained during winter and summer seasons from upstream and downstream locations but with a severe degree for the sample collected from downstream.Noticeably, samples collected from the downstream location during summer are specified with the occurrence of parasites (Fig. 2). In the present study, the histological alterations in skin and muscle may be attributed to the increasing level of ammonia, trace elements and turbidity in addition to depletion in oxygen concentrations in the surrounding water especially at downstream; forming an observation that matches with that obtained by **Ahmed (2012), Bayomy *et al*. (2017)**, **Hashem *et al*. (2020a)** and **Tayel *et al*. (2020)**.

3. Molecular Markers

Eight primers were used to study the effects of water pollution on SSR loci in the studied samples. The DNA fragments exhibited by the eight primers of SSR (P1-P8) from 20 samples (5 males and 5 females from each location of up and downstream) were separated using 3% agarose gel electrophoresis and are presented in Fig. (3).

All eight SSR markers loci primers of the 20 studied tilapia samples from the up and downstream of El-Rahawy Drain produced clear amplified fragments of DNA (Fig. 3). Most of the loci were polymorphic, with allele numbers that ranged from 1-4 alleles. Twenty alleles were obtained from females' genome samples and nineteen from males (Tables 3 & 4). The average of alleles per locus was 2.5 and 2.4 for females and males, respectively. It is worth mentioning that, the homozygous and heterozygous of locus do not depend on the sex. The dendrogram of SSR markers of males and females' samples are shown in Figs. (4 & 5), respectively. The dendrograms classified the studied samples of males and females into two clusters with respect to the studied locations. The up and downstream drain samples were in a separated cluster in the two sexes.

The two dendrograms indicate the genetic variances in SSR loci of fish samples from the same sex in the different studied locations. **Hashem *et al.* (2020a)** deduced the same results when they used ISSR markers and indicated that those variances were in a positive association with the concentrations of heavy metals and the quality of up and downstream of the drain. The previous authors assumed that, the concentrations of heavy metals in downstream water cause DNA mutilation.

Fishes are impacted strongly by environmental pollution, and the fishes potential to persist in contaminated water depends on their adaptive capacity, which is closely linked to their genome variability **(Gross *et al.*, 2010; Schneider *et al.*, 2013; Silva *et al.*, 2016; Araújo da Silva *et al.*, 2019)**.

In the present examination, the SSR markers were used to identify any molecular effects of polluted water from the El-Rahawy Drain on the *O. niloticus* DNA. Compared to ISSR, the SSR markers are specific and highly polymorphic (**Karp *et al.*, 1997; Jones**

***et al.*, 1997**), but they require information of the DNA sequence for specific primers' design.

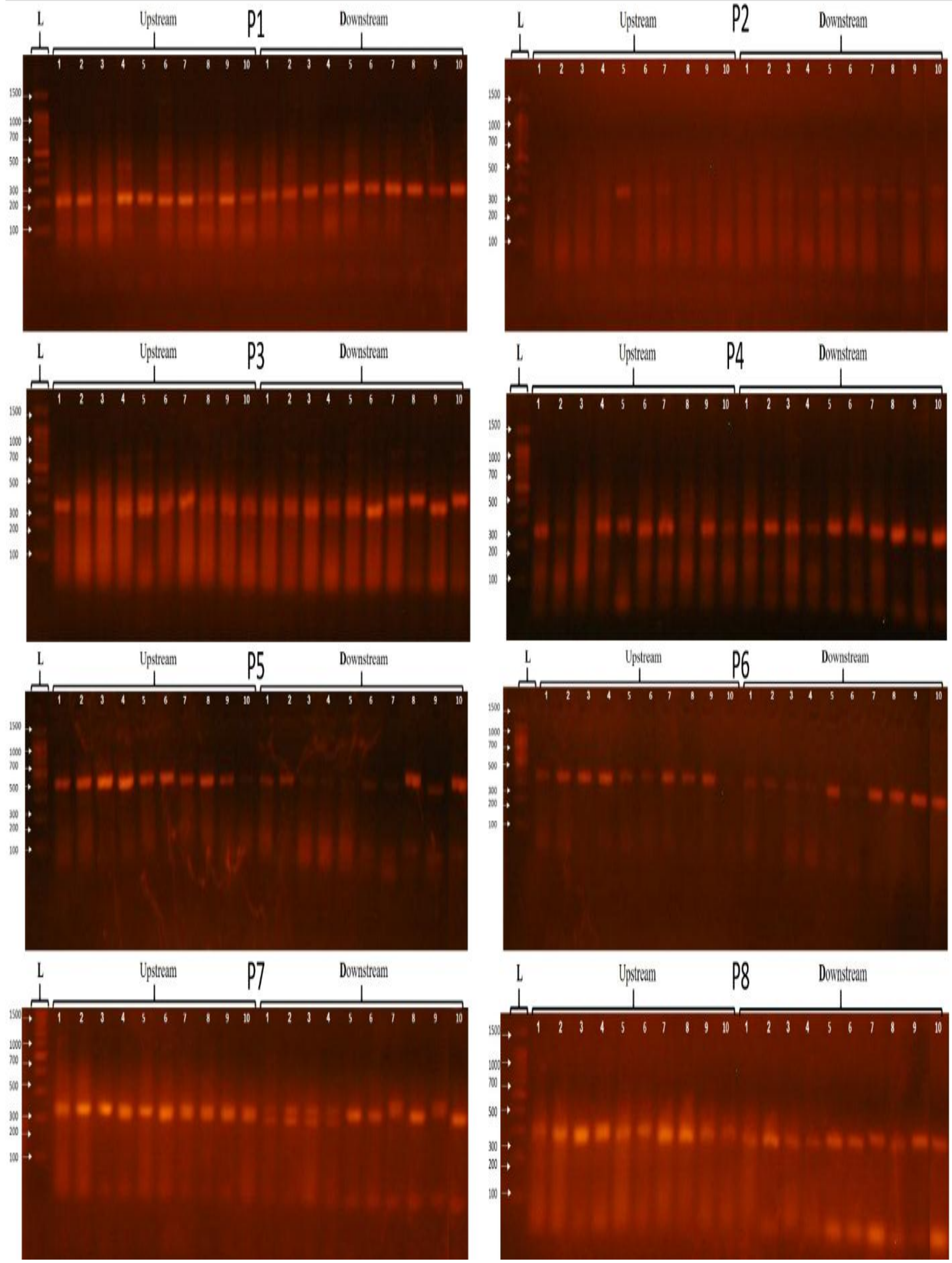

Fig. 3: Agarose Gel Electrophoresis for Amplified SSR Fragments Using (P1 - P8) Primers with Samples of the Two Different Locations of El-Rahawy Drain (Up and downstream).

Lines 1-5: males, Lines 6-10: Female, and L: DNA ladder.

Table 3: The Obtained Fragments and 0/1 Table of the Eight SSR Primers that were Used with *O. niloticus* Males' Samples from Up and Downstream of the Studied Drain.

Primers	Alleles	Males samples									
		Upstream					Downstream				
		1	2	3	4	5	1	2	3	4	5
P1	1	0	0	0	0	0	0	0	1	1	1
	2	0	0	0	0	0	1	1	0	0	0
	3	0	0	0	1	1	0	0	0	0	0
	4	1	1	1	1	1	0	0	0	0	0
P2	1	0	0	0	0	1	0	0	0	0	0
P3	1	0	0	0	1	1	1	1	1	1	1
	2	1	1	1	1	1	1	1	1	1	1
P4	1	0	0	0	1	1	0	0	0	0	0
	2	1	1	1	1	1	1	1	1	1	1
P5	1	0	1	1	1	1	0	0	0	0	0
	2	1	1	1	1	1	1	1	1	1	1
P6	1	1	1	1	1	1	0	0	0	0	0
	2	0	1	1	1	0	1	1	1	1	1
	3	0	0	0	0	0	0	0	0	0	1
P7	1	1	1	1	1	1	1	1	1	1	1
	2	0	0	0	1	0	0	0	0	0	0
	3	0	0	0	0	0	1	1	1	1	0
P8	1	1	1	1	1	1	0	0	0	0	0
	2	0	1	1	1	0	1	1	1	1	1

Table 4: The Obtained Fragments and 0/1 Table of the Eight SSR Primers that were Used with *O. niloticus* Females' Samples from Up and Downstream of the Studied Drain.

primers	Alleles	Females samples									
		Upstream					Downstream				
		1	2	3	4	5	1	2	3	4	5
P1	1	0	0	0	0	0	1	1	1	1	1
	2	0	0	0	0	0	1	1	1	1	1
	3	1	1	1	1	1	0	0	0	0	0
P2	1	1	1	0	0	0	1	1	1	1	1
P3	1	1	1	0	0	0	0	0	1	0	1
	2	1	1	0	0	0	0	1	0	0	0
	3	0	0	1	1	1	1	0	0	1	0
P4	1	0	1	0	0	0	1	0	0	0	0
	2	1	1	1	1	1	1	1	1	1	1
P5	1	1	1	1	1	0	0	0	1	0	1
	2	1	1	1	1	1	1	1	1	1	1
P6	1	1	1	1	1	1	0	0	0	0	0
	2	0	0	0	0	0	1	1	1	0	0
	3	0	0	0	0	0	1	1	1	1	1
	4	0	0	0	0	0	0	0	0	1	1
P7	1	0	0	0	0	0	0	1	0	1	0
	2	1	1	1	1	1	1	1	0	1	0
	3	1	1	1	1	1	0	0	1	0	1
P8	1	1	1	1	1	1	0	0	0	0	0
	2	0	1	1	0	0	1	1	1	1	1

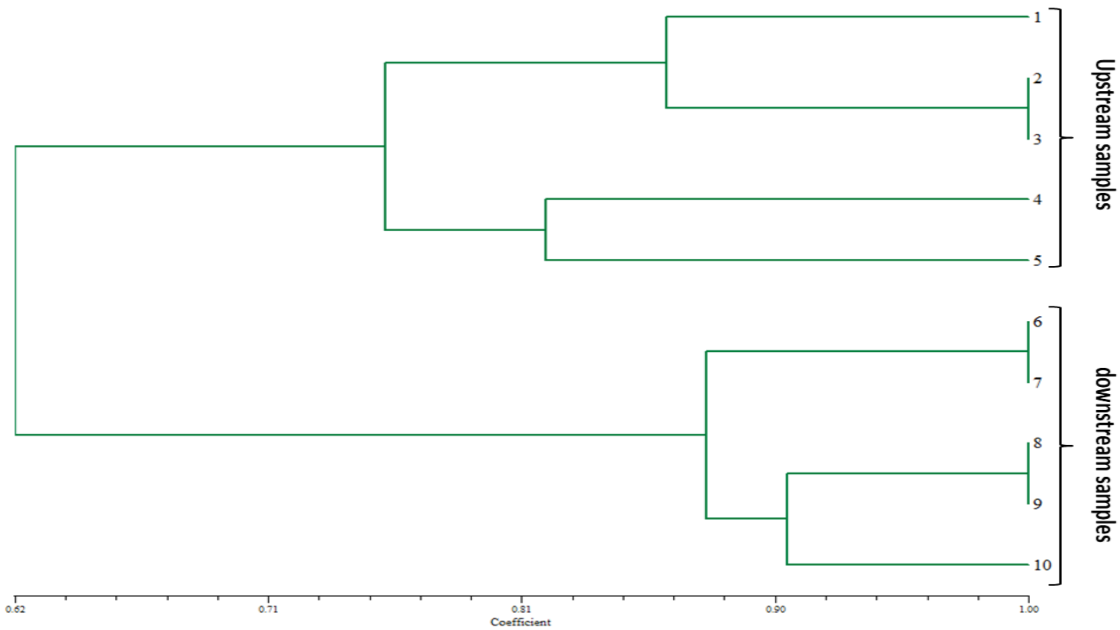

Fig. 4: The Dendrogram was Constructed from Eight Primers of SSR Markers Showing the Tilapia Males Samples' Genetic Relationships from Up and Downstream of El-Rahawy Drain.

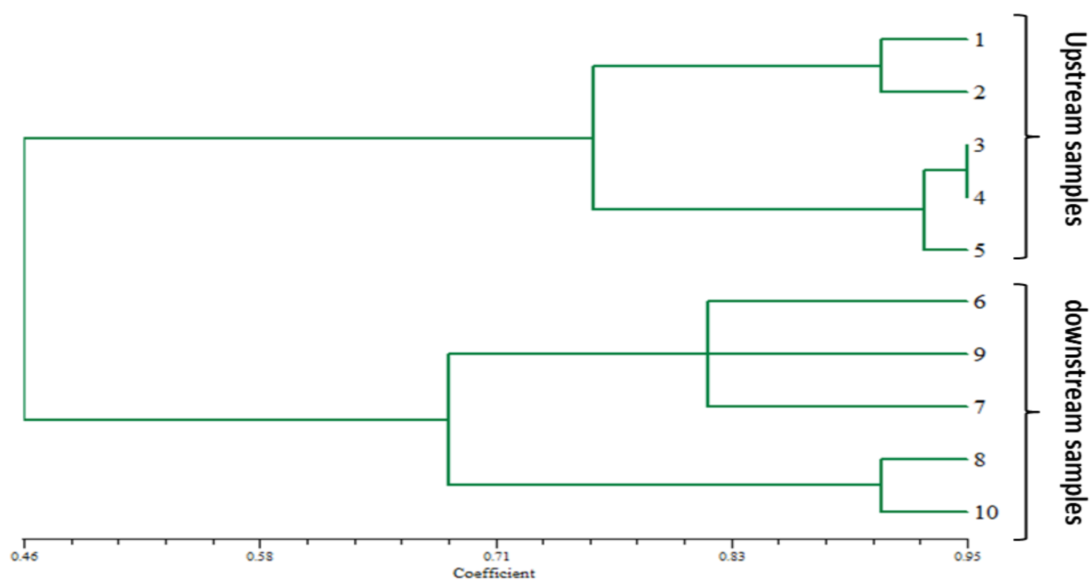

Fig. 5: The Dendrogram was Constructed from Eight Primers of SSR Markers Showing the Tilapia Females Samples' Genetic Relationships from Up and Downstream of El-Rahawy Drain.

SSR marker is a co-dominant marker that can differentiate between heterozygotic and homozygotic samples and have a large number of alleles. Since the use of a single SSR marker cannot provide authentic information (**Shehata *et al.*, 2009**), eight different SSR primers were used in the current study for the differences reliable detection between the fish samples from up and downstream El-Rahawy Drain. The 8 different SSR primers was recommended by **Warburton *et al.* (2002)** and **Ravago-Gotanco *et al.* (2010**). To eliminate any effects of the sex, the different samples SSR results of every sex (males or females) from the two locations were compared separately. In the samples of males and females, a number of unique fragments was found between the two locations samples.

Eminently, those unique fragments point to mutation occurrence. The mutations lead to the form of loss of a new primer binding site and the DNA fragment will hence be classified (disappearance or appearance) based on the effects of pollutants. It was noticed that, the downstream water of the studied drain was polluted with high concentrations of heavy metals compared to the upstream water; an observasion that concurs with that of **Hashem *et al*. (2020a)**. To illustrate, **Livingstone (2003)**, **Sevcikova *et al*. (2011)** and **Kamollerd *et al*. (2019)** stated that the pollutants including heavy metals can cause an imbalance between the free radical species production and reduction in fish**.** Additionally, the free radicals can attack DNA molecules, protein, and lipid to induce oxidative stress products and may cause DNA damage **(Castano & Becerril, 2004; Vilela *et al*., 2018; Kamollerd *et al*., 2019).**

The genetic variations in fish samples from the same sex in different locations as observed from dendrograms of SSR results indicated a positive correlation with metals concentrations detected by **Hashem *et al*. (2020a)** in the same studied area. The samples from the two different locations are separated into two different clusters as shown in Figs. (4 & 5). The aforementioned results suggest that the pollutants in the downstream of El-Rahawy Drain cause DNA damage. This finding matches with that of **Wood *et al*. (2001)**, **Vilela *et al*. (2018**) and **Kamollerd *et al.* (2019)** who reported that, in the polluted water, heavy metal exposure could cause DNA damage in fish as double and strand single breakages, alterations in the mechanism of DNA repair and DNA-protein crosslinks. Heavy metals can induce oxidative stress, DNA damage, point mutations, and several other indirect genotoxic effects **(Waalkes, 2003; Castano & Becerril, 2004; Suhartono *et al.*, 2013, Kamollerd *et al*., 2019).**

CONCLUSION

As a result of the persistent discharge of water containing high concentrations of organic waste and salts from agricultural runoff of the El-Rahawy Drain, Rosetta Branch is suffering from severe pollution and consequently impacts the Nile aquatic life. The fish samples collected downstream of El-Rahawy displayed more extreme histopathological and genetic variations than those collected upstream. It is recommended to make a master plan for sewage treatment with an identified target in rural areas. Additionally, the awareness of people who live on the banks of the River Nile valley must be increased.

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